ISSN 0972-8872

Volume 16
(December 2017 : Issue 2)

JOURNAL OF VETERINARY PHARMACOLOGY AND TOXICOLOGY

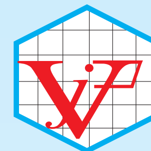
A PUBLICATION OF THE INDIAN SOCIETY OF VETERINARY PHARMACOLOGY AND TOXICOLOGY

On line Availability : www.isvpt.org

JOURNAL OF VETERINARY PHARMACOLOGY AND TOXICOLOGY

(Official Publication of the Indian society of Veterinary Pharmacology and Toxicology)

Indexed/Abstracted in ISA (Indian Science Abstract), MAPA (Medicinal and Aromatic Plant Abstract), CABI DATA BASE and AGRIS (FAO) DATA BASE



December 2017 Volume 16 issue 2

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Subscription Information : Journal of Veterinary Pharmacology and Toxicology is published biannually and the annual subscription is Rs. 2000 (India) and US$ 150 (other countries) for institutions. The journal is despatched within India by surface mail and to other countries by air mail.

Business Correspondence : Enquiries concerning subscription, advertisement etc. should be addressed to Dr. S.P. Singh, Chief Editor, Journal of Veterinary Pharmacology and Toxicology, C.V.A.Sc., G.B. Pant University of Agriculture and Technology, Pantnagar - 263 145 (Uttarakhand), India. Claims for missing issues should be sent within 2 months of issue date of the journal.

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Published by: Dr. S.P. Singh, Professor & Head, Department of Veterinary Pharmacology and Toxicology, C.V.A.Sc., G.B. Pant University of Agriculture and Technology, Pantnagar - 263 145 (Uttarakhand), India.
e-mail : sppharma@rediffmail.com, Mobile : 07500241448

Computer type setting and printed at Ocean Publication, Rampur-244 901, U.P.

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URL : www.isvpt.org

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Review Article

PESTICIDE RESIDUES IN MILK AND THEIR HEALTH HAZARDS

P.B. KAPURIYA, K. A. SADARIYA* AND A. M. THAKER

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ABSTRACT

Pesticides are chemicals or substances intended for preventing, destroying, repelling or mitigating pests. India is one of the largest manufacturers of different pesticides in Asia. The increasing number of pesticides entering our environment are potentially hazardous to health and ecosystem. Pesticides have high bioaccumulation potential due to chemical inertness, persistence, lipophilic nature and very less biodegradability. Pesticides are directly applied on animal body as in form of ectoparasiticides, sprayed in animal shelter and sheds of milk producing animals or used in agriculture sector that may be transferred to animal bodies through feed and fodder. Improper and indiscriminate use of pesticides creates a problem of residues in animal milk and other products. India is the largest producer of milk and one of the fastest growing exporters of dairy products in the world. The issues of pesticide residues in milk attain the instant attention of researcher because milk is being sucked by young ones of animals or consumed by people of different age groups which may impose risk of health hazards on man and animals. This review article focuses on the contamination of pesticides in milk and their health hazards on biotic system. Many studies reported that the pesticides residues of organochlorine, organophosphorus and pyrethroid are detected above the MRL set by FAO/WHO/EU/PFA in milk of animal origin in India and other countries. Many studies were reported on pesticides residues in milk causes serious, acute or chronic health hazards, depending on the length of exposure. Pesticides should be used only as a last resort or be recommended at the specified dose and frequency at the right time. This review article makes people particularly the users aware about the sources, health hazards caused by different levels and categories of pesticides.

Key Words: Milk, Pesticides Residues, Health hazards.

INTRODUCTION

Pesticides can be defined as any substance or mixture of substances intended for preventing, destroying, repelling or mitigating pests (Ecobichon and Klaassen, 2001). The most commonly applied pesticides are insecticides (to kill insects), herbicides (to kill weeds), rodenticides (to kill rodents) and fungicides (to control fungus and mold). Herbicides and insecticides are mainly used in the pre-harvest stages, rodenticides are used in the post-harvest storage stages and fungicides are applied at any stage on the crop. Pesticides can be transferred from plants to animals via the food chain. The pesticide residue is a substance or mixture of substances in feed, soil, water and air originating from the use of pesticides and includes its specified degradation, conversion, metabolites, reaction products and impurities (Tayade *et al.,* 2013). The maximum residue limit (MRL) is defined as the maximum concentration of a residue, resulting from the registered use of an agricultural or veterinary chemical that is recommended to be legally permitted or acceptable in food, agricultural commodity and animal feed (Richhariya *et al.,* 2017). MRLs for pesticide residues in milk and milk products are depicted in Table 1.

The use of synthesized pesticides started in 1949 with the application of DDT (Dichlorodiphenyl-trichloroethane) in malaria control and HCH (Hexachlorocyclohexane) to control locust. Pesticides are also useful to society by killing the harmful organisms, control insects, weeds and other pests which causes potential diseases. DDT, HCH, aldrin and dieldrin were used in agriculture and public health programmes in early 1980 in various countries. Most of countries now have put a ban on many pesticides but some of them are still being used by the local farmers and health department due to its versatile nature against various pests (Amoah *et al.,* 2006). The report on percent wise Status of Pesticides (Figure 1), Pesticides Uses: Indian Crop Protection Market Split (Figure 2) and state wise consumption of pesticides in India (Figure 3) were depicted and taken from Industry reports-2016, Analysis by Tata Strategic (Indian Agrochemical Industry, July 2016).

The amount of pesticides used in India is very low as compared to other developed countries even though we have much higher pesticide residues in food of our country (Abhilash and Singh, 2009). India is one of the largest manufacturer of different pesticides in Asia. Pesticides are mostly used in agriculture and health department (Sarkar *et al.,* 2012). The insecticides act, 1968 and insecticides rules, 1971 regulate the import, registration, manufacture, sale, transport and use of pesticides with a view to prevent risk to human and animals throughout India. All pesticides have to necessarily undergo the registration process with the Central Insecticides Board and Registration Committee (CIB and RC) in India.

Presence of pesticides in milk was reported by several authors in different countries over the last few decades, and use of most of these chemicals had been banned in many countries. The list of pesticides / formulation banned in India are aldicarb (17th July 2001), aldrin, benzene hexachloride, calcium cyanide,

chlorbenzilate (17th July 2001), chlordane, chlorofenvinphos, copper acetoarsenite, dibromochloropropane (25th July 1989), dieldrin (17th July 2001), endrin, ethyl mercury chloride, ethyl parathion, ethylene dibromide (17th July 2001), heptachlor, lindane (gamma-HCH), maleic hydrazide (17th July 2001), menazon, metoxuron, nitrofen, paraquat dimethyl sulphate, pentachloronitrobenzene (25th July 1989), pentachlorophenol, phenyl mercury acetate, sodium methane arsonate, tetradifon, toxaphene (camphechlor) (25th July 1989), trichloro acetic acid (17th July 2001) (Central insecticides board and registration committee, 2017). The list of pesticides for restricted use in India are aluminium phosphide, captafol, cypermethrin, dazomet, ddt, fenitrothion, fenthion, sodium cyanide, methyl bromide, methyl parathion, diazinon, monocrotophos, methoxy ethyl mercuric chloride (Central insecticides board and registration committee, 2017).

India is the largest producer of milk and one of the fastest growing exporters of dairy products in the world. Milk is an ideal fluid to dissolve pesticide residues since most of them have lipophilic properties. Milk can be contaminated by mainly 3 group of pesticide residues such as organochlorine (OC), organophosphorus (OP) and pyrethroid (Battu *et al.,* 2004). Animal origin food is responsible for 90% pesticides entry in the human body (Tecles *et al.,* 2013). Milk has nutritional value and it is consumed by peoples as well as young ones of animals therefore, the contamination of milk with residue considered as one of the main dangerous issues in the last few years. To control the presence of pesticide residues in fresh milk and milk products is a big issue for producer, consumer and government due to the potential risk.

CLASSIFICATION OF PESTICIDES

Pesticides have been divided into several major classes depending on their use in agricultural, veterinary and related fields. Toxicological point of view, Insecticides is further subdivided into chemical or other classes as mentioned below (Sandhu and Brar, 2009).

1) Insecticides:
a) Organochlorines: DDT, Aldrin, Dieldrin and Lindane, Endosulphan, HCB, HCH, Methoxychlor, Heptachlor, Endrin
b) Organophosphates: Parathion, Malathion, Phosphonate and Chlorpyriphos, Monocrotophos
c) Carbamates: Carbaryl, Aldicarb and Propoxur
d) Pyrethrins and Pyrethroides: Deltametharin, Cypermethrin, Flumethrin, Permethrin, Allethrin and Fenvalerate
2) Herbicides e.g. 2,4-D, Paraquat, Atrazine,
3) Fungicides e.g. Zineb, Captan, Dinocap.
4) Fumigants e.g. Aluminium Phosphide, Ethylene, Dibromide.
5) Rodenticides e.g. Zinc Phosphide, Warfarin, Red Squill.
6) Avicides e.g. 4-aminopyridine, Chloralose, Endrin.
7) Acaricides e.g. Azobenzene, Amitraz, Clofentezine.
8) Algicides e.g. Bethoxazin, Dichione, Hydrated Lime.
9) Bird repellents e.g. Guazatine, Thiram, Copper Oxychloride.
10) Mammal repellents e.g. Ziram, Copper Naphthenate, Trimethacarb

SOURCES OF PESTICIDES RESIDUES IN MILK

Contamination of milk with a pesticide depends on its stability, mode of application, duration of intake and exposure in the animal. The animals absorb pesticides mainly by inhalation, ingestion and dermal absorption and they can excrete through milk (Gazzotti *et al.,* 2009). These pesticides are applied pre-harvest, post-harvest and storage stages. They have ability to transfer from lower plants and animals to the higher plants and animals among the food chain and can accumulate in the higher organisms. In addition to this, sometimes pesticides are directly sprayed to the animal accommodation to infest the pest lead to the bioaccumulation of pesticides in the animal products like milk, meat, fat and eggs. Breeding animals can accumulate pesticides from contaminated feed and water and from pesticide application in animal production areas (treatment of cowsheds, pigsties, sheepfolds, warrens and/or treatment of animals themselves). Animal exposed to contamination of ponds, lakes, rivers due to spray of insecticides or discharge of effluents from the pesticide industries. Pesticide compounds are mostly stored in the fat and muscle of animals, they can also reach other compartments such as the brain, liver and lungs and the active ingredient is mostly metabolized in the liver or stored in the fat. Organochlorides compounds are lipophilic and are hardly metabolized to environmental exposure results in their accumulation and persistence in fat tissues. Excretion through milk can lead to residues in milk and milk products. Improper and indiscriminate use of pesticides is the main sources for pesticides residues in milk.

ANALYTICAL METHOD FOR DETECTION OF PESTICIDES RESIDUES

Gas chromatography (GC) and liquid chromatography (LC) are being used as separation, quantification and identification of pesticides residue in milk (LeDoux, 2011). GC used with electron capture detection (ECD) and nitrogen-phosphorus detection (NPD) for non-polar pesticides. Nowadays, it is preferable to use mass spectrometry (MS) or tandem mass spectrometry (MS/MS) detection in order to verify peak identities (Alder *et al.,* 2006). LC has been used for the analysis of polar or non-volatile or thermally labile pesticides. Few references on use of HPLC and HPTLC for the identification and determination of pesticides residue in milk were also reported.

TOXICITY OF PESTICIDE RESIDUES IN ANIMALS AND

HUMAN

Many studies have been reported for presence of pesticides residues in milk and its products which may cause serious, acute or chronic health hazards depending on the length of exposure. Certain pesticides like carbaryl, propoxur, parathion, 2,4-D, lindane and diazinon causes teratogenic defects in animals. Most of the OPs, OCs and carbamates may cause neurotoxic effects in man and animals including increase irritation, loss of memory, incoordination, ataxia, delayed response, convulsions, spasms and paralysis. Pesticides cause abnormalities in sperms leading to decrease their ability for fertilization in animals. The pesticide residues in food, may cause sterility, early abortion, still births or repeat breeding in animals. Pesticides residues also cause mutagenicity, neuropathy, nephropathy hepatotoxicity, immuno-deficiency, autoimmunity and hypersensitivity in animals and human (Chauhan and Singhal, 2006). Pesticide residues having toxic effects such as interfering with the reproductive systems, foetal development and also cause cancer and asthma in animals and human (Neme and Satheesh, 2016). Organochlorine pesticides have been clinically proven as endocrine disrupting and carcinogenic substances in animals and other living organism (Divya, 2011).

REPORTED STUDIES OF PESTICIDES RESIDUES IN MILK IN INDIA

Study 1: Haryana

Pesticide residues of HCH, DDT, endosulfan and aldrin were detected in 147 bovine milk samples from Haryana, India and samples were analysed through gas liquid chromatography. The HCH, DDT, endosulfan and aldrin were detected in 100%, 97%, 43% and 12% samples and with mean values of 0.0292, 0.0367, 0.0022 and 0.0036 µg/ml, respectively. Eight percent samples exceeded the maximum residue limit (MRL) of 0.10 mg/kg as recommended by WHO for HCH, 4% samples of 0.05 mg/kg for α-HCH, 5% samples of 0.01 mg/kg for γ-HCH, 26% samples of 0.02 mg/ kg for β-HCH as recommended by PFAA and 24% samples of 0.05 mg/kg as recommended by FAO for DDT. Concentrations of β-HCH and DDE were more as compared to other isomers and metabolites of HCH and DDT. According to data generated during sampling, no DDT and HCH were sprayed on wheat crop, the main source of dry fodder in the fields. As the organochlorine insecticides persist for a long time in the environment and appear even decades after their use has ceased, bovine milk contamination with these chemicals could be an environmental concern in many countries like India for many years to come (Sharma *et al.,* 2007).

Table 1:
MRLs for Pesticide Residues in Milk and Milk Products

Pesticides	FAO/WHO (mg/kg)	EUUS (mg/kg)	FDA (mg/kg)	PFA (mg/kg)
Alachlor	0.01	-	0.02	-
Aldrin	0.006	0.006	-	0.15
Aldicarb	0.01	0.01	-	-
Captafol	0.01	0.01	-	-
Carbaryl	0.05	-	0.01	-
Carbofuran	0.1	0.1	0.1	0.05
Chlordane	0.002	0.002	0.05	0.05
Chlorpyrifos	0.01	0.01	-	0.01
Parathion	0.05	-	-	-
Permethrin	0.05	0.05	-	-
Propoxur	0.05	0.05	-	-
Cypermethrin	0.05	0.02	-	0.01
Deltamethrin	0.05	-	0.1	-
Diazinon	0.01	0.01	-	-
Endrin	-	0.0008	-	-
Endosulfan	-	0.004	-	-
Ethion	-		0.5	0.5
Heptachlor	0.004	0.004	-	0.15
HCB	0.01	0.01	-	-
Imidacloprid	0.1	-	-	-
Lindane	0.001	0.001	0.05	0.01

Study 2: Punjab

Pesticides residue detect in 313 milk samples from eleven districts and one town of Punjab state. Chlorpyrifos residues were detected with highest mean levels of (2.158 ng/g) in 6.4% of the milk samples. Residues of DDT including DDE and DDD were the second most predominant pesticide with mean level of 1.594 ng/g and these were detected in 10.54% of the samples. Mean residue level of endosulfan, cypermethrin and HCH was recorded as 1.367, 1.001 and 0.985 ng/g, respectively. In this study, residues of fipronil, butachlor, cyhalothrin, fenvalerate, deltamethrin, malathion, profenophos and ethion were also observed in some milk samples. The milk samples exceeded the MRLs for HCH, DDT, endosulfan, fipronil, cypermethrin, chlorpyrifos and profenophos pesticide residues were reported. In the present study, current status of pesticide residues in milk is indicating the leading contaminant as chlorpyrifos followed by DDT and endosulfan. Out of twelve milk powder samples, four were found to be contaminated with DDT, HCH, cypermethrin and chlorpyrifos pesticide residues. In comparison to previous studies in India, change in pattern of occurrence of pesticide residues in milk and milk products was observed in present study with decrease in organochlorines and increase in organophosphorus and pyrethroids due to bans/restricted uses of HCH and DDT in agriculture. The proportion of organochlorines, organophosphorus and synthetic pyrethroids pesticides residues in milk samples were 46%, 30% and 24% reported, respectively (Bedi, 2012).

Study 3: Delhi

Organochlorine pesticide residues were detected in buffalo milk from different localities of Delhi, India. There were 20 milk samples collected in various farms. Residues

of Lindane exceeded the Maximum Residual Limit values in 50% of the samples is a cause of serious concern. The DDT was detected in 70% of the samples with DDE (dichlorodiphenyldichloroethylene) in 80% of the milk samples of different parts of Delhi state. DDD (Di-chlorodiphenyldichloroethane) another metabolite of DDT was detected in 65% of the milk samples whereas the α and β endosulfan were detected in 35% and 40% of the samples. The analysis indicates that DDT is the major contaminants in different parts of Delhi state. It could be concluded that the OC pesticide residues were detected in buffalo's milk as they were persistent in nature due to their slow decomposition rate, long half-life and high stability in the environment. In most cases, the values of detected OC pesticides were exceeded the tolerance levels of FAO/WHO (Aslam *et al.,* 2013).

Study 4: Andhra Pradesh

Isolation and identification of pesticides residue in raw milk and chilled milk samples were detected from coastal districts of andhra pradesh. A total 600 samples of milk were collected from different region of andhra pradesh. Samples were detected by spectrophotometer. The pesticide residues were found in raw milk samples includes lindane, endosulfan, chlorane, heptachlor and methoxychlor. The percentage of positive raw milk samples in various regions like in visakhapatnam, lindane, chlorane and methoxychlor were found in 20%, endosulfan and heptachlor were found in 40% samples. In srikakulam, lindane, chlorane and methoxychlor were found in 40% of samples. About 50% of samples were positive for endosulfan and 46% of samples positive for heptachlor. In vizianagaram, 40% of samples were positive for lindane and heptachlor. About 36% of samples were positive for chlorane and mehtoxchlor. endosulfan was found in 46% of samples. In east godavari, 50% of samples contained lindane, 40% of samples contained endosulfan, 34% of samples contained chlorane and 30 and 38% samples were positive for heptachlor and methoxychlor respectively. In west godavari, 50% of samples were positive for lindane and methoxychlor, endosulfan was present in 46% of samples, chlorane was present in 40% of samples and 36% of samples contained heptachlor. In vendor's milk samples, 40% of lindane, chlorane, 50% of endosulfan and heptachlor and 56% of samples were positive for methoxychlor. The pesticide residues were found in chilled milk samples includes lindane, endosulfan, chlorane, heptachlor and methoxychlor. The percentage of positive chilled milk samples in various regions were shown in table 8. In Visakhapatnam, lindane, chlorane and methoxychlor were found in 20%, endosulfan in 40% and heptachlor in 22% of samples. In Srikakulam, lindane was found in 36% of samples. Chlorane and methoxychlor were found in 40% of samples. About 48% of samples were positive for endosulfan and 42% of samples positive for heptachlor. In Vizianagaram, 38% of samples were positive for lindane and heptachlor. About 36% of samples were positive for mehtoxchlor. Endosulfan was found in 46% of samples and chlorane was found in 34% of samples. In east godavari, 48% of samples contained lindane, 38% of samples contained endosulfan, 34% of samples contained chlorane and 28% and 38% samples were positive for

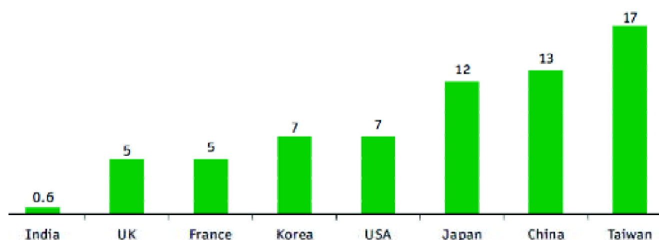

Fig. 1:
Status of Pesticides (%) (Source: Indian Agrochemical Industry report, 2016).

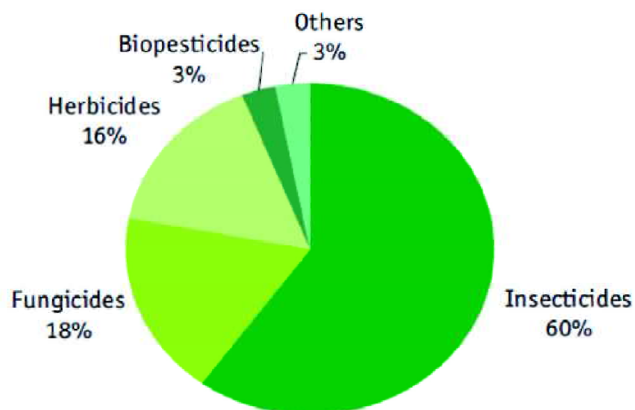

Fig. 2:
Pesticides Uses: Indian Crop Protection Market Split (Source: Indian Agrochemical Industry report, 2016).

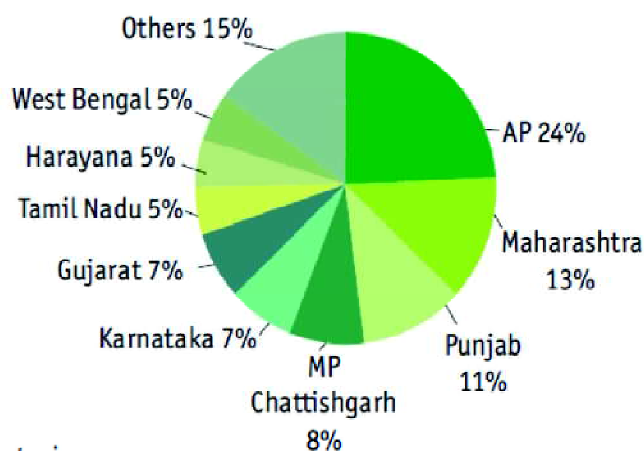

Fig. 3:
State wise Consumption of Pesticides in India (Source: Indian Agrochemical Industry report, 2016).

heptachlor and methoxychlor respectively. In west godavari, 50% of samples were positive for lindane and methoxychlor, endosulfan was present in 44% of samples, chlorane was present in 36% of samples and 24% of samples contained heptachlor.

Study 5: Gujarat

Detection of pesticides in buffalo milk from different areas of Gandhinagar and Ahmedabad by thin layer chromatography (TLC) for 3 pesticides residues namely dichlorovos, chlorpyrifos and DDT were reported. The 30 milk sample were collected from different local Street vendor and dairies from different areas of Gandhinagar and Ahmedabad and confirmation of pesticides residue was done through GC-MS. Results showed that chlorpyrifos residue was most significantly present in 40% of the collected milk samples. The intake of the pesticide contaminated milk might pose health hazardous to human. The findings of the study might help in extending awareness in dairy farmers and local people about pesticides and their hazardous effects on humans (Makadiya and Pandey, 2017).

REPORTED STUDIES OF PESTICIDES RESIDUES IN MILK IN OTHER COUNTRIES

Study 1: Italy

Determination of fifteen organophosphorus pesticides in Italian raw milk were reported. The milk samples were collected directly from tank trucks during delivery of 3,974 tonnes of raw milk at nine Italian dairy plants. The pesticide most frequently found was chlorpyriphos ethyl (9 positive samples), followed by parathion ethyl (2 samples) and disulphothon and acephate (1 sample each). The chlorpyriphos ethyl was mostly found in milk due its wide use as an insecticide and ectoparasiticides on livestock (Gazzotti *et al.,* 2009).

Study 2: Brazil

Organophosphorus and carbamates residues were detected in cattle milk samples in Brazil were reported. There were 30 raw milk samples collected from several farms and analysed through GC-MS. Result indicated that 6 (20%) sample were contaminated with OP, 5 (16.7%) with carbamates and 1 (3.33) milk sample contaminated with both pesticides. In four dairy farms the pesticides detected in milk were compatible with the active principles found in water and/or foodstuff, suggesting them to be the source of contamination. The transfer of pesticides from feed to milk is influenced by quantity ingested, absorption, pesticide metabolism and excretion by animals in production. Thus, the best risk management tool is prevention of exposure, either through feed or from the environment, of the animals to these contaminants, as well as the application of a withdrawal time (Fagnani *et al.,* 2011).

Study 3: Turkey

Organochlorine pesticide residues in cow, buffalo and sheep milk from Turkey were reported. The results indicated that milk is contaminated by 21 different pesticides out of that 16 OC pesticides residues were detected in sheep milk followed by 14 OC pesticides in buffalo milk and 11 OC pesticides in cow milk. Dominant pesticides in all samples examined were beta-HCH in buffalo cow and sheep milk in the concentrations of 63.36, 91.32, and 122.98 ng/ml, respectively. Total OCP levels were 243.81 ng/ml in sheep's milk, 151.02 ng/ml in cow's milk, and 133.38 ng/ml in buffalo's milk founded. Total OCP levels were found to be higher in sheep's milk than that of both buffalo's and cow's milk ($p < 0.05$), and there was no significant differences between buffalo's and cow's milk's OCP levels ($p > 0.05$). Some of the pesticides detected were found to be in the excess amount of the acceptable level regarding the EU regulations are beta HCH and methoxychlor were in all milk types examined. Alpha-HCH, beta HCH, gamma HCH, heptachlor-endo-epoxide (trans-isomer), trans-chlordane (γ), cis-chlordane (α), endrin, and methoxychlor were in sheep's milk; beta HCH, endrin, and methoxychlor were in cow's milk; beta HCH, heptachlor-endo-epoxide (transisomer), cis-chlordane (α), and methoxychlor were reported in buffalo's milk excess amounts of MRL in regards to the EU food codex (Bulut *et al.,* 2011)

Study 4: Mexico

The study on organochlorine contaminants in the milk of Alpine and Saanen goats breed were reported in the central region of Mexico. About 40 milk samples from these two breeds of goats were collected. The values for heptachlor + heptachlor epoxide in 13% of samples of Alpine breed and delta HCH and heptachlor + heptachlor epoxide in 17.3% and 50% in saanen breed goat above the upper permissible limits of Codex Alimentarius were evaluated. It may be concluded that the milk of these goat breeds showed contamination of pesticides residue. There were high values for HCH isomers in the Saanen breed (63.3 %) and the Alpine breed (85.8 %), probably as a result of application of this pesticide for pest control, which is common in animal shelters in Mexico (Schettino *et al.,* 2013).

Study 5: Pakistan

Identification of pesticides residues in different milk samples of cow, buffalo, goat, camel and sheep in Lahore, Pakistan were reported. The identification and quantification of pesticides was done through GC-MS. The 50% of milk samples were contaminated with pesticides of OP (chlorpyrifos), pyrethroid (bifenthrin, deltamethrin and lambda cyhalothrin), insecticides (imidacloprid) and carbamate (carbofuran) residues. Result showed that 73% of milk samples contaminated with deltamethrin (0.80 mg/kg) in all animals compare to other pesticides and out of that mainly sheep milk samples were mostly contaminated with all this pesticides residues compare

to other animals. The findings of the study might help in extending awareness in dairy farmers and local people about pesticides and their hazardous effects on humans (Shahzadi *et al.,* 2013).

Study 6: Pakistan

Organochlorine and pyrethroid residue were determined in dairy milk samples near Punjab in Pakistan. All 150 raw milk samples collected from the cotton growing belt and analysed on HPLC system. About 70% of the samples were found contaminated with pesticides like aldrine, cypermethrin, bifenthrin, permethrin, DDT, DDE, endosulfan and detlamethrin in 35%, 23%, 21%, 18%, 10%, 9%, 7%, and 5% of the milk samples respectively, showed pesticides residues. Pesticides presence in analyzed milk samples indicates the application of chlorinated pesticides in the past and its sparse application to some extents up to dates, as well as recent use of pyrethroid pesticides for agricultural purposes. Additionally, pyrethroids concentration level exceeds to maximum residue limit (MRL) in number of milk samples which is also matter of concern for consumer health. This study provides some basic information regarding the pesticide residues level in milk in the areas where huge amount of pesticides are applied every year but the pesticides monitoring activities are very limited (Hassan *et al.,* 2014).

Study 7: Egypt

Organochlorine and organophosphorus pesticide residues in buffalo milk from agro-industrial areas in Assiut, Egypt were reported. About 45 samples of buffalo milk were collected at random from local vendors, dairy farms and shops out of those 5 Organochlorine pesticides (alachlor, dieldrin, HCB, lindane and methoxychlor) and 3 organophosphorus pesticides (chlorpyrifos, malathion and methyl parathion) were detected in the milk samples. The residue levels of lindane and malathion exceeded European Commission MRLs (2008) in 44% of the samples. In addition, the residue levels of chlorpyrifos, methoxychlor and HCB exceeded EC MRLs (2008) in 33, 66 and 88% of the examined samples, respectively. The results of this survey demonstrate the need to establish pesticide residue monitoring programs in milk for human consumption to improve food safety and decrease exposure risks for consumers (Shaker and Elsharkawy, 2015).

PREVETION AND CONTROL OF PESTICIDES RESIDUE

There is need to break the habit of using harmful pesticides and switch to rising organic ones. Need to investigate the pesticides residues in milk in order to provide a baseline to health department or governing bodies to make safety regulations. Always use only recommended pesticides at the specified doses and frequency at the right time. Never use banned pesticides. The improper use or misuse through lack of understanding creates residue problems. Avoid indiscriminate use of pesticides. Proper precautions must be taken for the control of house hold insects / stored grain pests. Always read product labels carefully before applying any pesticide, mix and apply as directed. Pesticides residues monitoring program is very essential for the safety of consumer health. Organic food is grown and treated with natural pesticides.

CONCLUSIONS

Pesticides are any substance or mixture of substances intended for preventing, destroying, repelling or mitigating pests. India is one of the largest manufacturer of different pesticides in Asia. Pesticides are mostly used in agriculture and health department. GC/MS and LC/MS is widely used for separation, quantification and identification of pesticides residue in milk. Many studies reported that the pesticide residues of OP, OC and pyrethroids are detected above the MRL set by FAO/WHO/PFA/EU in milk of animal origin in India and abroad. Pesticides residues above MRL in milk may cause serious acute or chronic health hazards, depending on the length of exposure. Pesticides residue causes carcinogenicity, mutagenicity, teratogenicity, neuropathy, nephropathy, hepatotoxicity and reproductive disorder in animals and human. MRL of pesticides residue in milk should be taking into consideration before use of animal milk. Pesticides should be used only as a last resort.

FUTURE PROSPECTS

Immediate need to strengthen the pesticide residue monitoring programmed there by ensuring the safety and quality of milk for consumers and for sale. Use integrated pest management like grow pest-resistant crop varieties, use predatory insects to kill pests, employ mechanical pest traps and eliminate pest nesting areas as an alternative to the heavy use of pesticides. Official control laboratories are required to handle a large number of analyses facilities with validated, quick and easy methods to detect pesticides residues in milk.

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Received on : 12.08.2017
Revised on : 27.10.2017
Accepted on : 28.11.2017

PHARMACOKINETIC AND BIOEQUIVALENCE STUDY OF BUPARVAQUONE IN CATTLE CALVES

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ABSTRACT

The present study was undertaken to investigate the pharmacokinetics and bioequivalence of two formulations of buparvaquone (Theilex® and Butalex®) @ 2.5 mg/kg body weight in cattle calves. The plasma concentration of buparvaquone was determined by High Performance Liquid Chromatography (HPLC). The decay in plasma concentration of drug was biexponential in cattle calves. The C_{max} value of 0.16 µg/ml was obtained at T_{max} of 1.50 h in cattle calves following i.m administration of Theilex®. C_{max} value of 0.31 µg/ml was obtained at T_{max} of 1.92 h in cattle calves, following i.m administration of Butalex®. The elimination half life ($\beta t_{1/2}$), volume of distribution (V1_F) and AUC were calculated as 48.02 h, 11.77 L/kg,7.62 µg.h /ml in cattle calves following i.m administration of Theilex®. The elimination half life ($\beta t_{1/2}$), volume of distribution (V1_F) and AUC were calculated as 35.44 h, 3.62 L/kg and 9.45 µg.h /ml in cattle calves following i.m administration of Butalex®. Bioequivalence of Theilex® with respect to Butalex® in cattle calves was 82.34% following i.m administration.

Keywords: Bioequivalence, buparvaquone, cattle calves, pharmacokinetic.

INTRODUCTION

Buparvaquone is a second-generation hydroxynaphthoquinone antiprotozoal drug and belongs to the group of naphthoquinones. Naphthoquinone compounds have antimicrobial as well as fungicidal properties (Kayser and Kiderlen, 2003). Buparvaquone is a therapeutic drug for theileriasis and its efficacy in the treatment of East Coast Fever was first reported by McHardy *et al.* (1985). The pharmacokinetics of buparvaquone has been studied in cattle (Kinabo and Bogan, 1988; Muraguri *et al.,* 2006), rabbit (Venkatesh *et al.,* 2008) and rat (Pinjari *et al.,* 2008).

MATERIALS AND METHODS

The study was conducted in four cross-bred male cattle calves (1.0-1.5 yrs in age, weighing 110±5.0 kg). Animals were kept in animal house of the college and were reared under standard managemental and husbandry conditions, maintained on standard ration and were provided water *ad libitum* as per CPCSEA guidelines. Animals were kept on pre-experimental period for one month before the commencement of experiment to acclimatize them to new environment. During the pre-experimental period, all the animals were dewormed with ivermectin @ 0.2 mg/kg body weight and albendazole @ 10mg/kg body weight. An intervening wash out period of one month was given before administration of other formulation.

In this study, Theilex® was taken as test formulation and Butalex®, as reference formulation and were administered at a single dose @ 2.5 mg/kg body weight intramuscularly in rump region of cattle calves. Blood samples were collected from jugular vein in heparinized microcentrifuge tubes at time intervals of 0, 0.25, 0.50, 1, 2, 4, 8, 12, 24, 48, 72, 96, 120 h.The extraction of buparvaquone from plasma sample was carried out as per the method of Venkatesh *et al.* (2008) with slight modifications. Deproteinization of plasma sample was carried out by adding 0.5 ml of acetonitrile to 0.5 ml of separated plasma followed by vortex mixing at high speed for 1 min and subsequent centrifugation at 12,000 rpm for 15 min. The clear supernatant was collected into a micro centrifuge tube. This supernatant was buffered with 0.5 ml of 0.05 M KH_2PO_4 and extraction using solid phase extraction (SPE) procedure. Elute obtained from SPE was evaporated and reconstituted in mobile phase.

An isocratic mobile phase consisting ammonium acetate (0.02 M, pH 3.0) and acetonitrile in the ratio of 18:82 (v/v) was used.The flow rate was kept at 0.7 ml/min and the elution was monitored at 45 ^{0}C with UV detection at 251 nm. The limit of quantification (LOQ) and limit of detection (LOD) for buparvaquone was 0.025 µg/ml and 0.016 µg/ml, respectively. The pharmacokinetic analysis of the plasma concentration obtained following i.m. administration in this study was done by a pharmacokinetic software. Bioequivalence of both formulations was calculated by comparing area under curve (AUC) of both formulations (AUC i.m. Theilex®/ AUC i.m. Butalex®)

RESULT AND DISCUSSION

A two-compartment model adequately described plasma concentration-time profile of Theilex® and Butalex® in cattle calves following single dose i.m. administration. In the present study, the peak plasma concentration in cattle calves was attained at 2h post i.m administration of

Theilex® and Butalex®, respectively. The values of C_{max} were 0.16 µg/ml at T_{max} 1.50 h for Theilex® and 0.31µg/ml at T_{max} 1.93 h for Butalex® in cattle calves following i.m administration. These findings could be well corroborated with C_{max} (0.253µg/ml) and T_{max} (2.62h) in cattle (Muraguri *et al.,* 2006). The mean elimination half -lives were 48.02 and 35.44 h in cattle calves following i.m route of administration

Table 1:
Pharmacokinetic parameters of Theilex® and Butalex® in plasma following its single dose (2.5 mg.kg^{-1}) i.m. administration in cattle calves (n=4)

Parameters	Units	Mean± S.E.	
		Theilex®	Butalex®
V1_F	L/kg	11.77±1.09	3.62±0.38
V2_F	L/kg	10.17±2.24	8.93±0.35
αt½	h	2.21±0.60	1.04±0.17
βt½	h	48.02±6.75	35.44±4.75
C_{max}	µg/ml	0.16±0.01	0.31±0.02
T_{max}	h	1.50±0.15	1.93±0.27
AUC	µg. h /ml	7.62±1.04	9.45±1.50

V1_F = Volume of distribution of central compartment; V2_F=Volume of distribution of peripheral compartment; αt½ = Distribution half-life; βt½= Elimination half-life; C_{max}= Peak plasma concentration; T_{max}= maximum time required to attain peak plasma concentration; AUC= Area under curve.

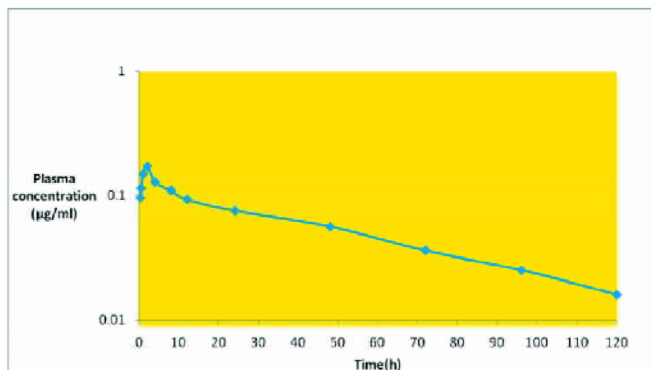

Figure 1:
Semilogarithmic plot of buparvaquone (Theilex®) concentrations in plasma versus time following i.m administration of Theilex® (2.5 mg.kg^{-1}) in male cattle calves (n=4).

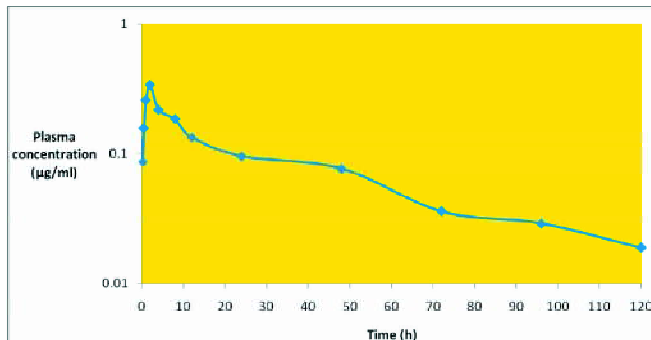

Figure 2:
Semilogarithmic plot of buparvaquone (Butalex®) concentrations in plasma versus time following i.m administration of Butalex®(2.5 mg.kg^{-1}) in male cattle calves (n=4).

of Theilex® and Butalex®, respectively. Long half life could be attributed to infiltration of buparvaquone into muscle leading to slow elimination from the body(Mc Hardy *et al.,* 1985).

The findings of the present study vary from the half-life value reported by Kinabo and Bogan (1988) in cattle calves following i.m administration (26.44 h), 9.13 h in rabbits (Venkatesh *et al.,* 2008) and 4.0 h in rats (Pinjari *et al.,* 2008) following i.v administration. Volume of distribution (Vd) in cattle calves in this study was found to be 11.77 and 3.62 L/kg. However, low volume of distribution (1.55 L/kg) has been reported in Sprague Dawley rats (Pinjari *et al.,* 2008), whereas in rabbits, total volume of distribution (12.57 L) following i.v administration as reported by Venkatesh *et al.,* 2008 is found to be equivalent to that determined in the present study. High lipophilicity and low plasma protein binding of drug contribute to high volume of distribution. The area under curve (AUC) was 7.62 and 9.45 µg.h /ml in cattle calves following i.m administration of Theilex® and Butalex®, respectively. A low value (4.785 µg.h /ml) has been reported in cattle (Muraguri *et al.,* 2006) following i.m administration as compared to the findings in the present study. Comparison of AUC values revealed that Bioequivalence of Theilex® with respect to Butalex® in cattle calves was 82.34% following i.m administration.

The plasma concentration time curve for Theilex® and Butalex® following single dose i.m. administration in cattle calves is depicted in Fig.1. Pharmacokinetic values describing the disposition kinetics of Theilex® and Butalex® following single dose (2.5mg.kg^{-1}) i.m. administration in cattle calves are presented in Table1.

ACKNOWLEDGEMENT

The authors would like to thank Dean, College of Veterinary & Animal Sciences and Directorate Experiment Station, Govind Ballabh Pant University of Agriculture and Technology, Pantnagar for providing necessary facilities in carrying out these experiments and also to M/S Montajat Vet. Pharmaceutical Ltd., for providing Theilex® and Butalex®.

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Received on : 26.07.2017
Accepted on : 29.09.2017

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*The full name of the Organization should be indicated by the Industries.
**Not applicable to Industries.

ANTIULCEROGENIC POTENTIAL OF ETHANOLIC EXTRACT OF WHOLE PLANT OF *CLERODENDRUM INDICUM* LINN.

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ABSTRACT

In the present study, antiulcerogenic activity of the ethanol extract of *Clerodendrum indicum* was evaluated using several standardized anti-ulcer models: HCl-ethanol, indomethacin, swimming stress ulcer and cold restraint stress induced ulcer in rats. The ulcer protective activity of the plant extract was analyzed at doses, 100 and 200 mg/kg body weight, orally by determining the ulcer score and index, gastric volume and pH along with the estimation of antioxidants *viz.* reduced glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT) in liver and stomach homogenates. Estimation of serum levels of certain enzymes *viz.* aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP) and confirmed by histopathological studies. Results indicated an antiulcerogenic activity of the extract at 200 mg/kg, (p.o), with the near normalization of the levels of the functional and the biochemical parameters after pre-treatment with the extract. Histopathological evidences supported the findings with a near normal arrangement of gastric mucosal cells.

Keywords: Antiulcerogenic activity, *Clerodendrum indicum*, Indomethacin, Peptic ulcer

INTRODUCTION

Peptic ulcer is one of the most common gastrointestinal disorders, which causes high rate of morbidity particularly in the population of non-industrialized countries (Falk, 2001). Peptic ulcer occurs due to an imbalance between the aggressive (acid, pepsin and *Helicobacter pylori*) and the defensive (gastric mucus and bicarbonate secretion, prostaglandins, innate resistance of the mucosal cells) factors (Tripathi, 1999). The agents that have been implicated in the pathogenesis of gastric ulcer includes increased gastric acid and pepsin secretion, decreased gastric blood flow, the suppression of endogenous generation of prostaglandins, inhibition of mucosal growth and cell proliferation and alteration of gastric mobility (Konturek *et al.,* 1998). Management of peptic ulcers involves H_2 receptor blockers, proton pump inhibitors, antimuscarinic drugs and drugs that afford cytoprotection by virtue of their effects on mucosal defensive factors. Although these drugs have brought about a remarkable advancement in ulcer therapy, they have been implicated to produce adverse reactions such as hypersensitivity, arrhythmia, impotence and haemopoetic changes with a possibility of a relapse within one year after cessation of treatment (Ariyphisi *et al.*,1986). Therefore, a search for cheaper alternative with minimal side effects and a lesser relapse possibility is rapidly gaining ground. In this perspective, herbal medicines are being increasingly explored as they presumably have no side effects. Also, there are several reports pertaining to their gastro protective functions.

Clerodendrum indicum L. (family Verbenaceae), commonly known as 'Bamanhati', grows up to 2 meters in height with soft succulent branches and simple broad leaves. It is an annual shrub and is cultivated as an ornamental plant throughout India (Kirtikar and Basu, 1975; Chopra *et al.*,1950). In traditional system of remedies, the plant is mainly used in the treatment of asthma, bronchitis, cold fever, intestinal worms, epilepsy, febric convulsion, gastric tumour, hematuria, hysteria, impotence, lipoma, nasal polyps, painful maturation and rheumatism (Uddin, 2006 ; Kirtikar and Basu, 1964 and Watt, 1972). The paste of the leaves of this plant has been employed in traditional medicine in treating external wounds and inflammation (Bhattacharya, 1980). Also, juice of the leaves and the roots are used as blood purifier and treat digestive disorders and other gastro intestinal ailments. Reports pertaining to the antinociceptive, anti-diarrheal and antimicrobial activities of leaf extracts of *C. indicum* have been found (Pal *et al.,* 2012).

Despite evidences suggesting the traditional use of *C.indicum* in gastric disorders, scientific data pertaining to the same have not yet been reported ; hence it was thought worthwhile to study the antiulcerogenic property of the ethanolic extract of whole plants of *C.indicum* using different models as described above.

MATERIALS AND METHODS

Whole plants of *C.indicum* were collected and

authenticated by Dr. I.C. Barua, Taxonomist, AAU, Jorhat. Voucher specimens were deposited (voucher specimen no: 4915) at the Central National Herbarium (CAL), Howrah and Kanjilal Herbarium, Shillong. The experimental plant material was shade dried and ground to a fine powder. The powdered plant part was macerated with ethanol at room temperature for 72 h. The mixture was then filtered with Whatman No. 1 filter paper. The filtration process was repeated three times. The solvent obtained was evaporated in Rotary evaporator (Buchi, Rotavapor R210, Switzerland) and stored at 4^0C until further use.

Healthy Wistar rats of either sex, approximately of the same age and weighing about 180-200 g were used for the study. Breeding and maintenance of the animals were done according to the guidelines of committee for the purpose of control and supervision of experiments on animals (CPCSEA) and animal welfare division, Government of India for the use of laboratory animals. This work has been carried out as per approval of the institutional animal ethical committee (No 770/ac/CPCSEA/ FVSc,AAU/IAEC/11-12/118). All the animals were housed in polypropylene cages using paddy husk bedding at 28±1°C temperature and 50 ± 5% humidity. Animals were maintained on a diet of laboratory feed and tap water *ad libitum*.

Six animals were randomly divided into 4 groups. Group I served as vehicle (distilled water) treated control group. Group II, as standard drug treated group (omeprazole at 4 mg/kg/rat,p.o.) (Perumal *et al.,* 2010). Group III and Group IV, *C.indicum* at 100 and 200 mg/kg/ rat,p.o., respectively.

Acute toxicity study was carried out according to the organization of Economic Corporation Development (OECD) guidelines 425. *C.indicum* was administered at a dose of 2 g/kg to a group of 3 mice and the percentage mortality was recorded for a period of 24 h. After the first hour of dose administration, the mice were observed for any behavioural changes suggesting toxicity. The animals were subsequently observed for a period of 14 days for any abnormalities and mortality. As no mortality was observed up to a dose of 2 g/kg, two doses at 100 mg/kg and 200 mg/kg were chosen for the study.

HCl/Ethanol induced ulcer

Gastric ulceration was induced in 24 hour starved rats allowing free access to drinking water. Omeprazole (4 mg/kg, p.o.) and the test extract (100 mg/kg, 200 mg/ kg, p.o.) were administered orally 1 hour prior to the oral administration of 1 ml of the necrotizing solution (150 mM HCl in 60% ethanol) to each rat. The animals were euthanized after 1 hour with diethyl ether, the stomachs removed and observed for ulcers in the glandular region (Kokate, 2000).

Indomethacin induced ulcer

Gastric ulcers were induced in rats kept on overnight fasting by administering indomethacin (40 mg/ kg, p.o.), with slight modification (Süleyman *et al.,* 2001), an hour after pre-treatment with the standard drug (omeprazole at 4 mg/kg, p.o.) and *C.indicum* (100 and 200 mg/kg, p.o.). Four hours later, the animals were euthanized with diethyl ether for estimation of various parameters described hereunder.

Swimming stress ulcer

Male wistar rats fasted for 24-36 hours are forced to swim inside the vertical cylinders (height 30 cm, diameter 15 cm) containing water up to 15 cm height, maintained at 23°C. Three hours after the stress, they were removed from the cylinder and euthanatized for determination of various parameters (Parmar and Desai, 1993).

Ulcer index: The number of ulcers was counted and scoring was done (Desai and Parmar, 1995).

Cold restraint stress

Animals in this group were subjected to cold stress after 45 min of treatment of test extract (100 and 200 mg/kg body weight) and omeprazole (4 mg/kg, p.o.) treatment, respectively (Dharmani *et al.,* 2005). All the animals were immobilized in restraint cages that were kept at 4^0C for 2 hours. Thereafter, the animals were euthanatized ; ulcers were detected and scored (Süleyman *et al.,* 2001).

Determination of ulcer score and index

The stomach was opened along the greater curvature observed macroscopically for gastric lesion and scored arbitrarily according to the severity of the lesions (*Süleyman et al.,* 2001). The ulcer scores were assigned as follows: 0= normal coloration, 1= red coloration, 1= spot ulcers, 1.5= haemorrhagic streak, 2= ulcers, 3= perforation.

Ulcer index was determined as follows: 10/X where X= total mucosal area/total ulcerated area.

Determination of gastric volume and pH

Gastric juice was collected from the stomach of the rats and subjected to centrifugation (Remi, R-8C) at 2000 rpm for 5 min. Following centrifugation, the volume and pH of the collected gastric juice were measured (Nagar *et al.,* 2012).

Biochemical estimation of antioxidant enzymes

A homogenate of the stomach (200 mg/ml in 20mM Tris buffer, pH 7.8) and liver (100 mg/ml in 20mM Tris buffer, pH 7.8) were used for the estimation of reduced glutathione (GSH) by the method of Beutler *et al.*(1963), superoxide dismutase (SOD) by Marklund and Marklund, (1974), catalase (CAT) by the method of Aebi (1984); alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP) were estimated in serum using reagent kits (Siemens).

Histopathological study

For histopathological studies, tissues were

collected and fixed in 10% neutral formalin solution and dehydrated with a series of ethanol-xylene solutions. The materials were processed by conventional paraffin embedding method. Microtome sections were prepared at 6 μm thicknesses, mounted on glass slides, stained with hematoxylin and eosin (H&E) followed by observation for histopathological changes under light microscope (Lee and Luna, 1968).

Statistical analysis

Results are presented as Mean ± SEM. Statistical analysis was performed by one-way analysis of variance (ANOVA) among different treatment groups followed by Dunnett's multiple comparison test (Graph Pad Prism 5.0).P <0.05 was considered for statistical significance.

RESULTS AND DISCUSSION

Oral administration of *C.indicum* up to a dose of 2 g/kg of *C.indicum* did not produce any visible signs of toxicity or mortality. Accordingly, 1/10th and 1/20th of the safe dose were taken for antiulcerogenic study in different models.

A significant reduction (P <0.05) in the ulcer score, ulcer index, gastric volume and an increase in the pH (P <0.05) was observed following pre treatment with *C.indicum* and omeprazole as compared to the control group in the HCl-ethanol induced ulcer model (Table 1). Elevation in the enzymatic antioxidants such as SOD and CAT and non enzymatic antioxidant GSH was also significant (P <0.05) (Table 2). A decline in the serum enzymatic parameters (ALT, AST and ALP) was observed in the extract treated as well as omeprazole treated groups (P <0.05) when compared to control group (ALT-3768.83±293.99, AST-3665.5±214.7 and ALP-88.00±7.470) (Fig. 1–3). ALT, AST and ALP levels were found to be 2137.5±116.50, 2101.50±203.70, 52.33±3.69 IU/L respectively in the *C.indicum* treated group (200 mg/kg, po) and 1693.5±293.99, 1754±194.99 and 36.30±3.06 IU/L respectively in the omeprazole treated group.

Histopathological examination of the control group showed severe necrosis of the gastric mucosa in addition to discontinuous villi (Fig 4A). The group treated with standard drug omeprazole exhibited a better gastroprotective activity in comparison to the extract treated group with a near normal gastric mucosa (Fig 4B). The group treated with *C.indicum* (200 mg/kg, p.o.) showed a reduction of mucosal damage with mild necrosis (Fig 4C).

In the Indomethacin- induced ulcer model, dose dependant reduction in the ulcer score, index, gastric volume and a rise in the pH were noted in the *C.indicum* (P <0.05) treated and omeprazole treated groups as compared to the control group (Table 1). An augmented antioxidant mediated defence was supported by a significant (P <0.05) dose dependant increase in GSH, SOD and CAT (Table 2) levels in stomach and liver tissues as well. A significant decline (P <0.05) in the levels of serum ALT, AST and ALP was observed in standard and extract treated groups when compared to control group (Fig. 1–3). Histopathological evidences support the ulceroprotective activity of the extract with mild disruption of the gastric mucosa (Fig. 5C) in contrast to an immensely eroded gastric mucosa of the control animals with shortened villi and edema (Fig. 5A). Omeprazole treated group showed superficial necrosis (Fig. 5B).

In the swimming stress model, antiulcerogenic activity of the extract was supported by a reduction in the gastric volume, ulcer score and index and an increase in the gastric pH (P <0.05) in comparison to the control group (Table 1).The effect was better with the highest dose i.e. 200 mg/kg GSH, SOD and CAT (Fig. 1–3) levels were also found to be elevated (P <0.05) in *C.indicum* as well

Table 1.
Antiulcer activity of ethanol extract of *C.indicum* in different models of ulcer.

ULCER MODEL	TREATMENT	ULCER SCORE	ULCER INDEX	GASTRIC CONTENT(ml)	PH
Hcl ethanol induced ulcer	Control	3.583±0.229	1.687±0.249	7.100 ± 0.366	2.660 ± 0.220
	Standard prazole)	0.498±0.050	0.340±0.025	1.130 ± 0.074	3.960 ± 0.145
	C.indicum (100)	1.187±0.098	0.543±0.083	4.129 ± 0.218	2.984 ± 0.237
	C.indicum (200)	0.953±0.061	0.380±0.032	2.180 ± 0.304	3.960 ± 0.105
Indomethacin induced ulcer	Control	2.950±0.170	1.410±0.177	6.840 ± 0.111	2.420± 0..256
	Standard (Omeprazole)	0.312±0.033	0.160±0.015	1.570 ± 0.290	4.040 ± 0.213
	C.indicum (100)	0.754± .987	0.345±0.084	3.007 ± 0.187	3.468±0.195
	C.indicum (200)	0.440±0.057	0.210±0.032	2.020 ± 0.024	4.180 ± 0.172
Swimming stress	Control	1.080±0.211	0.550±0.065	7.110 ± 0.273	2.920 ± 0.265
	Standard (Omeprazole)	0.220±0.019	0.158±0.020	0.993 ± 0.069	4.800 ± 0.146
	C.indicum (100)	0.754±0.987	0.345±0.084	3.007 ± 0.187	3.468± 0.195
	C.indicum (200)	0.305±0.027	0.220±0.029	1.230 ± 0.215	4.760 ± 0.148
coldrestrain stress induced ulcer	Control	0.697±0.065	0.337±0.024	3.239±0.149	2.870 ± 0.378
	Standard(Omeprazole)	0.138±0.019	0.125±0.019	0.887 ± 0.037	5.650 ± 0.193
	C.indicum (100)	0.345± .043	0.245±0.030	1.976± 0.167	3.478±0.189
	C.indicum (200)	0.242± .025	0.180±0.012	0.972 ± 0.052	4.810 ± 0.160

Table 2:
Biochemical parameters after treatment of ethanol extract of *C.indicum* in various ulcer models.

Ulcer Model	Treatment	SGPT	SGOT	ALKP	GSH		SOD		PRO		CAT	
					Stomach	Liver	Stomach	Liver	Stomach	Liver	Stomach	Liver
Hcl ethanol	Control	3768.83±293.99	3665.5±214.70	88±7.47	63.33±2.77	105.33±4.58	0.038±.004	0.075±.006	155.6±8.17	155.6±8.14	0.025±0.002	.019±0.002
induced ulcer	Standard	1693.5±293.99	1754±194.99	36.33±3.06	411.33±12.30	500.16±7.61	0.18±.003	0.2±.006	292.5±7.43	407.5±4.11	0.071±.002	0.066±.003
	C.indicum (100)	2392.5±97.32	2358.33±47.90	60.33±2.23	185.54±10.32	164.25±2.76	0.107±.008	0.153±.008	256.25±7.43	547±52.84	0.056±.003	0.042±.002
	C.indicum (200)	2137.5±116.51	2101.5±203.75	52.33±3.69	191.67±12.99	180.66±4.97	0.107±.008	0.153±.008	458.75±19.29	592.5±35.5	0.064±.003	0.057±.002
	Control	1348.63±128.97	1081.25±106.54	52.66±5.70	136.66±3.36	151.33±4.91	0.032±.004	0.088±.012	80±4.12	206.25±6.88	0.021±.001	0.013±.001
indomethacin	Standard	535.86±82.58	473.58±43.53	38±3.23	302.66±5.43	327.5±6.82	0.217±.012	0.232±.014	168.5±4.64	311±10.09	0.059±.003	0.056±.002
induced	*C.indicum (100)*	403.29±5.30	471.83±15.82	36.16±2.18	164.25±3.89	210.24±10.08	-	-	136.5±6.07	289.5±7.63	0.033±.001	0.036±.002
ulcer	*C.indicum (200)*	367.2±50.86	325.86±35.55	31.83±2.57	178.33±4.33	190.66±8.08	0.143±.002	0.27±.001	211.25±11.96	372.5±17.97	0.038±.002	0.03±.002
Swimming	Control	2619.71±44.13	2246.87±44.13	82±3.18	96.33±4.43	126.66±2.58	0.113±.01	0.197±.022	58±4.39	92.75±6.29	0.035±.002	0.027±.002
stress	Standard	2075.61±80.74	1726.9±69.67	62.16±3.10	116±5.47	137.17±3.80	0.303±.02	0.395±.01	118.75±18.75	271.25±17.83	0.079±.003	0.059±.003
	C.indicum (100)	1932.84±84.56	2016.9±54.54	70.66±1.02	102.45±5	114.21±60	-	-	99.25±8.45	218.75±6.25	0.06±.001	0.053±.002
	C.indicum (200)	1868.33±54.00	1850.26±50.30	63.5±2.28	104.83±5.64	112.17±5.72	0.218±.02	0.305±.01	137±4.31	200.25±7.19	0.065±.002	0.055±.002
	Control	2375±277.04	2094.66±262.10	99.33±10.74	130±4.58	195±6.85	0.096±.004	0.109±.004	30.16±4.12	157.16±5.36	0.047±.001	0.056±.002
Cold restrain	Standard	1044.83±86.39	979±77.39	42±3.99	192±6.02	250±7.84	0.377±.06	0.400±.03	72.6±3.90	205.8±28.55	0.092±.003	0.107±.003
stress	*C.indicum (100)*	1350.66±40.75	1517.±68.27	71.16±2.07	185±6.25	235±4.76	-	-	32.25±3.03	171.25±3.70	0.068±.002	0.089±.003
	C.indicum (200)	1150.83±123.26	1228.63±178.06	47.33±6.48	221±7.96	286.45±7	0.224±.01	0.306±.02	91±6.50	217.4±27.39	0.083±.003	0.112±.003

as omeprazole treated groups as compared to the control group.

Histopathological study shows sloughing, necrosis and reduced height of villi in the control group (Fig. 6A). Omeprazole treated group showed near normal arrangement of the gastric mucosal cells (Fig. 6B), whereas the extract treated group (200 mg/kg) showed very less superficial sloughing (Fig. 6C).

In the cold restraint stress model, a dose dependent elevation ($P < 0.05$) in the pH (Table 1) and antioxidants (GSH, SOD and CAT) in the extract treated groups as compared to the control group were recorded (Fig. 1–3). In contrast, a significant decline ($P < 0.05$) in the gastric volume, ulcer score, ulcer index (Table 1) and serum enzymatic parameters was noticeable (Fig. 1–3). Histopathological study showed that the control group had gastric mucosal necrosis and degeneration (Fig. 7A). In the treated group, the damage was restricted to superficial sloughing and focal necrosis (Fig. 7C). In this model too, omeprazole treated group exhibited the best results (Fig. 7B).

The present study was conducted with an aim to validate the folklore claims of the usage of *C. indicum* in treatment for gastric ulcers. The finding of this study demonstrates the ulceroprotective potential of the ethanol extract of *C.indicum* in several standardized models for antiulcerogenic activity in Wistar rats. A dose dependent response was noted. The extract was also found to be devoid of any hepatotoxicity in the animals.

Ethanol induced gastric ulcers have been widely used to assess gastro protective activity. Ethanol has been reported to cause ulceration by breakdown of mucosal barrier, alterations in the mucosal permeability, gastric mucus depletion and free radical generation. The necrosis and the haemorrhagic aspect of the tissue injury may be attributed to the stasis in the gastric blood flow (Mitra *et al.,* 2010).

Gastric ulcers induced by cold-restraint stress or swimming stress ulcer models are widely used for assessing the healing effects of compounds in ulcers. Stress is reported to inactivate mucosal prostaglandin synthesis by accumulating hydrogen peroxide in addition to reactive oxygen species (ROS) generation (Izzo *et al.,* 1994). The ulcers are reported to be produced due to the release of histamine, leading to an increase in acid secretion, a reduction in mucus production (Borrelli and Izzo, 2000), pancreatic juice reflux and poor flow of gastric blood (Asuzu and Onu, 1990).

Reduction in the ulcer lesions, ulcer score, ulcer index and gastric volume whereas, an increase in the pH of the gastric content in the groups pre-treated with *C.indicum* in the ulcerogenic models in the present study signifies an ulceroprotective effect of the extract in ulcers of different etiology.

Preventive antioxidants, both enzymatic and non-enzymatic such as superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH) are the first line of defence against reactive oxygen species. Previous reports have shown that the administration of ethanol and NSAIDs decreased the levels of SOD, CAT and GSH in tissues (Goel *et al.,* 2001). A significant increase in the levels of SOD, GSH and CAT was noted on pre-treatment with the extract in all the studied models as compared to the control animals. This could be suggestive of a decreased oxidative stress and an increase in cellular antioxidant defence in the gastric mucosa.

Liver cell contain several enzymes which may be released into

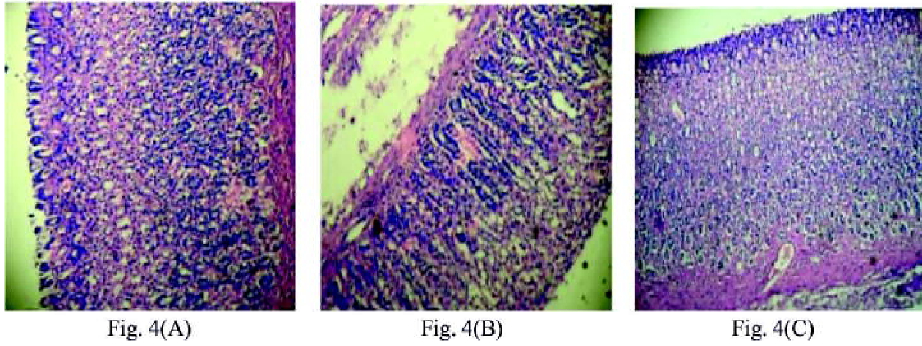
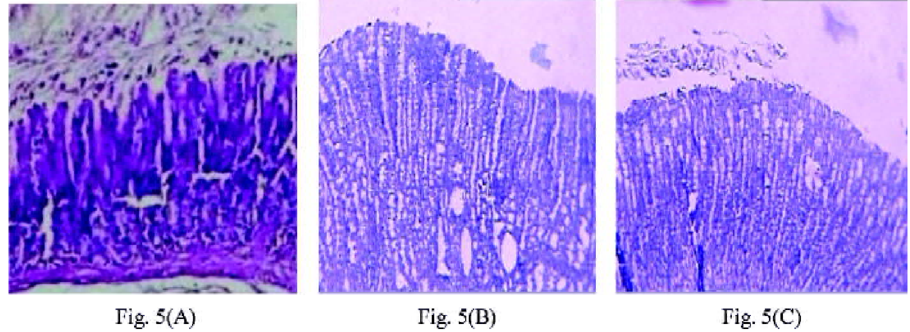


Fig 4
Histopathological changes in stomach of rats in HCl-ethanol induced ulcer model (H&E stain, 10X). (A)Control group showing sloughing, necrosis and reduction in the height of the villi;(B) Standard drug (omeprazole) treated group showing near normal mucosal surface;(C) *C.indicum* treated (200 mg/kg) group showing mild necrosis and erosion.

Fig 5
Histopathological changes in stomach of rats in indomethacin induced ulcer model (H&E stain, 10X). (A) Control group showing necrosis and congestion in sub mucosa and lamia propia;(B) standard drug (omeprazole) treated group showing superficial necrosis;(C) *C.indicum* treated (200 mg/kg) group showing mild necrosis and erosion.



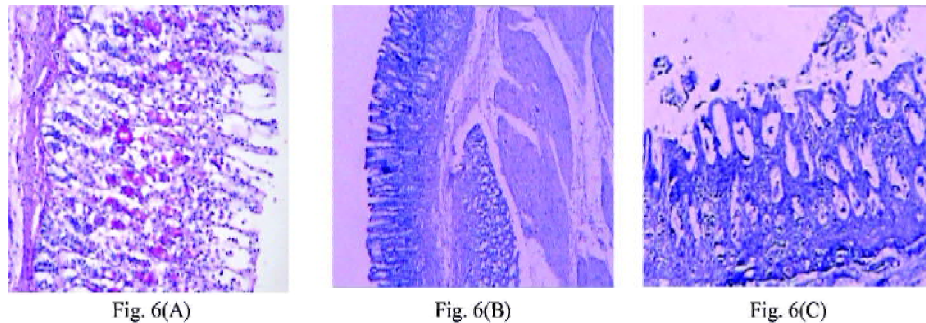
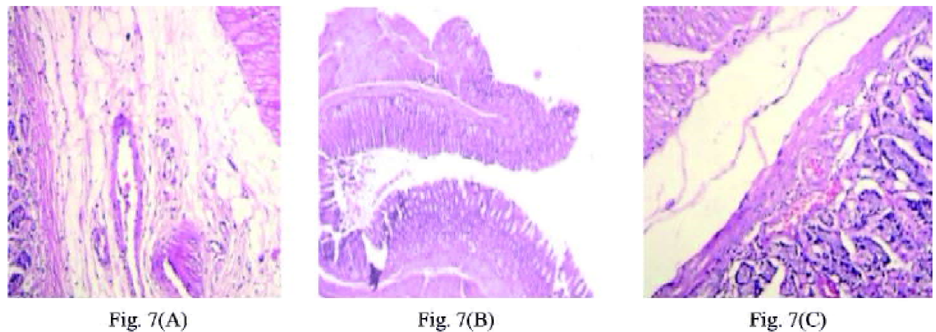


Fig 6
Histopathological changes in stomach of rats in swimming stress ulcer model (H&E stain, 10X) (A)Control group showing sloughing, necrosis and reduction in the height of the villi;(B) standard drug (omeprazole) treated group normal arrangement of gastric mucosal cells;(C) *C.indicum* (200 mg/kg) treated group showing very less sloughing.

Fig 7
Histopathological changes in stomach of rats in cold restraint stress ulcer model (H&E stain, 10X). (A) Control group showing massive necrosis and sloughing of gastric mucosa ;(B) near normalization of the mucosal cells; (C) *C.indicum* (200 mg/kg) treated group showing small erosions and superficial sloughing.



the blood circulation during hepatic damage or injury from different types of diseases or conditions resulting in increase in serum concentrations (Cella and Watson, 1991). Measurement of such selected marker enzymes in serum can be used as sensitive markers of cellular integrity and cellular toxicity induced by pathological conditions and is often used to assess the liver function. The administration of *C. indicum* significantly prevented the elevation of AST, ALT and ALP in the serum of the extract treated animals as compared to the ulcer induced control animals. The decreased serum level of the hepatic enzymes emphasizes the organ protective role of the extract. Presence of tannins in the extract can be presumed to offer a cytoprotective role as tannins have been widely reported to be associated with anti-ulcer activity (Martin *et al.*,1993). A low concentration of tannins

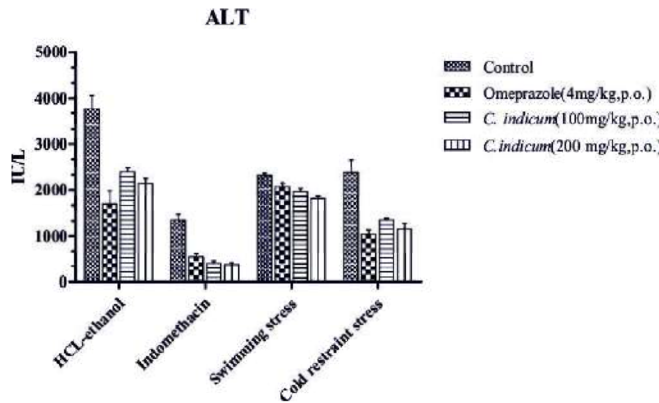


Fig 1
Effect of *C.indicum* on serum ALT in HCl-ethanol induced ulcer model, indomethacin induced ulcer, swimming stress ulcer and cold restraint stress ulcer model. (Mean ± SEM, n=6) *P* *<0.05 significant from control.

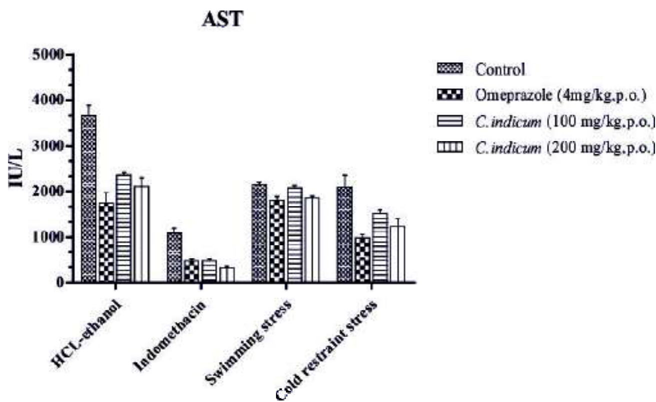


Fig. 2
Effect of *C.indicum* on serum AST in HCl-ethanol induced ulcer model, indomethacin induced ulcer, swimming stress ulcer and cold restraint stress ulcer model (Mean ± SEM, n=6) *P* *<0.05 significant from control.

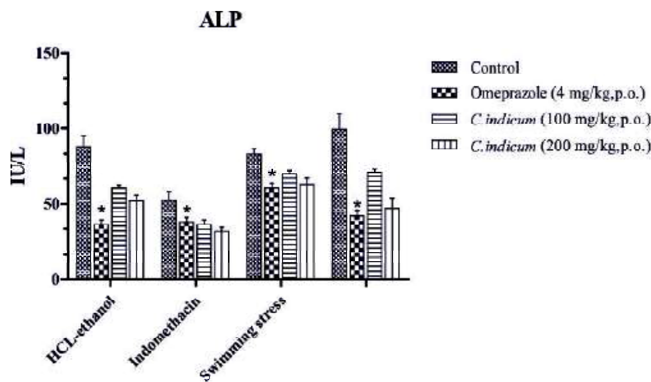


Fig 3
Effect of *C.indicum* on serum ALP in HCl-ethanol induced ulcer model, indomethacin induced ulcer, swimming stress ulcer and cold restraint stress ulcer model (Mean±SEM, n=6), *P* *<0.05 significant from control.

renders the mucosal layer less permeable and affording an increased protection against the action of bacteria, chemical irritation, and to a certain extent, against mechanical irritation (Di Carlo *et al.*,1994).

Therefore, on the basis of the above findings, it can be concluded that the ethanol extract of *C. indicum* has significant antiulcerogenic activity as evidenced by functional, biochemical and histopathological parameters. Omeprazole, a pure compound, a proton pump inhibitor, used as a standard drug in the study exhibited better results in all the gastric ulceration models in comparison to the test extract.

ACKNOWLEDGEMENT

The authors are grateful to the Department of Biotechnology (DBT), New Delhi for financial help and the Director of Research (Vety), College of Veterinary Sciences, Khanapara for providing the necessary facilities.

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Received on : 12.09.2017
Accepted on : 10.10.2017

PROTECTIVE ROLE OF β-TOCOPHEROL AGAINST FLUBENDIAMIDE AND COPPER-INDUCED CHRONIC TOXICITY IN WISTAR RATS

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ABSTRACT

The objective of the present study was to evaluate the ameliorative effect of β-tocopherol (100 mg/kg) against copper (33 mg/kg) and/or flubendiamide (200 mg/kg)-induced toxicity in Wistar rats following 90 days oral exposure. Fifty four male rats of 130-150 g were divided into nine groups of six animals each. Groups I (negative control), II (vehicle control-corn oil) and III (β-tocopherol) were control groups, while animals of groups IV to IX treatment groups received copper sulphate, flubendiamide, flubendiamide + copper sulphate, copper sulphate + β-tocopherol, flubendiamide + β-tocopherol and flubendiamide + copper sulphate + β-tocopherol, respectively. Body weight and per cent weight gain in rats of copper and flubendiamide alone and in combined exposed groups were markedly lower. Significant reduction in Hb, PCV, WBCs count and granulocytes count was observed in flubendiamide and copper + flubendiamide intoxicated groups while lymphocytes % was significantly increased in rats of flubendiamide alone treated group. RBCs, MCV, MCH, MCHC, LY%, MO%, GR% and PCT values did not differ significantly in any of the xenobiotics treatment groups except significant increase in RDWC and decrease in PLT count, respectively, in copper or flubendiamide or copper + flubendiamide-intoxicated rats. Lungs of xenobiotics exposed group showing alveolar emphysema, rupture of alveolar septa and exudation of serous fluid in bronchial lumen. Co-treatment with β-tocopherol + xenobiotics indicating improvement in the feed intake, body weight and per cent weight gain, values of Hb, RBCs count, RDWC, WBC, LY%, MO%, GR%, absolute monocytes count and platelet count were found to be almost comparable to those in control groups; and reverse the degenerative changes in lung; thus suggesting β-tocopherol hold promising potential to prevent toxic effects of these environmental pollutants on human and animal health.

Key words: Flubendiamide, copper sulphate, haemopoietic, toxicity, Wistar rats.

INTRODUCTION

Flubendiamide (3-iodo-*N*ⁱ-(2-mesyl-1,1-dimethylethyl)-*N*-{4-[1,2,2,2-tetrafl uoro-1-(trifl uoromethyl)ethyl]-*o*-tolyl}- phthalamide) is a novel class insecticide, provide favourable and selective toxicological profile (Ebbinghaus-Kintscher *et al.,* 2005) due to its specific action through activation of insects (lepidopterist) ryanodine-sensitive intracellular receptors (Nauen, 2006). It does not bind to mammalian ryanodine receptors type1, 2 and 3. Due to low acute oral LD_{50} (>2000 mg/kg) of flubendiamide makes widely acceptance in uses to control infestation of insects on fruits, vegetables, nuts, corn and rice etc. Flubendiamide has been reported to toxic to liver, thyroid, haemopoietic system (JMPR, 2010) and testis (Mandil *et al.,* 2016). Copper (Cu) is an essential trace element of the transition series namely zinc, manganese, cobalt, iron and chromium. About 40 to 60% of ingested copper is absorbed across from the upper small intestine. As it is required for functioning of several metalloenzymes involved in normal functioning of various system of body. Copper sulphate pentahydrate is water soluble salt of copper and widely used as pesticides, fungicides and nutritional suppliment (Gamakaranage *et al.,* 2011). Animals and human beings mainly exposed to copper through water, feed, inhalation (copper dusts and fumes) and dermal (USEPA, 1986). Accidental exposure of copper to field workers through skin and self-harm by oral ingestion has been reported (Franchitto *et al.,* 2008). Excess copper interfere ability of body to use several vitamins and minerals (Tabrizian, 2002). Signs of acute copper toxicity include salivation, epigastric pain, nausea, vomiting and diarrhoea, Wilson's disease in humans as well as in dogs. Both pesticides and metals including copper tend to produce toxic effects through generation of free radicals and hence, increasing oxidative stress.

β-tocopherol (β-TOH) is a lipid soluble natural forms of vitamin E has free radical chain-breaking antioxidant property. Protective effect of β-tocopherol on pesticides induced adverse effect has been reported by (Durak *et al.,* 2008; Sodhi *et al.,* 2008). There is a significant lack of published report on individual toxicity of flubendiamide as well as its concurrent exposure with copper in animals and human beings. Therefore, present study was planned to investigate toxic effects of flubendiamide and copper alone and in combination on haematology and histopathological of lungs and spleen of Wistar rats following 90 days continuous exposure and its amelioration with β-tocopherol.

MATERIALS AND METHODS

Procurement and maintenance of experimental animals

Male Wistar rats were procured from Laboratory Animal Resource Section, Indian Veterinary Research Institute, Izatnagar and maintained under standard ethical condition in the Departmental Experimental Animal House. Animals had free access to pelleted feed (Ashirwad Industries, Chandigarh) and clean and deionized drinking water. Daily light and dark cycle of 12 h was ensured. Before start of the experiment, an acclimatization period of 15 days was allowed. Animals were kept under constant observation during the entire period of study and were undertaken in study after approval by Institutional Animal Ethics (vide No.79 IAEC/13). The research work was conducted in Department of Veterinary Pharmacology and Toxicology, College of Veterinary Science and A.H., DUVASU, Mathura.

Experimental design

Fifty four male Wistar rats weighing between 130-150 g were divided in nine groups of six animals each. Animals of I (negative control), II (vehicle controls-corn oil) and III (β-tocopherol-@ 100 mg/kg) groups were controls, while animals of other groups namely- IV (copper sulphate-@ 33 mg/kg), V (flubendiamide-@ 200 mg/kg), VI (copper sulphate-@ 33 mg/kg + flubendiamide-@ 200 mg/kg), VII (copper sulphate-@ 33 mg/kg + β-tocopherol-@ 100 mg/kg), VIII (flubendiamide-@ 200 mg/kg + β-tocopherol- @100 mg/kg) and IX (copper sulphate-@ 33 mg/kg + flubendiamide-@ 200 mg/kg + β-tocopherol-@ 100 mg/kg) were treatment groups. Solutions of copper sulphate (Copper sulphate pentahydrate; $CuSO_4.5H_2O$; SDFCL) and flubendiamide (commercial grade; FAME®; Bayer) were prepared by dissolving in deionized water while á-tocopherol (Sigma-Aldrich) was dissolved in corn oil and administered once daily orally continuously 90 days. The selection of doses of flubendiamide and copper sulphate were 1/10th of the values of LD_{50} of flubendiamide (> 2000 mg/kg) and copper sulphate used as a copper salt (> 333 mg/kg) (JMPR, 2010; USEPA,2001).

Toxicity parameters

Rats of all the groups were closely observed during the entire 90 days experimental period for any apparent signs of toxicity including discomfort, gait, loss of hair, diarrhoea or mortality etc. Feed and water intake in rats of different treatment groups were recorded daily and computed on weekly basis during the entire duration of study. Weekly body weights of rats of all the groups were recorded and weekly body weight gain were calculated. At the 91st days after collection of blood, rats were humanely sacrificed by cervical dislocation. Heart, brain, spleen, lungs and adrenals were collected, blotted with tissue paper, and weighed to determine the absolute and relative organ weights.

Hematology and clinical biochemistry

Blood from experimental rats was collected on 91st day in heparinised tubes from inner canthus of eye after overnight fasting for determination of haematological parameters by Abacus Haematology Analyzers (Diatron). Blood samples were centrifuged at 2000 rpm for 15 min. Plasma was separated and transferred into another eppendorf tube for estimation of glucose, cholesterol and HDL-cholesterol by using the commercially available kits (Span Diagnostic Ltd.) with the help of auto-analyser (Erba, Manheim).

Histopathology

A small piece of lungs from each rat of the group was collected and preserved in 10 % formalin saline and washing was done under tape water then after alcoholization in increasing concentration, were embedded into paraffin as per standard technique. Tissue sections of 6µm thickness were cut using microtome and stained with haematoxylin-eosin. Slides were examined under light microscope to observe the histoarchitectural changes in lungs, if any.

Statistical analysis

Statistical analysis for various parameters was done by using one-way ANOVA followed by Tukey's multiple post hoc test with help of SPSS® 16 software. Comparisons were made among the treatment groups. Data are presented as mean ± SE and significant difference was considered at $P<0.05$.

RESULT AND DISCUSSION

Toxicity parameters

Chronic exposure of rats to copper and flubendiamide alone and both these in combination over a period of 90 days did not produce any apparent change in behaviour of animals or produce any clinical signs of toxicity. No loss of hair or treatment-related mortality was observed during the entire experiment period. No toxicity symptoms of copper and flubendiamide in the present study are in agreement with the findings of Scheinberg and Sternlieb (1994) and Enomoto (2003), respectively. Rats of all the treatment groups exhibited normal activities and behaviour throughout the period of study. Feed intake in copper sulphate alone exposed group was significant reduced ($P<0.05$) during 9th and 11th weeks as compared flubendiamide alone and in combined exposed groups. However, Feed intake in flubendiamide alone and flubendiamide + copper sulphate exposed groups were significantly ($P<0.05$) higher. Co-treatment with β-tocopherol and copper sulphate, significant ($P<0.05$) increase in feed and water intake was observed during 8th to 12th weeks. Similarly, co-treatment of β-tocopherol in flubendiamide alone and flubendiamide + copper sulphate improved feed and water intake during the entire period of 13 weeks (Table 2 & 3). Body weight of rats of different

treatment groups did not differ significantly from each other except in flubendiamide + copper sulphate exposed rats on 13th week in which, compared to control group, marked decrease in body weight was observed. However, body weight of β-tocopherol alone treated rats was significantly (P<0.05) higher compared to combined exposed group (flubendiamide + copper sulphate) and copper sulphate + β-tocopherol treated groups at the end of exposure period. Final body weight and percent gain in body weight of rats on 91st day was less in copper sulphate (60.52%) and flubendiamide (69.85 %) alone and in combined exposed groups (58.44 %) as well as β-tocopherol + xenobiotics-treated groups i.e. copper sulphate + β-tocopherol (58.76 %) and copper sulphate + flubendiamide + β-tocopherol (49.10 %) as compared to control group (94.57 %) and flubendiamide + β-tocopherol (88.30 %) groups (Table 1). Decrease feed intake in copper sulphate exposed groups which is due to anorectic and gastrointestinal irritant property of copper sulphate or due to direct toxicity or stressogenic activity of toxicants in gastrointestinal tract as a result diminished appetite and absorption (Bhelonde and Gosh, 2004) it lead to decrease in body weight and per cent weight gain in copper-intoxicated groups was agreement with findings of Bataineh *et al.* (1998). Thus decrease in body weight is a predictor of poor general health and excessive breakdown of tissue proteins (Mladenoviæ *et al.,* 2014).

No significant alterations were observed in absolute and relative organs weights in different treatment groups except significant increase in relative weight of lungs and slight increase in brain weight in copper alone intoxicated group is in accordance with Ashish *et al.* (2013) and Kumar *et al.* (2015). Similarly in combined exposed group brain weight was significantly higher but relative liver weight was slight increased and it is agreed with observation of Barceloux, (1999) and JMPR (2010) report. Increase in brain and liver weights may be due to cumulative effect of copper in brain and flubendiamide-induced hepatocellular necrosis and hepatocytes hypertrophy. Boveris *et al.* (2012) and Musacco-Sebio *et al.* (2014) suggested that copper-induced damage to brain may be mediated by formation of hydroxyl radical with subsequent oxidative damage. Relative weight of brain in rats of copper sulphate + flubendiamide was found to be normal following treatment with β-tocopherol + copper sulphate + flubendiamide (Table 4 and 5).

Haematology and clinical chemistry

Result on haematological examination revealed nonsignificant decrease in total erythrocytes count (RBCs) in copper sulphate and flubendiamide alone and combined exposed groups (Table 6) and this observation is in agreement with Ranjan *et al.* (2014) who reported marginal alterations in haematological parameters in buffalo calves following flubendiamide alone and combined exposure with

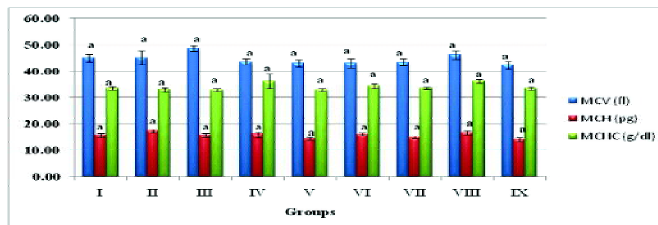

Fig. 1
Effect of oral administration of β-tocopherol (100mg/kg) on erythrocytes indices MCV, MCH and MCH of rats of different treatment groups following 90 days oral exposure to copper (33 mg/kg) and flubendiamide (200 mg/kg) alone and both in combination (copper 33 mg/kg + flubendiamide 200 mg/kg). Different superscripts in histograms show significant (P<0.05) differences. Different superscripts in histograms show significant (P<0.05) differences

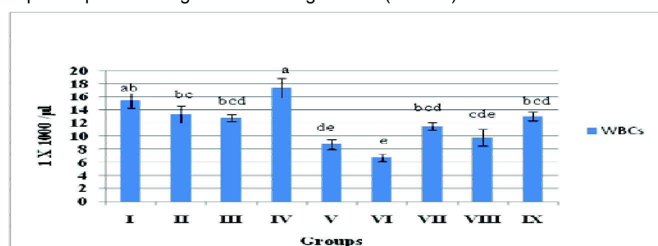

Fig. 2
Effect of oral administration of β-tocopherol (100 mg/kg)on absolute leucocytes counts of rats of different treatment groups following 90 days oral exposure to copper (33 mg/kg) and flubendiamide (200 mg/kg) alone and both in combination (copper 33 mg/kg + flubendiamide 200 mg/kg). Different superscripts in histograms show significant (P<0.05) differences. Different superscripts in histograms show significant (P<0.05) differences

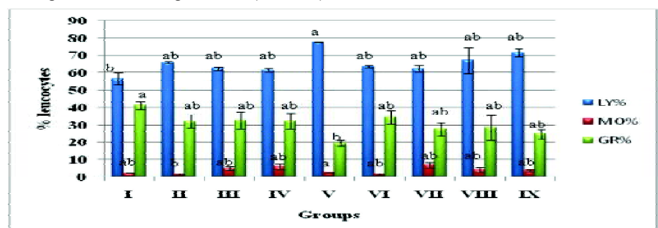

Fig. 3
Effect of oral administration of β-tocopherol (100 mg/kg) on lymphocytes, monocytes and granulocytes percentage of rats of different treatment groups following 90 days oral exposure to copper (33 mg/kg) and flubendiamide (200 mg/kg) alone and both in combination (copper 33 mg/kg + flubendiamide 200 mg/kg). Different superscripts in histograms show significant (P<0.05) differences. Different superscripts in histograms show significant (P<0.05) differences

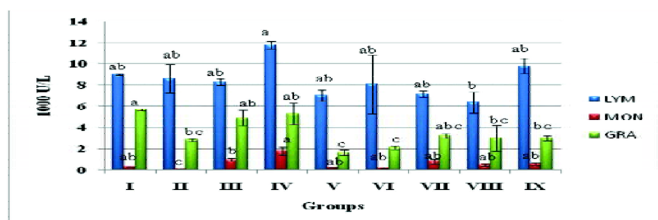

Fig. 4
Effect of oral administration of β-tocopherol (100 mg/kg)on absolute lymphocytes, monocytes and granulocytes counts of rats of different treatment groups following 90 days oral exposure to copper (33 mg/kg) and flubendiamide (200 mg/kg) alone and both in combination (copper 33 mg/kg + flubendiamide 200 mg/kg). Different superscripts in histograms show significant (P<0.05) differences. Different superscripts in histograms show significant (P<0.05) differences

lead. Flubendiamide alone and combined intoxicated

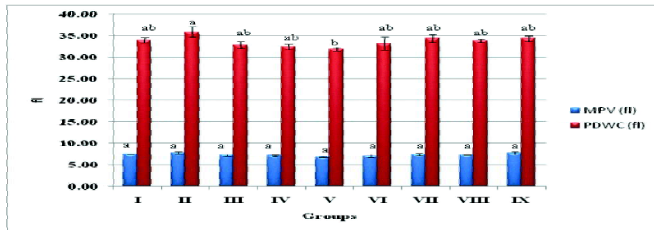

Fig. 5
Effect of oral administration of β-tocopherol (100mg/kg) on MPV and PDWC of rats of different treatment groups following 90 days oral exposure to copper (33 mg/kg) and flubendiamide (200 mg/kg) alone and both in combination (copper 33 mg/kg + flubendiamide 200 mg/kg)
Different superscripts in histograms show significant (P<0.05) differences

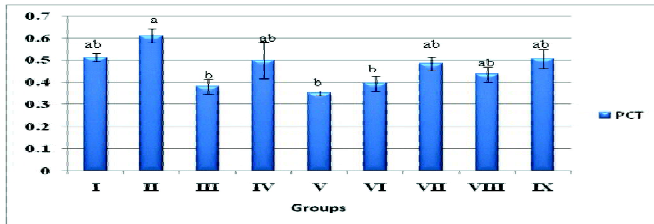

Fig. 6
Effect of oral administration of β-tocopherol (100mg/kg) on PCT of rats of different treatment groups following 90 days oral exposure to copper (33 mg/kg) and flubendiamide (200 mg/kg) alone and both in combination (copper 33 mg/kg + flubendiamide 200 mg/kg). Different superscripts in histograms show significant (P<0.05) differences

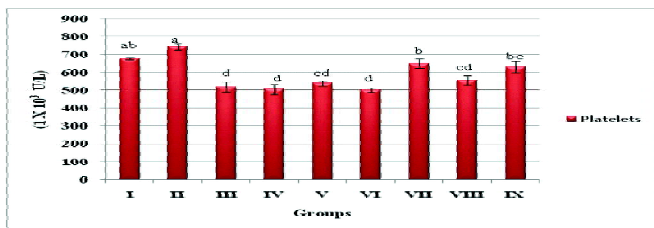

Fig. 7
Effect of oral administration of β-tocopherol (100mg/kg) on platelets of rats of different treatment groups following 90 days oral exposure to copper (33 mg/kg) and flubendiamide (200 mg/kg) alone and both in combination (copper 33 mg/kg + flubendiamide 200 mg/kg) . Different superscripts in histograms show significant (P<0.05) differences

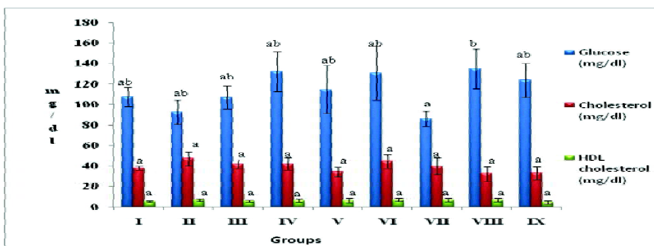

Fig. 8
Effect of oral administration of β-tocopherol (100 mg/kg) on blood glucose, cholesterol and HDL-cholesterol in rats of different treatment groups following 90 days oral exposure to copper (33 mg/kg) and flubendiamide (200 mg/kg) alone and both in combination (copper 33 mg/kg + flubendiamide 200 mg/kg). Different superscripts in histograms show significant (P<0.05) differences.

groups also showing significant (P<0.05) reduction in haemoglobin (Hb) and PCV values, respectively, are similar to those in observed in JMPR (2010) report. However, Ranjan *et al.* (2014) observed insignificant decrease in Hb values in flubendiamide exposed rats and buffalo calves. Reduction in Hb values is due to increased breakdown of red blood cells and /or reduction in rate of formation of RBCs (Mossa, 2004) and decreased ability of kidney to secrete erythropoietin as erythropoietin stimulates bone marrow to produce red blood cells. Young and Maciejewski (1997) suggested that xenobiotics showing effect on RBCs and Hb have adverse effects on bone marrow, kidneys and haemoglobin metabolism. Red cells distribution width (RDWC) was significantly higher in copper, flubendiamide and copper + flubendiamide exposed groups. Increase in RDWC reflects heterogeneity in the size of circulating red blood cells (anisocytosis), microcytic (caused by iron deficiency) anemia, modify the shape of red blood cells due to the premature release of immature cells into the bloodstream (severe blood loss), abnormal hemoglobin, hemolysis or hemolytic anemias (Evans and Jehle, 1991; Perkins, 2003) or chronic heart failure, as well as fatal and nonfatal cardiovascular disease events (O'Leary *et al.,* 1999). Increase RDWC is observed in chronic lower respiratory tract infection and lungs cancer associated mortality in human beings (Patel *et al.,* 2010; Koma *et al.,* 2013). In the present study, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) values did no differ significantly in xenobiotics treatment groups (Fig. 1) and this observation is in agreement with the findings of Akomolafa *et al.* (2014) and Ranjan *et al.* (2014) following exposure to copper (100 mg/kg) in rats and flubendiamide and lead acetate to buffalo calves. Direct haemotoxic effect of pesticides manifested as reduction in total RBCs, PCV and Hb concentration has been reported by several workers (Khalaf-Allah, 1999; Mossa, 2004; Mossa and Abbassy, 2012).

Total leucocytes count (WBCs) in flubendiamide alone and in combination with copper exposed groups was significantly (P<0.05) lower, while in copper alone-exposed group it was exceptionally and significantly (P<0.05) higher as compared to control groups. This finding was in accordance with JMPR (2010) report and observations of Vemu and Dumuka (2015) who have reported similar alterations in WBC in male rats following 28 days exposure of flubendiamide to 250 mg/kg. White blood cells play important role in defense against invading microbes and any alteration in their number would result in degradation of immune response. Co-treated with β-tocopherol in copper and flubendiamide alone and both together groups were found to be almost comparable to those of control groups (Fig. 2). Percentage of lymphocytes (LY%), monocytes (MO%) and granulocytes (GR%) and absolute counts of lymphocytes and granulocytes did not differ significantly in copper sulphate exposed group except absolute monocytes count significantly increase which is agreed with observations of Akomolafa *et al.* (2014) who reported appreciable but insignificant increase in absolute

WBCs count in copper sulphate (200 mg/kg) exposed rats to two weeks. Flubendiamide alone group showing significant increase in lymphocytes percentage and decrease in granulocytes percentage and absolute granulocytes count is in agreement with the report of JMPR (2010) indicates potential of flubendiamide to induce leucopoenia and thrombocytopenia. Flubendiamide alone and combined with copper exposed groups were showing significant ($P<0.05$) decrease in granulocytes count as compared to control group (Fig. 3 and 4). Lymphocytosis is the most common condition in which leucopoenia and neutropenia are observed (Benjamin, 2001).

In the present study, mean platelet volume (MPV), plateletcrit (PCT), platelet distribution width (PCWC) values did not differ significantly in any of the xenobiotics treatment

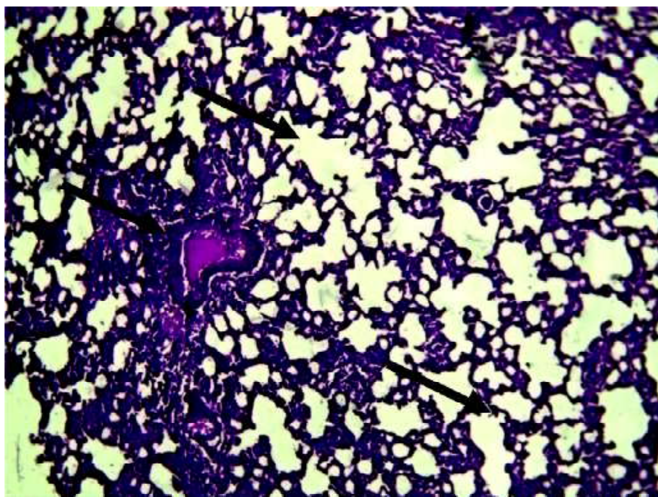

Fig. 9
Lung section of copper sulphate (33 mg/kg)-treated group (IV) showing rupture of alveolar septa, emphysema and exudation of serous fluid in bronchial lumen. (10 X H&E stain).

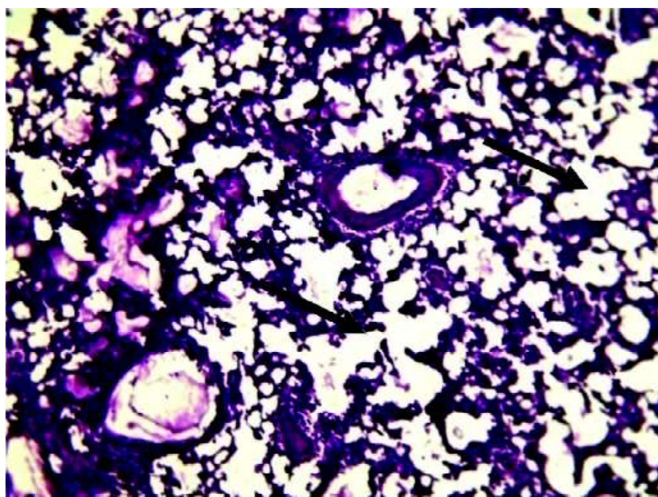

Fig. 10
Lungs section of flubendiamide (200 mg/kg)-exposed group (V) showing ruptured alveolar septa suggestive of emphysematous changes (10 X H&E).

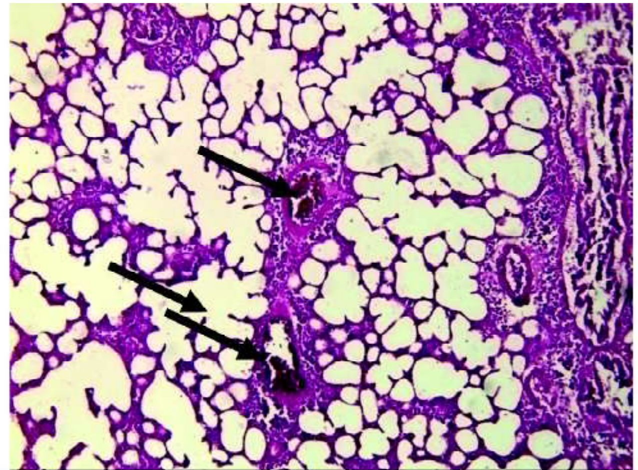

Fig. 11
Lung of flubendiamide (200 mg/kg) + copper sulphate (33 mg/kg) exposed group (VI) showing ruptured alveolar septa, alveolar emphysema with congestion. (10X H&E stain)

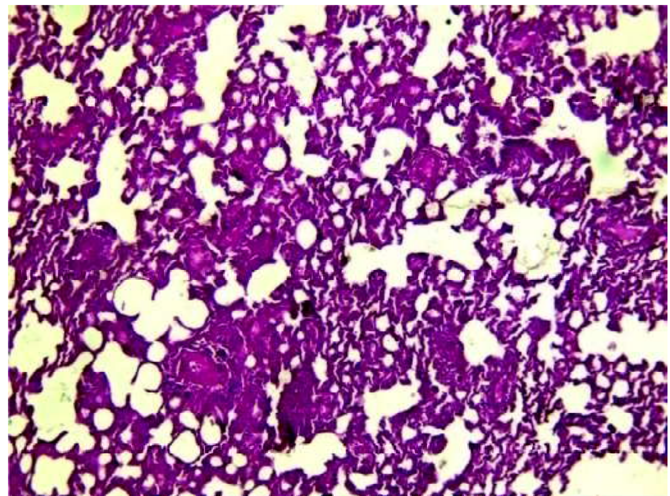

Fig. 12
Lung of rats treated with β-tocopherol (100 mg/kg) + copper sulphate (33 mg/kg) group (VII) revealed reparative changes and few areas of mild exudation and alveolar rupture. (10X H&E stain).

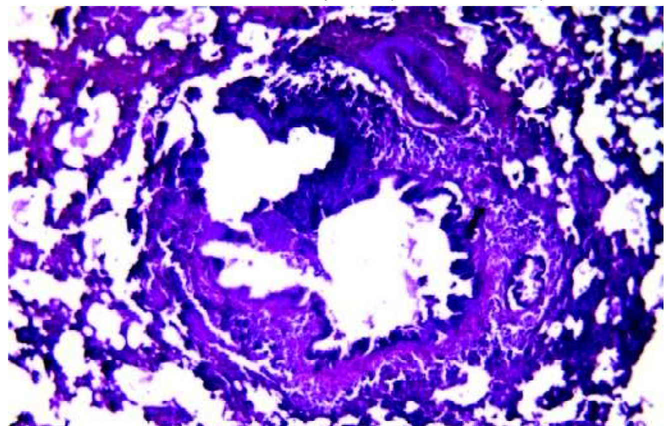

Fig. 13
Lung of β-tocopherol (100 mg/kg) + flubendiamide (200 mg/kg)-treated group showing apparently healthy bronchiolar epithelium and alveoli with few areas of emphysema (10 X H&E satin).

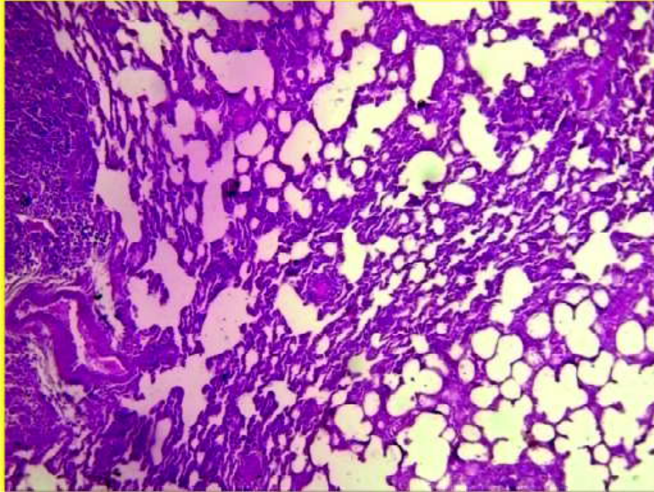

Fig. 14
Lung of concurrent treatment of β-tocopherol (100 mg/kg) + flubendiamide (200 mg/kg) + copper sulphate (33 mg/kg) showing reparative changes and few areas mild exudation and alveolar rupture (10 X H&E satin).

groups. However, significant (P<0.05) decrease in platelets count in rats of copper-treated group was contrary to the observation of Akomolafa *et al.* (2014) who reported insignificant alteration in platelet count following 14 days of exposure to copper sulphate (100 and 200 mg/kg). PLT count in flubendiamide alone (V) and in concurrent exposure group with copper (VI) showed significant decrease (P<0.05) and it is in agreement with data of Vemu and Dumuka (2015) who have reported similar findings of PLT alterations in male rats following 28 days exposure to flubendiamide at 250 mg/kg. Platelets play an important role in blood coagulation mechanism and any defect in the structure, number and membrane stability may lead to bleeding or thrombic disorders. In addition platelets play important role in assessing the risk of cardiovascular health using parameters like platelet distribution width (PDW-for thrombocytsis) and mean platelet volume (MPV-for overall vascular mortality, ischemic heart diseases, myocardial infarction and unstable angina etc) (Yaghoubi *et al.,* 2013). Increased PLT counts in rats simultaneously co-treated with á-tocopherol and xenobiotics was found to be comparable with control groups (Fig. 5-7).

Increase in blood glucose level was insignificant (22.84%) in copper sulphate group is in agreement with observations of Kumar and Sharma (1987) in rats exposed to sub-lethal concentration of copper sulphate. Increase in blood glucose in flubendiamide intoxicated groups is in agreement with public release summary on flubendiamide (JMPR, 2010) in which 10% increase in blood glucose level has been reported in flubendiamide exposed rats. Our results further reveal that flubendiamide has almost insignificant effect on blood glucose while copper has moderate to marked potential to cause hyperglycaemic effect. Copper and flubendiamide-induced hyperglycaemic effect is due to stress and resultant increase in glucocorticoids and catecholamines secretion which

Table 1
Effect of oral administration of β-tocopherol (100mg/kg) on body weight of rats of different treatment groups following 90 days oral exposure to copper (33 mg/kg) and flubendiamide (200 mg/kg)alone and both in combination (copper 33 mg/kg + flubendiamide200 mg/kg)

Groups	Treatment	Weekly body weights (g)													
		0 day weight(g)	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII
I	Control	131.83± 10.76[a]	124.83± 13.54[a]	130.50± 13.96[a]	145.00± 10.27[a]	170.67± 18.40[a]	185.33± 15.86[a]	201.50± 17.78[a]	211.83± 18.05[a]	214.67± 21.20[a]	210.33± 23.69[a]	241.50± 18.73 [a]	246.33± 17.10[a]	248.83± 16.38[ab]	256.50± 14.67[ab] (94.57)%
II	Vehicle control (Corn oil)	138.00± 5.27[a]	125.17± 8.59[a]	131.33± 10.69[a]	142.83± 12.67[a]	163.00± 15.50[a]	190.17± 15.35[a]	199.00± 15.43[a]	209.17± 17.93[a]	213.50± 17.81[a]	223.83± 15.39[a]	232.83± 18.03[a]	232.67± 20.0[a] (74.88%)	237.83 ± 21.42[ab]	241.33 ± 20.64[ab]
III	±-tocopherol (100 mg/kg)	137.00± 11.66[a]	132.83± 14.87[a]	118± 8.87a	137.67± 10.60[a]	166.83± 13.01[a]	197± 14.35[a]	210± 13.86[a]	225.67± 12.68[a]	233.83± 11.72[a]	240.33± 10.47[a]	250.17± 14.65[a]	258± 13.37[a]	263.5± 16.01[a]	274.5± 18.25[a] (100.36%)
IV	Copper sulphate (33 mg/kg)	141.00± 3.38[a]	136.83± 8.28[a]	133.50± 13.25[a]	154.50± 14.42[a]	159.50± 17.06[a]	180.00± 16.14[a]	185.67± 12.24[a]	198.67± 15.71[a]	201.67± 14.06[a]	212.67± 13.18[a]	213.00± 14.88[a]	216.33± 15.41[a]	225.50± 15.95[ab]	226.33± 11.81[ab] (60.52)%
V	Flubendiamide (200 mg/kg)	137.67± 10.02[a]	125.00± 9.95[a]	119.50± 7.48[a]	120.33± 6.35[a]	147.00± 6.48[a]	174.67± 5.83[a]	195.17± 6.75[a]	207.00± 14.07[a]	214.33± 11.32[a]	213.67± 13.11[a]	222.33± 11.66[a]	218.50± 6.85[a]	234.33± 7.09[ab]	233.83± 4.28[ab] (69.85%)
VI	Copper sulphate (33 mg/kg) + Flubendiamide (200 mg/kg)	128.33± 7.20[a]	118.00± 10.62[a]	114.67± 7.28[a]	110.00± 12.14[a]	127.00± 11.32[a]	142.33± 12.22[a]	168.33± 12.76[a]	169.00± 9.20[a]	191.67± 13.15[a]	189.00± 7.88[a]	201.00± 7.32[a]	194.00± 7.96[a]	195.33± 4.97[b]	203.33± 7.98[b] (58.44%)
VII	Copper sulphate (33 mg/kg) + ±- tocopherol (100 mg/kg)	133.33± 6.74[a]	137.33± 7.18[a]	146.67± 11.81[a]	147.67±. 12.25[a]	166.33± 11.30[a]	184.67± 18.66[a]	200.67±. 16.42[a]	193.67± 10.99[a]	198.33± 8.32[a]	208.67± 17.52[a]	200.67± 7.86[a]	203.67± 10.29[a] (58.75%)	218.50± 12.85[ab]	211.67± 11.57[b]
VIII	Flubendiamide (200 mg/kg) + ±-tocopherol (100 mg/kg)	131.00± 11.41[a]	134.33± 11.97[a]	124.33± 6.54[a]	140.50± 8.84	173.33± 10.30[a]	195.33± 9.67[a]	196.00± 8.87[a]	212.67± 10.68[a]	217.00± 8.21[a]	230.33± 8.83[a]	232.67± 10.35[a]	235.67± 18.39[a] (88.29%)	243.17± 13.5	246.67± 10.69[ab]
IX	Copper sulphate (33 mg/kg) + Flubendiamide (200 mg/kg)) + ±-tocopherol (100 mg/kg)	148.67± 14.31[a]	130.33± 8.51[a]	127.33± 12.41[a]	133.67± 14.25a	159.00± 13.72[a]	169.00± 15.60[a]	185.33± 12.37[a]	195.33± 9.39[a]	205.33± 10.60[a]	202.67± 10.81[a]	210.33± 11.56[a]	214.00± 9.31[a]	223.33± 12.37[ab]	221.67± 9.44[ab] (49.10%)

Values(mean ± SEM; n=6) bearing different superscripts in the same column differed significantly (P<0.05). Figures in parentheses indicate per cent increase in body weight.

Table2:
Effect of oral administration of β-tocopherol (100mg/kg) on weekly feed intake in rats of different treatment groups following 90 days oral exposure to copper (33 mg/kg) and flubendiamide (200 mg/kg) alone and both in combination (copper 33 mg/kg + flubendiamide 200 mg/kg)

Groups		Weekly body weights (g)												
	Treatment	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII
I	Control	205.0± 13.04^{ab}	167.2± 33.54^{ab}	100.7±7.51^{a}	126.4±3.89^{a}	117.14± 5.2^{ab}	132.1± 2.6^{a}	138.5±2.6^{ab}	142.1± 1.01^{b}	139.29 ± 0.71^{a}	141.43 ± 2.36^{ab}	142.14 ± 1.48^{a}	128.57 ± 4.59^{b}	135.00 ± 2.97^{a}
II	Vehicle control (Corn oil)	223.57± 15.22^{ab}	200.29± 33.54^{ab}	93.57 ± 4.96^{a}	125.00 ± 1.89^{a}	112.14 ± 8.98^{ab}	134.29 ± 2.02^{a}	140.00 ± 2.44^{ab}	140.71 ± 2.02^{b}	142.14 ± 1.84^{a}	141.43 ± 1.42^{ab}	142.14 ± 1.01^{a}	140.00 ± 1.89^{a}	128.57± 8.28^{a}
III	β-tocopherol (100 mg/kg)	218.57± 11.49^{ab}	219.57 ± 33.54^{ab}	90.71 ± 9.02^{a}	115.00 ± 7.94^{ab}	122.14 ± 4.98^{ab}	137.14 ±1.48^{a}	134.29 ± 3.16^{a}	142.14 ± 1.48^{b}	141.43± 2.36^{a}	146.43 ± 1.79^{abc}	146.43 ±1.79^{a}	144.29 ± 2.97^{a}	143.57 ± 2.82^{a}
IV	Copper sulphate (33 mg/kg)	167.14± 12.09^{b}	204.29±36.91^{ab}	95.00 ±10.17^{a}	125.71± 1.70^{a}	121.43 ± 6.04^{ab}	133.57 ± 3.89^{a}	141.43 ± 0.92^{ab}	141.43 ± 6.04^{b}	127.86 ± 7.05^{c}	130.71 ±8.55^{a}	131.43 ± 2.36^{b}	129.29 ± 4.14^{b}	126.67 ± 6.61^{a}
V	Flubendiamide (200 mg/kg)	210.71 ± 16.19^{ab}	248.14±16.18^{ab}	113.57 ±14.86^{a}	116.43 ±6.24^{ab}	126.43 ±3.57^{ab}	136.43 ± 1.42^{a}	140.71± 2.02^{ab}	160.00 ± 3.08^{a}	165.71 ± 1.70^{b}	161.43 ± 1.79^{cd}	150.00 ± 1.54^{a}	144.29 ± 1.70^{a}	139.17 ± 3.52^{a}
VI	Copper sulphate (33 mg/kg) + Flubendiamide (200 mg/kg)	200.71± 15.29^{ab}	255.00± 38.44^{a}	104.29 ± 8.62^{a}	115.00 ±2.88^{b}	118.57 ± 4.04^{ab}	137.86 ± 1.84^{a}	142.86 ± 1.48^{ab}	160.00 ± 3.08^{a}	158.57 ± 1.79^{b}	166.43 ± 1.79^{d}	142.86 ± 3.05^{a}	145.71± 2.76^{a}	143.57 ± 2.82^{a}
VII	Copper sulphate (33 mg/kg) + β -tocopherol (100 mg/kg)	232.14 ± 2.40^{a}	180.71± 12.88^{ab}	116.43 ± 4.59^{a}	100.00 ±0.0^{ab}	126.43 ± 4.96^{ab}	140.71± 1.30^{a}	150.71± 4.14^{bc}	162.14 ± 2.64^{a}	165.00 ± 1.89^{b}	158.57 ± 5.53^{cd}	144.29 ± 3.16^{a}	147.14 ± 1.84^{a}	147.14 ± 1.84^{a}
VIII	Flubendiamide (200 mg/kg) + β-tocopherol (100 mg/kg)	210.0 ±11.39^{ab}	221.71± 26.46^{ab}	119.29 ± 7.51^{a}	111.43 ±5.60^{ab}	100.00 ± 10.91^{b}	137.14 ± 3.05^{a}	133.57 ± 4.35^{a}	161.43 ± 3.57^{a}	163.57 ±1.42^{b}	165.00 ± 3.93^{d}	150.00 ± 2.44^{a}	146.43 ± 2.02^{a}	145.00 ± 2.44^{a}
IX	Copper sulphate (33 mg/kg) + Flubendiamide (200 mg/kg)) + ±-tocopherol (100 mg/kg)	185.57± 14.60^{ab}	119.00 ± 21.40^{b}	100.00 ± 7.79^{a}	115.71 ± 8.16^{ab}	135.00 ± 2.88^{a}	142.14 ± 1.84^{a}	158.57 ± 4.04^{c}	167.86 ± 3.24^{a}	167.86 ± 1.79^{b}	160.71 ± 1.79 ^{bcd}	151.43 ± 1.70^{a}	147.14 ± 1.48^{a}	132.86 ± 6.44^{a}

Values (mean ± SEM; n=6) bearing different superscripts in the same column differed significantly (P<0.05).

Table 3:
Effect of oral administration of β-tocopherol (100mg/kg) on weekly water intake of rats of different treatment groups following 90 days oral exposure to copper (33 mg/kg) and flubendiamide (200 mg/kg) alone and both in combination (copper 33 mg/kg + flubendiamide 200 mg/kg)

Groups		Weekly body weights (g)												
	Treatment	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII
I	Control	75.14± 7.96a	42.54 ± 4.58ab	45.71 ± 3.19a	43.57± 4.18a	34.29 ± 8.69a	58.57± 3.40ab	58.57±2.60a	52.86±4.20a	55.71 ± 4.80a	63.57 ± 8.50b	70.71 ± 4.14a	72.14 ± 8.59b	102.14 ± 7.22a
II	Vehicle control (Corn oil)	87.43 ± 10.23ab	72.71 ± 6.82b	30.86 ± 7.69ab	42.86 ± 5.65a	27.14 ± 5.21a	50.0 ± 4.36a	56.43 ± 3.89a	60.71 ± 5.16a	62.86 ± 5.96a	82.14 ± 4.34ab	77.14 ± 3.42a	77.14 ± 6.06b	90.71± 4.93a
III	β-tocopherol (100 mg/kg)	93.86 ± 10.44ab	69.43 ± 7.47b	39.29 ± 6.52a	25.71 ± 6.58a	37.14 ± 6.44a	57.14 ±3.75ab	55.71 ± 2.97a	67.86 ± 4.06a	54.29± 4.80a	88.56 ± 1.42a	68.57 ± 4.59a	104.29 ± 8.95ab	102.29 ± 10.71a
IV	Copper sulphate (33 mg/kg)	104.29± 6.94ab	54.86 ± 6.78ab	47.14 ± 4.72ab	36.43± 6.78a	48.57 ± 7.45a	60.0 ± 5.34abc	62.86 ± 4.61ab	62.86 ± 3.05a	47.14 ± 5.21a	73.57 ± 4.72ab	68.57 ± 7.13b	100.0 ± 4.88ab	110.0±6.17a
V	Flubendiamide (200 mg/kg)	108.57± 15.84ab	60.14 ± 6.08ab	35.43±13.13ab	30.86 ±9.16a	27.86 ± 7.22a	67.86 ± 1.84bcd	65.71± 3.16ab	63.57 ± 4.72a	59.29 ± 9.15a	81.43 ± 1.42ab	72.86 ± 4.34a	104.29 ± 11.30ab	94.29 ± 16.49a
VI	Copper sulphate (33 mg/kg) + Flubendiamide (200 mg/kg)	95.0 ± 8.92ab	36.43 ± 7.06a	48.29 ± 5.12ab	24.29 ± 8.62a	22.14 ± 2.85a	70.71 ± 4.42bcd	76.43 ± 1.79b	73.57 ± 2.82a	59.29 ± 8.19a	72.86 ± 6.44ab	71.43 ± 4.59a	91.43± 9.86ab	101.43 ± 11.0a
VII	Copper sulphate (33 mg/kg) + β-tocopherol (100 mg/kg)	147.43 ± 4.51c	107.86± 10.29c	68.57 ± 10.39ab	32.14 ± 6.71a	48.57 ± 10.16a	75.71± 1.70cd	74.29± 2.97b	64.29 ± 7.74a	72.14 ± 3.42a	77.14 ± 2.14ab	74.29 ± 3.16a	105.71 ± 11.51ab	114.29 ± 8.12a
VIII	Flubendiamide (200 mg/kg) + β-tocopherol (100 mg/kg)	105.0 ±10.44ab	66.71± 8.21ab	42.0 ± 6.81ab	28.57 ±6.78a	40.0 ± 6.9a	62.14 ± 4.34abcd	65.0 ± 5.45ab	70.0 ± 5.66a	65.71 ±5.71a	77.86 ± 2.64ab	75.71 ± 1.70a	97.48± 11.48ab	118.00 ± 12.96a
IX	Copper sulphate (33 mg/kg) + Flubendiamide (200 mg/kg)) + ±-tocopherol (100 mg/kg)	125.57±7.92bc	38.29 ± 4.90a	22.86 ± 5.54b	23.57 ± 3.22a	48.57 ± 8.57a	77.14 ± 3.42d	75.71 ± 2.02b	65.71 ± 5.81a	76.43 ± 9.04a	85.71 ± 2.29a	117.14 ± 8.60b	125.14 ± 12.48a	121.43 ± 10.33a

Values (mean ± SEM; n=6) bearing different superscripts in the same column differed significantly (P<0.05).

stimulate glycogenolysis and gluconeogenesis in liver from non carbohydrate sources (Benjamin, 2001; Almeida *et al.,* 2001).

Cholesterol level insignificantly decrease (16.56%) in flubendiamide alone-exposed group (Fig. 8) is in aggrement with JMPR report (2010) and with the other observation in experimental animals following exposure to acephate, dichlorvos, deltamethrin, dimthoate and diazinon (Damodar *et al.,* 2015). Hypocholesterolemia in present study is due to adverse effect on liver of flubendiamide group which is confirmed by histological examination of liver revealed mild fatty changes in the present study (data not shown). Multiple mechanisms influence hypocholesterolemia like down regulation of hepatic synthesis (Giovannini *et al.,* 1999), decreased production of cholesterol (Bakalar *et al.,* 2003), severe chronic hepatic insufficiency (D'Arienzo, 1998) and increased cholesterol catabolism (Giovannini *et al.,* 1999). Hypocholesterolemia might be the cause of anaemia as cholesterol deficiency leads to rigidity of erythrocytes and making them more prone to destruction (Pok and Deutsch, 1980).

Histopathology

Histopathological examination of lungs of groups I, II and III revealed normal histoarchitectural with presence of normal alveolar system, alveolar ducts and saccules. Alveoli were surrounded by dense capillary net and separated from each other by thin alveolar septum. Lungs of copper sulphate exposed group showing alveolar emphysema, rupture of alveolar septa and exudation of serous fluid in bronchial lumen. Lungs of flubendiamide-treated group (V) revealed ruptured alveolar septa with emphysematous changes. Flubendiamide + copper sulphate exposed group lungs

Table 4:
Effect of oral administration of β-tocopherol (100mg/kg) on absolute organ weights of rats of different treatment groups following 90 days oral exposure to copper (33 mg/kg) and flubendiamide (200 mg/kg) alone and both in combination (copper 33 mg/kg + flubendiamide200 mg/kg)

Groups	Treatment	Absolute organ weight (g)						
		Initial body weight (g)	Final body wt (g)	Spleen	Heart	Lungs	Adrenals	Brain
I	Control	131.83± 10.76[a]	256.50 ± 14.67[ab]	0.48 ± 0.02	0.99 ±0.05[ab]	1.47 ± 0.07[ab]	0.04 ± 0.00 [a]	1.49 ± 0.09[a]
II	Vehicle control (Corn oil)	138.00± 5.27[a]	241.33 ± 20.64ab	0.65± 0.06[a]	0.93± 0.03[ab]	1.28± 0.04[b]	0.04± 0.00[a]	1.50± 0.10[a]
III	β-tocopherol (100 mg/kg)	137.00± 11.66[a]	274.50 ± 18.25[b]	0.37± 0.01[a]	1.07± 0.17[a]	1.41± 0.07[ab]	0.05± 0.01[a]	1.33± 0.13[a]
IV	Copper sulphate (33 mg/kg)	141.00± 3.38[a]	226.33 ± 11.81[ab]	0.34± 0.03[a]	0.83± 0.04[ab]	1.54± 0.21[ab]	0.04± 0.04[a]	1.49± 0.08[a]
V	Flubendiamide (200 mg/kg)	137.67± 10.02[a]	234.17 ± 4.32[ab]	0.54± 0.04[a]	0.96 ± 0.08[ab]	1.40 ± 0.04[ab]	0.03 ± 0.00[a]	1.34 ± 0.06[a]
VI	Copper sulphate (33 mg/kg) + Flubendiamide (200 mg/kg)	128.33± 7.20[a]	202.00 ± 8.26[a]	0.35 ± 0.04[a]	0.82± 0.02[ab]	1.13 ± 0.08[b]	0.03 ± 0.00[a]	1.63 ± 0.04[a]
VII	Copper sulphate (33 mg/kg) + β-tocopherol (100 mg/kg)	133.33± 6.74[a]	211.67 ± 11.57[a]	0.35 ± 0.04[a]	0.78± 0.03[b]	1.97± 0.19[a]	0.07± 0.04[a]	1.59 ± 0.04[a]
VIII	Flubendiamide (200 mg/kg) + β-tocopherol (100 mg/kg)	131.00± 11.41[a]	250.00 ± 12.14[ab]	.503 ± 1.02[a]	1.00 ± 0.06[ab]	1.55 ± 0.10[ab]	0.04 ± 0.00[a]	1.67 ± 0.03[a]
IX	Copper sulphate (33 mg/kg) + Flubendiamide (200 mg/kg)) + β-tocopherol (100 mg/kg)	148.67± 14.31[a]	227.67 ± 11.85[ab]	0.37 ± 0.04[a]	0.92 ± 0.05[ab]	1.98 ± 0.17[a]	0.03± 0.00[a]	1.56 ± 0.10[a]

Values (mean ± SEM; n=6) bearing different superscripts in the same column differed significantly (P<0.05).

Table 5:
Effect of oral administration of β-tocopherol (100mg/kg) on relative organ weights of rats of different treatment groups following 90 days oral exposure to copper (33 mg/kg) and flubendiamide (200 mg/kg) alone and both in combination (copper 33 mg/kg + flubendiamide200 mg/kg)

Groups	Treatment	Relative organ weights (g/100 g)						
		Initial body weight (g)	Final body wt (g)	Spleen	Heart	Lungs	Adrenals	Brain
I	Control	131.83±10.76[a]	256.50±14.67[ab]	0.18±0.00[a]	0.38±0.00[a]	0.57±0.02[a]	0.015±0.00[a]	0.58±0.03[ab]
II	Vehicle control (Corn oil)	138.00± 5.27[a]	241.33 ± 20.64ab	0.28±0.04[a]	0.39±0.01[a]	0.54±0.03[a]	0.018±0.00[a]	0.64±0.06[abc]
III	β-tocopherol (100 mg/kg)	137.00± 11.66[a]	274.50 ± 18.25[b]	0.13±0.00[a]	0.38 ±0.01[a]	0.51 ±0.05[a]	0.019 ±0.00[a]	0.49 ±0.05[a]
IV	Copper sulphate (33 mg/kg)	141.00± 3.38[a]	226.33 ± 11.81[ab]	0.15±0.00[a]	0.37 ±0.00[a]	0.92 ±0.06[c]	0.016 ±0.00[a]	0.66 ±0.03[ab]
V	Flubendiamide (200 mg/kg)	137.67± 10.02[a]	234.17±4.32[ab]	0.23±0.01[a]	0.40±0.03[a]	0.59±0.01[a]	0.016±0.00[a]	0.57±0.03[ab]
VI	Copper sulphate (33 mg/kg) + Flubendiamide (200 mg/kg)	128.33± 7.20[a]	202.00±8.26[a]	0.17±0.01[a]	0.40±0.01[a]	0.56±0.04[a]	0.014±0.00[a]	0.81±0.04[c]
VII	Copper sulphate (33 mg/kg) + β-tocopherol (100 mg/kg)	133.33± 6.74[a]	211.67 ± 11.57[a]	0.16±0.01[a]	0.36±0.01[a]	0.67±0.09[ab]	0.035 ±0.01[a]	0.76 ±0.03[bc]
VIII	Flubendiamide (200 mg/kg) + β-tocopherol (100 mg/kg)	131.00± 11.41[a]	250.00 ± 12.14[ab]	0.20±0.05[a]	0.40±0.02[a]	0.62±0.05[a]	0.014±0.00[a]	0.67±0.03[ab]
IX	Copper sulphate (33 mg/kg) + Flubendiamide (200 mg/kg)+ β-tocopherol (100 mg/kg)	148.67± 14.31[a]	227.67 ± 11.85[ab]	0.16 ±0.01[a]	0.40 ±0.01[a]	0.86 ±0.04[bc]	0.015±0.001[a]	0.69±0.05[abc]

Values (mean ± SEM; n=6) bearing different superscripts in the same column differed significantly (P<0.05).

Table 6:
Effect of oral administration of β-tocopherol (100mg/kg) on certain erythrocytic indices of rats of different treatment groups following 90 days oral exposure to copper (33 mg/kg) and flubendiamide (200 mg/kg) alone and both in combination (copper 33 mg/kg + flubendiamide 200 mg/kg)

Groups	Treatment	RBCs (X 10^6U/L)	Hb (g/dl)	HCT/PCV (%)	RDWC (fl)
I	Control	8.00 ± 0.59[a]	12.60± 0.50[ab]	33.35 ± 0.16[c]	15.83± 0.70[c]
II	Vehicle control (Corn oil)	7.39 ± 0.62[a]	11.15 ± 0.41[abc]	29.33 ± 1.82[b]	18.28± 0.97[ab]
III	β-tocopherol (100 mg/kg)	8.34 ± 0.33[a]	13.50 ± 0.20[a]	41.17 ± 0.33[a]	17.60± 0.30[ab]
IV	Copper sulphate (33 mg/kg)	7.24 ± 0.68[a]	11.32 ± 0.77[bc]	33.90 ± 2.25[ab]	20.67± 1.72[a]
V	Flubendiamide (200 mg/kg)	7.12 ± 0.18[a]	10.34 ± 0.20[c]	31.12 ± 0.34[b]	18.83 ± 0.67[ab]
VI	Copper sulphate (33 mg/kg) + Flubendiamide (200 mg/kg)	7.07 ± 0.31[a]	10.83 ± 0.65[bc]	32.08 ± 1.62[b]	18.00 ± 0.37[ab]
VII	Copper sulphate (33 mg/kg) + β-tocopherol (100 mg/kg)	7.94 ± 0.48[a]	11.90 ± 0.66[abc]	36.05 ± 1.97[ab]	18.38 ± 0.27[ab]
VIII	Flubendiamide (200 mg/kg) + β-tocopherol (100 mg/kg)	6.79 ± 0.39[a]	11.32 ± 0.78[abc]	31.33 ± 2.15[b]	17.97 ± 0.62[ab]
IX	Copper sulphate (33 mg/kg) + Flubendiamide (200 mg/kg)) + β-tocopherol (100 mg/kg)	8.44 ± 0.37[a]	11.95 ± 0.51[abc]	31.10 ± 3.41[b]	18.54 ± 0.48[ab]

Values (mean ± SEM; n=6) bearing different superscripts in the same column differed significantly (P<0.05).
HCT-haemotocrit; Hb-Hemoglobin; PCV- Packed cell volume; RBCs-Red blood cells; RDW-Red cell distribution width.

exhibited ruptured alveolar septa, alveolar emphysema with congestion. Treatment of rats of group VII with β-tocopherol + copper sulphate revealed reparative changes and few areas mild exudation and alveolar rupture. β-tocopherol + flubendiamide-treated group showing mild degenerative changes like desquamation of bronchiolar mucosal epithelium, thickening of alveolar septa along with intact and active bronchial mucosal epithelium and aggregation lymphoid cells in peribronchiolar area. However, lungs of group IX rats treated concurrently with β-tocopherol + copper sulphate + flubendiamide showing emphysematous changes, ruptured alveolar septa with some areas of congestion and infiltration of serous exudate in the alveolar spaces and bronchial lumen with mild reparative changes (Figs. 9-14).

Results of present study on haematological and histological examinations indicated haemopoietic system, lungs and spleen are the organs for copper and flubendiamide-induced toxic effect therefore, assessment of these parameters is very vital in determining safety or

toxicity of the toxicant under study. β-tocopherol (β-TOH) is a natural form of vitamin E, and is major lipid soluble antioxidant, known to protect cellular membranes and lipoproteins from peroxidation (Yavuz *et al.,* 2004). Following co-treatment with β-tocopherol + xenobiotics i.e. β-tocopherol + copper sulphate, β-tocopherol + flubendiamide and β-tocopherol + copper sulphate + flubendiamide indicating improvement in the feed intake, body weight and per cent weight gain, values of Hb, RBCs count, RDWC, WBC, LY%, MO%, GR%, absolute monocytes count and platelet count were found to be almost comparable to those in control groups; thus suggesting reparative potential of β-tocopherol against toxicity manifested by copper and flubendiamide alone or both these in combination which is further substantiated by histological examination of lungs and spleen of β-tocopherol treated groups. The ameliorative potential of β-tocopherol against these xenobiotics due to that major free radical chain-breaking antioxidant property which interferes with the initiation and progression of copper and flubendiamide-induced oxidative damage in cells (Kaneko *et al.,* 1991; Sodhi *et al.,* 2008). These findings are in accordance with observations of several researchers to examine the protective effects of β-tocopherol on pesticide (Mandil *et al.,* 2016) and copper-induced oxidative stress (Mandil *et al.,* 2016).

It is concluded from this investigation that flubendiamide and copper revealed toxic potential on haemopoietic system and lungs and spleen in rats. β-tocopherol possessed the promising protective activity as it restored some of the haematological parameters closely towards control values and reversed the degenerative changes in lung and spleen. Findings suggested β-tocopherol to be a promising potential to prevent toxic effects of these environmental pollutants on human and animal health.

ACKNOWLEDGMENT

The authors are thankful to the Dean, College of Veterinary Science and Animal Husbandry, RKVY and Central Instrumentation Facility and Vice Chancellor of the University for providing the necessary laboratories facilities established under RKVY project in the Department of Pharmacology and Toxicology.

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Received on : 03.11.2017
Accepted on : 13.11.2017

Research Article

A MULTIPLE ONCE DAILY DOSE PHARMACOKINETICS OF AMIKACIN IN COW CALVES FOLLOWING INTRAVENOUS ADMINISTRATION

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ABSTRACT

A multiple once daily dose pharmacokinetic of amikacin (@ 10 mg/kg, i.v.) was carried out in healthy cow calves weighing between 80-100 kg. Concentrations of amikacin in blood plasma were estimated by microbiological assay techniques as well as various kinetic parameters were calculated by using two compartment open model for amikacin. Serial blood samples were taken on days 1 and 5 of treatment and at predose, 1 and 6 h on days 2, 3 and 4. Attempts were made to calculate the rational dosage regimens of amikacin on first and last dosing, on the basis of kinetic data and maintenance of therapeutic concentrations in plasma. Amikacin was administered separately in each of four healthy calves by intravenous (i.v) route once daily for five days. The drug was detectable up to 10 h. The minimum therapeutic concentration ($\geq$ 1.0 μg/ml) of amikacin was maintained up to 2 h in both 1st and 5th day of amikacin administration. Significantly higher plasma concentrations of the drug appeared from 0.042 to 6 h except 0.25 h in 5th day of amikacin administration as compared to 1st day amikacin administration. Mean trough amikacin plasma concentrations were 0.3, Significantly higher values of extrapolated zero time concentration of the drug during distribution phase (A), theoretical zero time concentration (C_p^o), area under curve (AUC), area under first moment curve (AUMC), mean residential time (MRT) and elimination of drug from central compartment (Kel) while significantly lower value of elimination rate constant (â) and total body clearance (Cl_B) are observed in 5th day of amikacin administration as compared to 1st day of amikacin administration. All other kinetic parameters differ non-significantly between 1st and 5th day of amikacin administration. It is concluded that once-daily administration of amikacin may provide adequate plasma levels to treat most susceptible gram-negative infections in cow calves.

Key Words: amikacin, Cow calves, multiple dose, pharmacokinetics.

INTRODUCTION

Amikacin, a recent aminoglycoside antibiotic derived from kanamycin by the process of acetylation, has proved its effective and greater clinical advantages over other aminoglycosides due to its high antibacterial spectrum against wide range of bacteria which are resistant to other aminoglycosides. It is most commonly used drug for the treatment of mixed bacterial infections which are unresponsive to other routine antibiotics, in medical and veterinary clinical practices. The aminoglycoside antibiotics have concentration-dependent bactericidal activity and the peak drug concentration (C_{max}) to minimum inhibitory concentration (MIC) ratio (C_{max}/MIC) is the pharmacokinetic/pharmacodynamic parameter best correlated with clinical efficacy (Blaser *et al.,* 1987). High drug concentrations of no less 10 fold the MIC of the bacterial pathogen are associated with successful treatments. However, because of its adverse effect i.e. ototoxicity and nephrotoxicity, plasma concentration must fall below a specific level before administering another dosage (Prins *et al.,* 1996). Thus, safe and effective dosage regimens must be carefully determined.

Once-daily administration of aminoglycosides may allow high peak levels, resulting in enhanced bacterial killing and post antibiotic effects and less nephrotoxic low trough levels, due to an extended interval between doses. Once-daily administration is currently applied in human clinical practice in order to reduce aminoglycosides renal toxicity (Prins *et al.,* 1996). Hence, the aim of the present study was to determine the pharmacokinetics of repeated doses of amikacin administered once daily in cow calves following intravenous route. However, no literatures are available for the pharmacokinetics of repeated doses of amikacin administered once daily in cow calves following intravenous route.

MATERIALS AND METHODS

The present study was undertaken to determine the pharmacokinetics of repeated doses of amikacin administered once daily in cow calves following intravenous route.

Experimental animals

In the present study, four clinically healthy female cow calves of non-descript breed between 4 to 6 months of age and 80 - 100 kg body weight were used. The animals were maintained at the Instructional Farm, College of Veterinary Science and Animal Husbandry, Mhow, Madhya Pradesh. They were kept under constant observation for fifteen days prior to commencement of the experiment. During this period they were subjected to clinical

examination in order to exclude the possibility of any disease. The animals were then housed in separate pen and were provided standard ration as per the farm schedule. Water was provided *ad libitum.* Fifteen days before the start of experiment deworming carried out with broad spectrum anthelmintics. All necessary managemental procedures were adopted to keep the animals free from stress. The experimental protocol for general procedure and use of animals for conducting the present study has been reviewed and approved by the Institutional Animal Ethics Committee (IAEC).

Administration of Drugs

Amikacin (AMIDAC®) - an injectable commercial preparation containing amikacin equivalent to 250 mg/ml, marketed by ZYDUS AHL., India was used. Amikacin was administered at the dose rate of 10 mg/kg body weight by i.v. route in each of four healthy calves. Intravenous injection of the drug was given through a jugular vein. Amikacin was administered separately in each of four healthy calves by intravenous (i.v) route once daily for five days.

Collection of blood samples

Blood samples (approx.1 ml) were withdrawn from jugular vein into heparinized glass centrifuge tubes on days 1 and 5 of treatment: at 0, 2.5, 5, 10, 15, 20, 30, 45 min and 1, 1.5, 2, 3, 4, 6, 8, 10, 12 and 24 h after administration of the drug. On days 2, 3 and 4 blood samples were taken 1 and 6 h after drug administration. Plasma was separated by centrifugation at 3,000 r.p.m. for 15 min at room temperature and kept at -4ºC until analysis, which was usually done within two days of collection of samples.

Estimation of amikacin

The concentration of amikacin in plasma was estimated by a rapid, specific microbiological assay technique using *Escherichia coli* as the test organism (Paul *et al.,* 1971).

Pharmacokinetic analysis

The plasma concentration-time profile of amikacin for each animal was used to determine the pharmacokinetics. The data of amikacin was subjected to two compartment open model. Kinetic parameters were calculated on the basis of Baggot (1977) and Gibaldi and Perrier (1982). Based on kinetic parameters, dosage regimen was derived using the following formula.

For calculation of D* (loading or priming dose) and D_0 (maintenance dose) the following formulae of Baggot (1977) were used

$$D^* = C_P(\min).\ Vd_{area}\ (e^{\hat{a}.\tau})$$

$$D_0 = C_P(\min).\ Vd_{area}\ (e^{\hat{a}.\tau}-1)$$

Where, C_P(min) = minimum therapeutic plasma drug concentration, Vd_{area} = volume of distribution based on total area under the plasma drug concentration versus time curve, â = elimination rate constant, τ = dosage interval, e = base of natural logarithm

Statistical analysis

Comparison of concentrations of the drugs in plasma, various kinetic parameters and dosage regimen of amikacin on first and last doses after multiple i.v. once daily dose administration in cow calves were compared by using paired ‘t’ test (Snedecor and Cochran, 1994).

RESULTS

Concentrations of amikacin in plasma at various time intervals following its multiple intravenous (i.v.) injection at the dose rate of 10 mg/kg body weight have been shown in Figure 1. On first dosing, the mean plasma concentration of the drug at 0.042 h was found to be 24.69 ± 0.011 μg/ml and the values ranged from 24.75 to 24.64 μg/ml. The drug was detectable in all the four animals up to 10 h and the mean concentration at 10 h was noted to be 0.08 ± 0.002 μg/ml however, the effective therapeutic concentration ($\geq$1 μg/ml) of amikacin was maintained up to 2 h of administration. While, on last dosing the mean plasma concentration of the drug at 96.042 h was found to be 25.56 ± 0.097 μg/ml and the values ranged from 25.79 to 24.98 μg/ml. The drug was detectable in all the four animals up to 10 h (i.e 106 h) and the mean concentration at 106 h was noted to be 0.09 ± 0.002 μg/ml however, the effective therapeutic concentration ($\geq$1 μg/ml) of amikacin was maintained up to 2 h of administration.

Plasma concentrations of amikacin in healthy cow calves after i.v administration between 1st and 5th days was also compared. The drug was detectable up to 10 h. The minimum therapeutic concentration (≥ 1.0 μg/ml) of amikacin was maintained up to 2 h in both 1st and 5th day of amikacin administration. Significantly higher plasma concentrations of the drug appeared from 0.042 to 6 h except 0.25 h in 5th day of amikacin administration as

Table 1:
Comparison of kinetic parameters of amikacin for i.v. administration in healthy cow calves between 1st and 5th day.

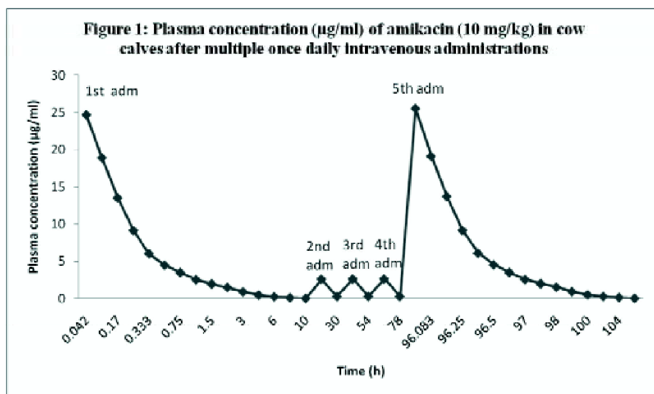
Parameter (Unit)	1st day	5th day
A (μg/ml)	15.439 ± 0.008	15.71 ± 0.023**
B (μg/ml)	3.23 ± 0.007	3.20 ± 0.012
C_p^o (μg/ml)	18.67 ± 0.002	18.92 ± 0.03*
β (h^{-1})	0.378 ± 0.002	0.362 ± 0.001*
α (h^{-1})	3.812 ± 0.005	3.81 ± 0.012
$t_{1/2}$ α (h)	0.182 ± 0.002	0.181 ± 0.005
$t_{1/2}$ β (h)	1.834 ± 0.012	1.91 ± 0.010
AUC (μg/ml.h)	13.3 ± 0.051	13.67 ± 0.042**
AUMC (μg/ml.h^2)	22.7 ± 0.266	24.57 ± 0.229**
MRT (h)	1.7 ± 0.020	1.82 ± 0.012*
K_{12} (h^{-1})	1.735 ± 0.002	1.770 ± 0.005
K_{21} (h^{-1})	0.972 ± 0.004	0.947 ± 0.004
Kel (h^{-1})	1.48 ± 0.005	1.458 ± 0.005*
Vd_{area} (L/kg)	1.99 ± 0.007	2.02 ± 0.007
Cl_B (ml/kg/h)	754.66 ± 2.68	732.994 ± 2.187**

A = The extrapolated zero time concentration of the drug in plasma during distribution phase, B = The extrapolated zero time concentration of the drug in plasma during elimination phase, Cp^0 = The theoretical zero time concentration, á = The distribution rate constant, $t_{1/2á}$ = The distribution half-life, β = The elimination rate constant, $t_{1/2â}$ = The elimination half-life, AUC = The area under curve in plasma, AUMC = The area under first moment curve, MRT = The mean residence time, K_{12} = The average rate of transfer of drug from central to peripheral compartment, K_{21} = The average rate of transfer of drug from peripheral to central compartment, K_{el} = The elimination from central compartment, Vd_{area} = The volume of distribution during area under curve, and Cl_B = The total body clearance.

Table 2:
Comparison of calculated dosage regimens for amikacin for i.v. administration in healthy cow calves between 1st and 5th day.

C_P^{∞}min(μg/ml)	τ (h)	Dose (mg/kg)	Amikacin on 1st day	Amikacin on 5th day
1	8	D*	2.21 ± 0.01	2.29 ± 0.13
		D_0	0.21 ± 0.03	0.12 ± 0.01*
	12	D*	3.01 ± 0.006	2.97 ± 0.01*
		D_0	1.02 ± 0.02	0.95 ± 0.01**
2	8	D*	4.42 ± 0.03	4.58 ± 0.26
		D_0	0.43 ± 0.05	0.25 ± 0.03*
	12	D*	6.03 ± 0.01	5.94 ± 0.02**
		D_0	2.04 ± 0.04	1.89 ± 0.03**

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



compared to 1st day amikacin administration.

Table 1 reveals the comparison of kinetic parameters of amikacin in healthy calves. Significantly higher values of extrapolated zero time concentration of the drug during distribution phase (A), theoretical zero time concentration (C_p^o), area under curve (AUC), area under first moment curve (AUMC), mean residential time (MRT) and elimination of drug from central compartment (Kel) while significantly lower value of elimination rate constant (â) and total body clearance (Cl_B) are observed in 5th day of amikacin administration as compared to 1st day of amikacin administration. All other kinetic parameters differ non-significantly between 1st and 5th day of amikacin administration.

Comparison of calculated dosage regimens of amikacin for different therapeutic levels (C_p^{∞} min = 1.0 and 2.0 μg/ml) and different dosage intervals (τ) of 8 and 12 h have been shown in Table 2. All calculated data for loading (D*) and maintenance (D_0) doses for different therapeutic levels at different dosage intervals (τ) were noted to be significantly lower except loading (D*) at dosage interval of 8 h in 5th day of amikacin administration as compared to 1st day of amikacin administration.

DISCUSSION

The semi logaritham plot of plasma levels of amikacin as a function of time after its multiple once daily dose i.v. administration of amikacin exhibited two distinct phases on 1st and 5th day of drug administration and the data obtained were adequately described by two compartment open model in the present study. Following multiple once daily intravenous dose of amikacin in healthy cow calves, mean peak plasma concentration at 2.5 min was 24.69 ± 0.011 μg/ml (1 day) & 25.56 ± 0.097 μg/ml (5 day) and amikacin was detected up to 10 h with a mean plasma concentration of 0.08 ± 0.002 μg/ml (1 day) & 0.09 ± 0.002 μg/ml (5 day). This value is comparatively higher in cross-bred bovine calves after a single intravenous administration of amikacin at dose rate of 10 mg/kg at 1 min. The concentration of amikacin in the plasma was 116.9±3.16 μg/ml and the minimum therapeutic concentration was maintained for 8 h (Saini and Shrivastava, 1998). The study conducted by Orsini *et al.* (1985) indicated that doses of amikacin at the rate of 4.4, 6.6 and 11.0 mg/kg show the concentrations 30.3 ± 0.3, 61.2 ± 6.9 and 122.8 ± 7.4 ìg/ml, respectively at 15 min following i.v. injection. Sumano *et al.* (2005) determined the pharmacokinetic variables of amikacin in cows after administration of amikacin sulphate either intravenously (i.v.) or intramuscularly (i.m.) at a dose of 25 mg/kg per day for three days and amikacin concentrations at time zero and maximum serum concentrations were found as 240.8 μg/mL and 122.53 μg/mL, respectively. According to Edward and Richard (1993), amikacin administration resulted in peak values of 27.3 ± 6.9 ìg/ml in study of the pharmacokinetic properties of gentamicin and amikacin in the cockatiel (*Nymphicus hollandicus*), a small (approximate body weight = 100 g) psittacine bird, utilizing treatment regimens developed in larger parrot species.

In the present study, plasma concentrations of amikacin *versus* time disposition curves after intravenous administration were best fitted to a two compartment open model. Similarly, (Kathryn *et al.,* 1995) reported two compartment open model after the intravenous dose of 5.8 mg/kg of amikacin in scimitar-horned oryx, also reported in dogs (Baggot *et al.,* 1985), in cats (Jernigan *et al.,* 1988) and in goats (Uppal *et al.,* 1992). Likewise first compartment model was fitted in mice according to Zhou *et al.* (1997).

The minimum therapeutic concentration of amikacin in plasma ranges from 1- 4 μg/ml (Leroy *et al.,* 1978). According to Brown *et al.* (1984) and Orsini *et al.,* (1985), for most of the susceptible bacteria, the therapeutic level of amikacin is 1-2 μg/ml maintained at time interval of 8 and 12 h. Therefore the Keeping in view the influence of certain unavoidable factor *in vivo*, the MIC of (≥ 1 $\mu g.ml^{-1}$) of amikacin has been maintained up to 6 h.

The elimination half-life ($t_{1/2}\beta$) is the time taken for plasma concentration in the body to be reduced by its half (50 per cent). Half-life provides a good indicator of time which is required to reach steady state after initiation of dosage regimen. The elimination half-life of amikacin in

cow calves following multiple once daily intravenous administration in the present study was 1.834 ± 0.012 h (1 day) and 1.91 ± 0.010 h (5 day). The elimination half-life of amikacin in cow calves is more or less similar to 3.09 ± 0.27 h in bovine calves (Saini and Shrivastava, 1998), in lactating sheep 1.64 ± 0.06 h (Haritova, 2004) and 2.16 h for goats (Uppal *et al.,* 1992).

The distribution half life ($t_{1/2}\,\alpha$) of amikacin in cow calves following multiple once daily intravenous administration in the present study was 0.182 ± 0.002 h (1 day) and 0.181 ± 0.005 h (5 day). These values are more or less similar to values reported in goat as 0.24 h (Uppal *et al.,* 1997), 0.36 h in calves and 0.43 h in sheep (Carli *et al.,* 1990).

The high values of AUC and AUMC reflect that most of the body area is covered with the drug concentrations. The AUC values of amikacin in cow calves following multiple once daily intravenous administration in the present study was 13.3 ± 0.051 μg/ml.h (1 day) and 13.67 ± 0.042 μg/ml.h (5 day), but significantly higher in goats 73.18 μg/ml.h (Agrawal *et al.,* 2001), in lactating sheep 94.09 ± 6.95 μg/ml.h (Haritova, 2004) and in Greyhounds dogs 79.97 h·ìg/ml (Kukanich and Coetzee, 2007). Similarly, the AUMC values of amikacin in cow calves following multiple once daily intravenous administration in the present study was 22.7 ± 0.266 μg/ml.h^2 (1 day) and 24.57 ± 0.229 μg/ml.h^2 (5 day). The mean residence time (MRT) of amikacin in cow calves following multiple once daily intravenous administration in the present study was 1.7 ± 0.02 h (1 day) and 1.82 ± 0.012 h (5 day), which is more in goat 4.67 ± 0.19 h (Agrawal *et al.,* 2001) and almost same in oryx 2.27 h (Kathryn *et al.,* 1995). This is in contrast to Witchel *et al.* (1992) who reported the increased values of AUC, AUMC and MRT in diseased foals after amikacin administration.

The volume of distribution (Vd_{area}) values of amikacin in cow calves following multiple once daily intravenous administration in the present study was 1.99 ± 0.007 l/kg (1 day) and 2.02 ± 0.007 l/kg (5 day). This value in healthy cow calves is higher than in bovine calves 0.40 ± 0.03 L/kg (Saini and Shrivastava, 1998), in man 0.27 ± 0.04 L/kg (Bauer and Blouin, 1983) and in Beagles dog 234.0 ml/kg (Kukanich and Coetzee, 2007)**.** This is reflecting good penetration of amikacin into various body fluids and tissues of cow calves. A very high value of Vd_{area} obtained in the present study may be attributed to wide distribution of amikacin in the body because of its polar organic base nature (Carli *et al.,* 1990). The total body clearance (Cl_B) values of amikacin in cow calves following multiple once daily intravenous administration in the present study was 754.66 ± 2.68 ml/kg/h (1 day) and 732.994 ± 2.187 ml/kg/h (5 day). It was observed that elimination of amikacin was altered by first and last dose, which plays an important role in the decrease of body clearance of drugs, including amikacin which is widely eliminated by the renal route. Similarly, lower value of total body clearance (Cl_B) was noted for goats 2.34 ± 0.17 ml/kg/min (Agrawal *et al.,*2001) and also in camel as 0.97 ml/kg/min (Wasfi *et al.*,1999) after i.m. administration, in dogs 2.66 ml/kg/min (Baggot *et al.,* 1985) and in cow calves 0.09 ± 0.002 L/kg/h in normal condition which is higher than that of febrile condition 0.05 ± 0.01 L/kg/h after i.v. administration of amikacin (Saini and Shrivastava, 1997).

This difference in the values of Cl_B amongst various species of the animals indicated that difference in their glomerular filteration rates of amikacin which is polar organic base hence weakly bound to serum proteins and is excreted as unchanged into the urine by glomerural filteration as supported by Carli *et al.* (1990).

The ultimate objective of the study of disposition kinetics is to determine an appropriate dose regimen of drugs. For any antimicrobial agent the dosage regimen is calculated to maintain the minimum therapeutic concentration (MIC) throughout the course of infections. An average plasma concentration of 1.0- 4.0 ìg/ml has been reported to be the minimum therapeutic concentration (MIC_{90}) of amikacin against most gram positive, gram negative and atypical bacteria (Leroy *et al.,* 1978, Agrawal *et al.,* 2001). Keeping in view of synergistic effects of the immune system and other *in vivo* factors as well as to cover most of the susceptible organisms, in this discussion, the MIC_{90} of 1.0 ìg/ml of amikacin has been taken into consideration. Amikacin possessed excellent antibacterial activity (MIC for 90% of tested strains i.e. MIC_{90} d" 2.0 µg/ml) against most common gram-negative aerobic pathogens, including *E. coli*, *K. pneumoniae*, *Enterobacter spp*, and *Brucella spp* (Shaffer *et al.,* 1953) *Mycobacteria* (Suter, 1952). Thus, in the present study dosage regimen was derived at MIC of 1.0 and 2.0 µg/ml for amikacin at dosage interval of 8 and 12 h.

All calculated data for loading (D*) and maintenance (D_0) doses for different therapeutic levels at different dosage intervals (τ) were noted to be significantly lower except loading (D^*) at dosage interval of 8 h in 5th day of amikacin administration as compared to 1st day of amikacin administration for intravenous route (Table 2) which suggested that amikacin dose has to be reduced for safe and effective for treating systemic microbial infections.

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Received on : 11.08.2017
Accepted on : 10.09.2017

Research Article

PHARMACOKINETICS OF GEMIFLOXACIN FOLLOWING SINGLE DOSE INTRAVENOUS AND INTRAMUSCULAR ADMINISTRATION IN BROILER BIRDS

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ABSTRACT

The present study was designed to investigate the detailed pharmacokinetics of gemifloxacin following its single dose intravenous and intramuscular administration at the dose of 10 mg/kg of body weight in broiler birds (n=6). Gemifloxacin concentration in plasma was determined using High Performance Liquid Chromatography (HPLC). Pharmacokinetic parameters were calculated using non-compartmental approach. Following intramuscular administration peak plasma concentration was 0.63 ± 0.011 µg/ml observed at 1h. Gemifloxacin concentration was not detected in plasma after 24h and 30h following IV and IM administration, respectively. After IV and IM administration, its lowest mean concentration 0.16 ± 0.001 and 0.13 ± 0.002 µg/ml were detected at 20 and 24 h, respectively. The mean values of distribution half-life ($t_{½α}$) and elimination half life ($t_{½β}$) following IV and IM administration of gemifloxacin at the dose rate 10 mg/kg in broiler birds were 1.23 ± 0.03, 4.70 ± 1.37h and 6.88 ± 0.05, 21.93 ± 2.40 h, respectively. Following IV and IM administration of gemifloxacin, the mean values of AUC were 18.14 ± 0.15 and 10.11 ± 0.52 µg.h/ml whereas the mean values of AUMC were 128.12 ± 1.24 and 293.68 ± 41.40 µg.h^2/ml. Following IV and IM administration of gemifloxacin, the mean values of volume of distribution $Vd_{(area)}$, volume of distribution at steady state (Vd_{ss}) total body clearance (Cl_B) and mean residence time (MRT) were 5.48 ± 0.04 and 30.82 ± 1.86 l/kg, 3.90±0.04 and 27.76 ± 1.21 l/kg, 0.55±0.004 and 1 ± 0.05 l/h/kg and 7.06±0.04 and 28.37 ± 2.62h, respectively. Following IM administration of gemifloxacin, Peak plasma concentration (C_{max}) and Bioavailability (F) were 0.63 ± 0.01 µg/ml and 55.75 ± 2.81%, respectively.

Key words: Broiler birds, gemifloxacin, pharmacokinetics.

INTRODUCTION

Gemifloxacin is a newer, fourth-generation fluoroquinolone drug with enhanced affinity for bacterial topoisomerase IV and is developed for the treatment of respiratory and urinary tract infections. It has shown potent antibacterial activity against clinical isolates and reference strains both *in vitro* studies and experimental models of infection in animals (Johnson *et al.,* 1999; Berry *et al.,* 2000). Gemifloxacin is particularly active against Gram-positive organisms including penicillin, macrolide and quinolone-resistant *Streptococcus pneumonia* (Hardy *et al.,* 2000). It has also shown potent activity against other major pathogens involved in respiratory tract infections, including *Haemophilus influenza* and *Moraxella catarrhalis* and the atypical organisms, *Legionella pneumophila*, *Chlamydia spp.* and *Mycoplasma spp.* (Felmingham *et al.,* 1999). Most of the pharmacokinetic parameters of gemifloxacin have been generated in humans. Studies on the pharmacokinetics of gemifloxacin in dogs and rats were reported (Ramji *et al.,* 2001). The pharmacokinetic data of gemifloxacin in broiler birds following single dose intravenous and intramuscular administration are completely lacking. Considering its great potential for its use in treatment of bacterial infections of broiler birds in future, the present study was planned to determine the pharmacokinetics of gemifloxacin in broiler birds following its single intravenous (IV) and intramuscular (IM) administration at the dose of 10 mg/kg body weight.

MATERIALS AND METHODS

Experimental animals

The study was conducted on 12 broiler birds of 5-6 weeks age weighing 1.5 to 2 kg. The birds were kept on Poultry Research Station, College of Veterinary Science and Animal Husbandry, Anand Agricultural University, Anand. The birds were kept under constant observation for at least 5 days before commencement of experiment. They were housed in clean cages and maintained on standard broiler ration as followed on the Station. The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC/GVC/VPT/248/2016). Water was provided *ad libitum.*

Drugs and chemicals

Gemifloxacin technical grade powder was purchased from open market (Hetero pharmaceuticals Pvt. Ltd., Hyderabad, Telangana). Acetonitrile, sodium acetate, methanol, triethylamine 70%, ortho-phosphoric acid min. 58% (analytical grade) and deionised water of HPLC grade were purchased from Merck Limited, Mumbai.

Experimental plan and drug administration

Twelve healthy broiler birds were used to investigate the pharmacokinetics of gemifloxacin following single dose IV and IM administration. Each treatment was given to 6 broiler birds. Gemifloxacin was administered at a dose rate of 10 mg/kg of body weight. Wing vein was employed to administer the drug by intravenous route. Intramuscular injection was carried out at pectoral/ breast muscle in broiler birds.

Collection of blood samples

Blood samples (1 ml) were collected in micro centrifuge tube at 0 minutes (before drug administration), 2, 5, 10, 20 and 40 minutes and at 1, 2, 4, 8, 12, 16, 20

and 24 hrs after IV administration of gemifloxacin and at 0 minutes (before drug administration), 5, 10, 20 and 40 minutes and at 1, 2, 4, 8, 12, 16, 20, 24 and 30 hrs after IM administration of gemifloxacin. Blood samples were collected in microcentrifuge tubes with 2ml capacity by fixing of IV cannula in wing vein and centrifuged at 10000 rpm for 10 minutes at 15°C (Eppendorf 5804 R, Germany). Separated plasma was transferred to labeled cryovials. Plasma samples were stored at - 60 °C in deep freezer until further analysis.

Gemifloxacin assay

Gemifloxacin was assayed in plasma by adopting procedure as described by Mohammad *et al.,* (2010) with minor modifications. The high performance liquid chromatography apparatus of Laballiance (USA) comprising of quaternary gradient delivery pump (model AIS 2000) and UV detector (model 500) was used for assay. Chromatographic separation was performed by using reverse phase C_{18} column (Whatman, PARTISIL 5 ODS-3 RAC-II; 4.6 × 100mm ID, UK) at room temperature. The HPLC data integration was performed using software Clarity (Version 2.4.0.190, Central Europe). The mobile phase consisted of a mixture of 0.49 % sodium acetate buffer (0.6 M solution) and acetonitrile (65:35 V/V) adjusted to pH 3.2 with ortho-phosphoric acid. Mobile phase was filtered by 0.45µ size filter and pumped into column at a flow rate of 0.6 ml/min at ambient temperature. The effluent was monitored at 272 nm wavelength.

For extraction of gemifloxacin from plasma, 306 µl plasma was added to each 2.0ml eppendorf tube and mix gently followed by 680 µl of methanol was added. Kept vial for minimum 15 minutes, vortex for 1 minute and then centrifuge at 10000 rpm for 10 minutes at 30°C. 800µl of supernent was transfered to drying tube. Then supernatant was evaporated at 56°C, 15 lbs pressures for 12 minutes using nitrogen evaporator. Reconstituted in 100µl of ACN: Methanol: Water (4:4:2) then vortex for 1 minute followed by transfer to HPLC vial. 20µl was loaded into HPLC system by using auto sampler. A standard curve of gemifloxacin was prepared using drug-free broiler bird plasma. The lower limit of quantification for gemifloxacin was 0.1 µg/ml. The assay was found to be sensitive, reproducible and its linearity was observed from 0.1 to 6.4 µg/ml with mean correlation coefficient (r^2) > 0.998.

Pharmacokinetic analysis

Non compartmental pharmacokinetic analysis was performed using software PK solution (Version 2.0, Summit Research Services, Colorado, USA) to calculate various pharmacokinetic parameters from plasma concentrations of gemifloxacin.

RESULTS AND DISCUSSION

All broiler birds remained in good health throughout the acclimatization and study periods. The plasma levels of gemifloxacin after its single IV and IM administration (10 mg/kg) in healthy broiler birds are presented as a semi logarithmic plot in Figure 1. The concentration of gemifloxacin ≥ 0.16 and 0.13 µg/ml was maintained in plasma from 0.033 h (2 minutes) to 20 hours after IV whereas 0.083 h (5 minutes) to 24 hours after IM drug administration. Ramji *et al.* (2001) reported maximum plasma concentration of gemifloxacin 1.20 ± 0.17 µg/ml in rats and 2.48±0.24 µg/ml in dogs following its intravenous administration.

Various pharmacokinetic parameters (Mean ± S.E.) calculated from plasma concentration Vs time profile after single dose IV and IM administration of gemifloxacin (10 mg/kg) in broiler birds are depicted in Table 1. The high values of distribution rate constants (0.57 ± 0.01µg/ml) and low values of elimination rate constant (0.10 ± 0.001 hours) observed in the present study following single dose IV administration of gemifloxacin indicated that the drug is rapidly distributed in the body and then relatively slowly eliminated from the body of birds.

The mean $t_{1/2\beta}$ after IV administration of gemifloxacin was 6.88 ± 0.05 hours which is found higher than the half lives of 1.5 ± 0.2 hours in rats and 5.0±0.9 hours in dogs (Ramji *et al.,* 2001). This reflects on relatively short duration of action of a single dose of the drug in broiler birds. The lower $t_{1/2\beta}$ values of 3.62 ± 0.03h for gatifloxacin at the dose rate 10 mg/kg (Devada *et al.,* 2012), 6.45 h for marbofloxacin (Urzua *et al.,* 2016), 4.25±0.13h for enrofloxacin (Ehmeza *et al.,* 2016) were reported in broiler birds following IV administration where as higher $t_{½\beta}$ values of 9.82± 0.97h for ciprofloxacin (Ivanova *et al.,* 2017) and 6.84± 0.15 h for enrofloxacin (Mekala *et al.,* 2014) were reported following IV administration of gemifloxacin in broiler birds.

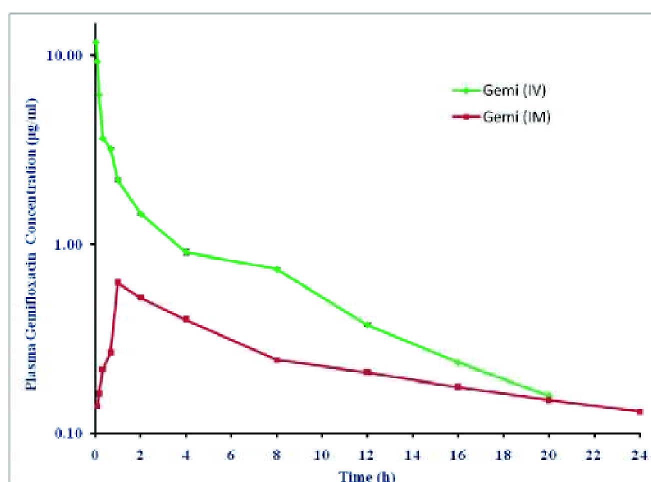

Fig. 1:
Semilogarithmic plot of Gemifloxacin concentration in plasma versus time following IV and IM administration (10 mg/kg) in broiler birds (Each point represents mean ± SE of six animals)

Table 1:
Pharmacokinetic parameters (Mean ± SE) of gemifloxacin following IV and IM administration (10mg/kg) in broiler birds (n=6)

Pharmacokinetic parameters	Unit	IV administration	IM administration
Cp^0	μg/mL	35.27 ± 4.19	-
K_a	h^{-1}	-	0.35 ± 0.03
α	h^{-1}	0.57 ± 0.01	0.20 ± 0.04
β	h^{-1}	0.10 ± 0.001	0.03 ± 0.004
$t_{½Ka}$	h	-	2.45 ± 0.39
$t_{½α}$	h	1.23 ± 0.03	4.70 ± 1.37
$t_{½β}$	h	6.88 ± 0.05	21.93 ± 2.40
C_{max}	μg/mL	-	0.63 ± 0.01
T_{max}	h	-	1.00 ± 0.00
$AUC_{(0-\infty)}$	μg.h/mL	18.14 ± 0.15	10.11 ± 0.52
AUMC	$μg.h^2/mL$	128.12 ±1.24	293.68 ± 41.40
Vd_{area}	L/kg	5.48 ± 0.04	30.82 ± 1.86
Vd_{ss}	L/kg	3.90 ± 0.04	27.76 ± 1.21
Cl_B	L/h/kg	0.55 ± 0.004	1.00 ± 0.05
MRT	h	7.06 ± 0.04	28.37 ± 2.62
F	%	-	55.75 ± 2.81

Cp^0: Concentration at time 0; Ka: Absorption rate constant; α: Exponential coefficient of distribution; β: Exponential coefficient of elimination; $t_{½Ka}$: Absorption half-life; $t_{1/2α}$: Distribution half-life; $t_{½β}$: Elimination half-life; C_{max}: Maximum drug concentration; T_{max}: Time of maximum observed concentration in serum; $AUC_{0-\infty}$: Area under plasma drug concentration-time curve; AUMC: area under first moment curve; Vd_{area}: Apparent volume of distribution; Vd_{ss}: Volume of distribution at steady-state; Cl_B: Total body clearance; MRT: Mean residence time; F: Bioavailability.

In the present study, the mean apparent volume of distribution and volume of distribution at steady state calculated following IV administration of gemifloxacin were 5.84 ± 0.04 L/kg and 3.90 ± 0.04 l/kg, respectively. Similarly, apparent volume of distribution value for broiler chicken following IV administration at dose rate of 10 mg/kg body weight was found 4.0 l/kg (Knoll *et al.,* 1999) and 4.044 ± 0.077 l/kg (Varia *et al.,* 2009) for enrofloxacin and levofloxacin respectively. Volume of distribution in the present study are indicative of good distribution of the drug in the body of birds, which is in agreement with similar values observed for other fluoroquinolones by other workers. Other reported study includes, the lower values of 2.5± 0.025 l/kg for gatifloxacin (Devada *et al.,* 2012), 3.88 l/kg for marbofloxacin (Urzua *et al.,* 2016), 3.04 ± 0.09 l/kg for enrofloxacin (Mekala *et al.,* 2014) and 4.86± 0.34 l/kg for ciprofloxacin (Ivanova *et al.,* 2017) were reported following IV administration of the drugs. Gemifloxacin has large volume of distribution owing to its high lipid solubility and low plasma protein binding which results in extensive penetration in tissues as seen for other members of fluoroquinolones.

The area under the curve following single dose IV administration of gemifloxacin was 18.14 ± 0.15 μg·h/ml in the present study. The higher AUC value of 20.89±0.10 μg.h/ml for gatifloxacin (Devada *et al.,* 2012), 32.72 ± 1.15 μg.h/ml for enrofloxacin (Mekala *et al.,* 2014) and 19.84 ± 0.42 μg.h/ml for ciprofloxacin (Ivanova *et al.,* 2017) were reported following IV administration in broiler birds while lower AUC values of 16.42± 2.23 μg.h/ml for enrofloxacin (Ehmeza *et al.,* 2016) was reported in Broiler Birds. The difference in AUC values may be related to species variation and difference in dosage.

The total body clearance of gemifloxacin in broiler birds following single dose IV administration was 0.55 ± 0.004 l/h/kg in the present study. Similarly, lower clearance values have been reported in chicken after IV administration at dose rate of 10 mg/kg body weight for pefloxacin (0.28 ± 0.012 l/h/kg) by Moutafchieva *et al.* (2009), for difloxacin (0.37 ± 0.10 l/h/kg) by Ding *et al.* (2008). Lower Cl_B values of 0.48 ± 0.002 l/h/kg for gatifloxacin (Devada *et al.,* 2012), 0.416 l/h/kg for Marbofloxacin (Urzua *et al.,* 2016), 0.31±0.01 l/h/kg for ciprofloxacin (Ivanova *et al.,* 2017) were reported following IV administration in broiler birds where as higher CL_B values of 0.62 ± 0.07 l/h/kg was reported by Ehmeza *et al.* (2016). The clearance found in the present study was very high as compared to the values found in other species and for other members of fluoroquinolones. The reasons of faster clearance of gemifloxacin in birds may be due to low protein binding, high lipid solubility, excretion as unchanged form and minimal tubular reabsorption (Tasso *et al.,* 2008).

In the present study, the mean peak plasma concentration (C_{max}) 0.63 ± 0.01 μg/ml was observed at 1h following IM administration of gemifloxacin in broiler birds. The mean bioavailability (F) was 55.75 ± 2.81 % following IM administration of gemifloxacin in broiler birds. Other reported mean bioavailability includes, 59.54 ± 1.97% for levofloxacin (Varia *et al.,* 2009), 72.96 ± 1.10% for gatifloxacin (Devada *et al.,* 2012), 77.47± 5.86% for enrofloxacin (Mekala *et al.,* 2014), 98% for marbofloxacin (Urzua *et al.,* 2016) after oral administration whereas 63.89 ± 0.34% for ciprofloxacin after intraingluvial administration (Ivanova *et al.,* 2017) were reported in broiler birds.

ACKNOWLEDGEMENT

Authors thank Dean/Principal of Veterinary College, AAU, Anand for the financial support, and Research Scientist, Poultry Research Station for providing bird house facilities for this research work.

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Received on : 12.11.2017
Accepted on: 28.12.2017

Research Article

IMMUNOMODULATORY EFFECT OF GIR COW URINE DISTILLATE IN CYCLOPHOSPHAMIDE INDUCED IMMUNOSUPPRESSIVE MICE

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ABSTRACT

To evaluate the effect of Cow urine distillate on humoral and cell mediated immunity in cyclophosphamide induced immunosuppressive mice, Swiss albino mice were randomly divided into 5 groups (numbered I to V), each containing 6 mice. Animals of groups I were administrated normal saline for 28 days. Group II were administered cyclophosphamide @ 60 mg/kg b.w. (body weight), daily once orally for 28 days. Group III, Group IV and Group V were administrated Cow urine distillate @ 2 ml/kg, 4 ml/kg and 6 ml/kg b.w., for 28 days, respectively along with cyclophosphamide @ 60 mg/kg b.w. for 28 days. On 29th day humoral and cell mediated immune responses, TLC (Total leukocyte counts), DLC (Differential leukocyte counts), serum total protein, globulin, albumin and histopathological examination of tissues were conducted. The result obtained clearly indicated that oral administration of Cow urine distillate in immunosuppressed mice having immunomodulatory effect in dose dependent manner by increasing both the cell mediated immune response and antibody titer. So the present study suggests that repeated oral administration of Cow urine distillate for 28 days in immunosuppressive mice showed immunomodulatory action.

Key words: Cow urine distillate, cyclophosphamide, immunomodulatory effect, mice.

INTRODUCTION

Cyclophosphamide is probably one of the most prescribed anticancer drugs used for treatment of various forms of cancer. Cyclophosphamide is converted to active metabolites in body, which alkylates DNA in both proliferating and non-proliferating cells; proliferating cells are more susceptible to alkylation. Cyclophosphamide also prevents B cells to renew their antigen receptors. Cyclophosphamide is used as an immunosuppressant primarily due to its immunosuppressive effects on lymphoid elements in bone marrow transplantation (Sandhu, 2013). Immunosuppression caused by cyclophosphamide and other anticancer drugs significantly complicates the course of cancer chemotherapy and worsens the condition of the patients. In regard to the immunosuppressive effects of anticancer chemotherapy, the stimulation of production of immune cells in an immunosuppression model has been classified as immunomodulation (Vigila and Baskaran, 2008). In Indian ancient literature, several herbal preparations have been described, which can be given in order to augment the immune response of individual (Bhargava and Singh, 1981). In this direction earlier some herbal preparations have been studied for their immunomodulatory properties (Chauhan, 1999; Chaterjee, 1994). Panchgavya therapy plays an important role in ayurvedic system of medicine. Panchgavya means the mixture of natural products of cow like milk, curd, ghee, urine and dung. In the ancient literature, it is mentioned that it may increase the resistance of the body, but there seems to be no/few authentic scientific report about the efficacy of panchgavya components. So the present study was carried out to investigate the immunomodulatory properties of “Kamdhenu ark (Cow urine distillate)”, prepared from Gir cow urine.

MATERIALS AND METHODS

Experimental animals

The present study was conducted on 30 healthy male albino mice that were of 6-8 weeks old. The mice were procured from Zydus Research Center, Ahmadabad and kept in cages at Laboratory Animal House, College of Veterinary Science & Animal Husbandry, Anand Agricultural University, Anand. The animals were kept under constant observation for at least 5 days before commencement of the experiment. Mice were provided with standard pellet diet. Diet and deionized water were provided *ad libitum*. All necessary procedures were adopted to keep mice free from stress. This study was performed after the approval from Institutional Animal Ethical Committee and all procedures were carried out in accordance with the Guidelines laid down by the International Animal Ethics Committee (IAEC/182/VPT/2014).

Preparation of cow urine distillate

Three apparently healthy pure Gir cows aged 3-4 years, maintained at Livestock Research Station, College of Veterinary Science & A. H., Anand raised under standard feeding and management conditions were selected as donor of the urine. Early morning cow urine was collected in sterilized plastic bottles and the samples were brought to the laboratory for preparation of Goumutra ark (Cow urine distillate). It was prepared by boiling cow urine at 100 °C in distillation apparatus, as per the method outlined

by Khanuja (2002).

Development of immunosuppressed mice model

Immunosuppressive mice model was developed by oral administration of cyclophosphamide @ 60 mg/kg body weight daily once for 28 days (Qi et al., 2010).

Experimental design

All the mice were randomly divided into five groups (I, II, III, IV and V) having 6 mice in each group. Animals of all groups were given different treatment for 28 days. Mice of group I was administered normal saline and served as vehicle control. The group II mice were administered cyclophosphamide (60 mg/kg) and served as immunosuppressed control group. Group III, IV and V were received Cow urine distillate @ 2 ml, 4 ml and 6 ml per kg body weight respectively along with cyclophosphamide (60 mg/kg, orally) daily once for 28 days.

On 29th day of the experiment, blood samples were collected from the retro-orbital plexus with the help of capillary tube before sacrificing the mice. Blood samples were collected in vials containing K_3EDTA (ethylene diaminetetra-acetate) for hematology (differential leucocyte counts and total leucocyte counts) and in plain vials for serum biochemical estimation (albumin, globulin and total protein) and sheep red blood cells (SRBCs) antibody titer by hemagglutination. After sacrificing the mice, spleen and thymus were collected for histopathological examination. Total leukocyte counts and differential leukocyte counts (lymphocyte, granulocyte and monocytes) estimated by hematology autoanalyzer (Mindray, BC – 2800 Vet, China).

Immunization of mice was done using SRBCs. SRBCs were collected in Alsever's solution (composed of 20.5 g of dextrose, 8 g of sodium citrate, 4.2 g of sodium chloride, 0.55 g of citric acid in one liter distilled water), washed in large volumes of sterile 0.9 % normal saline thrice and adjusted to a concentration of 5×10^9 cells/ml, were used for immunization. Animals were immunized by injecting 0.2 ml SRBC suspension intraperitoneally 7 days prior to sacrifice (on 21st day of the experiment). Blood was collected from retro orbital plexus under ether anesthesia on 29th day and serum was separated to determine the antibody titer by the Hemagglutination test (HA).

Antibody titer

Antibody titer was carried out according to the protocol as described by Puri *et al.* (1994). Antibody titer was carried out by diluting the test serum two fold times in 0.15 M phosphate buffer saline (PBS) and aliquoted in "U" bottomed microtiter plates. 1% SRBC suspended in PBS was dispensed in each well and mixed thoroughly. The plates were incubated for 4 h at 37°C and then observed visually for hemagglutination. The highest dilution of the test serum giving hemagglutination was taken as antibody titer.

Assessment of cell-mediated immune response

Cell-mediated immune response was assessed by the method as described by Lagrange *et al.* (1974). All the animals under various groups were immunized by injecting 20 µl of 5×10^9 SRBC/ml subcutaneously into the right footpad on 19th day of the treatment. Thickness of left footpad was measured using vernier callipers on 26th day of the treatment. The mice then challenged by injecting 20 µl of 5×10^9 SRBC/ml intradermally on the left hind foot pad (time 0). Foot pad thickness was measured at 24 and 48 h of challenge. The difference in mm was taken as a measure of delayed type hypersensitivity.

Histopathology

On 29th day of study, all the mice from each group were sacrificed by cervical dislocation and subjected to post mortem examination in the confined disinfected laboratory to determine the presence or absence of gross and histopathological lesions. Post mortem findings were made by systematic approach. Detailed post mortem lesions from all the mice were recorded. For gross (macroscopic) lesions thymus and spleen were collected and examined after opening the body of sacrificed mice. For histopathological examinations, tissues from spleen and thymus were collected in 10 % formalin and preserved for processing. The formalin fixed tissues were processed by paraffin wax embedding method of tissue sectioning. Sections were cut at 5–6 µ thickness with automatic section cutting machine (Leica, Automatic Microtome Machine, Germany), and were stained with Hematoxylin and Eosin (H and E) stains. The H and E stained slides were observed under microscope and lesions were recorded.

Statistical analysis

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

RESULTS

Hematological examination

Results obtained in relation to hematological assessment are presented in Figure 1. There was significant decrease ($P < 0.05$) in total leucocyte counts and lymphocyte counts in immunosuppressed control group II ($6.483 \pm 0.104 \times 10^3$ /µl and $2.550 \pm 0.105 \times 10^3$ /µl, respectively) as compared to vehicle control group I ($9.567 \pm 0.433 \times 10^3$ /µl and $5.667 \pm 0.242 \times 10^3$ /µl, respectively). There was significant increase ($P < 0.05$) in total leucocyte counts ($8.500 \pm 0.159 \times 10^3$/µl, $8.750 \pm 0.177 \times 10^3$/µl and $8.867 \pm 0.112 \times 10^3$/µl, respectively) and lymphocyte counts ($4.533 \pm 0.178 \times 10^3$/µl, 4.733 ±

0.196 × 10^3/µl and 5.050 ± 0.112 × 10^3/µl, respectively) in Cow urine distillate given immunosuppressed groups III, IV and V as compared to immunosuppressed control group II (6.483 ± 0.104 × 10^3/µl and 2.550 ± 0.105 × 10^3/µl, respectively). The increase in total leucocyte counts and lymphocyte counts were in dose dependent manner.

Biochemical examination

There was significant decrease in total protein and albumin level (P < 0.05) in immunosuppressed control group II (4.028 ± 0.073 g/dl and 3.157 ± 0.069 g/dl, respectively) as compared to vehicle control group I (6.953 ± 0.108 g/dl and 6.093 ± 0.106 g/dl, respectively). There was significant increase (P < 0.05) in total protein (6.200 ± 0.086 g/dl, 6.782 ± 0.126 g/dl and 6.825 ± 0.101 g/dl, respectively) and serum albumin level (5.332 ± 0.095 g/dl, 5.902 ± 0.127 g/dl and 5.932 ± 0.104 g/dl, respectively) in Cow urine distillate treated immunosuppressed groups III, groups III, IV and V were in dose dependent manner as shown in Figure 2.

Results obtained in relation to humoral and cell-mediated immune responses are presented in Figure 3 and 4 respectively. There was significant decrease (P < 0.05) in antibody titer in immunosuppressed control group II (16.00 ± 5.842) as compared to vehicle control group I (192.00 ± 28.622). There was significant (P < 0.05) increase in antibody titer in Cow urine distillate treated immunosuppressed group IV (160.00 ± 32.000) and group V (170.66 ± 26.985) as compared to immunosuppressed control group II (16.00 ± 5.842) as shown in Figure 3.

There was significant decreased (P < 0.05) in foot pad skin thickness in immunosuppressed control group II (0.298 ± 0.005 mm) as compared to vehicle control group I (0.328 ± 0.008 mm) at 24 h. There was significant increase (P < 0.05) in foot pad skin thickness in Cow urine distillate treated immunosuppressed treatment group III (0.322 ±

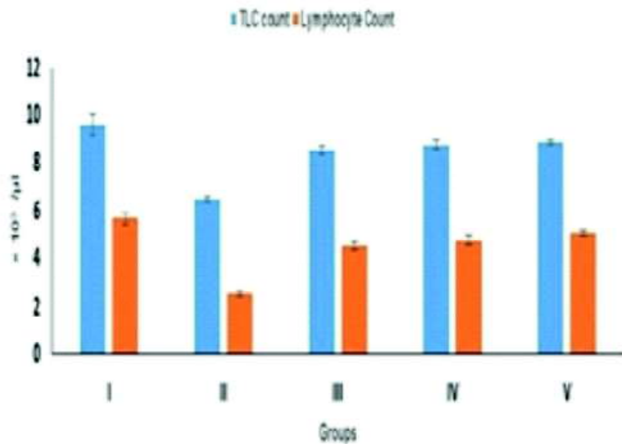


Fig. 1
Effect of daily oral administration of Cow urine distillate on total leucocytes count (TLC) and lymphocyte count in immunosuppressed mice

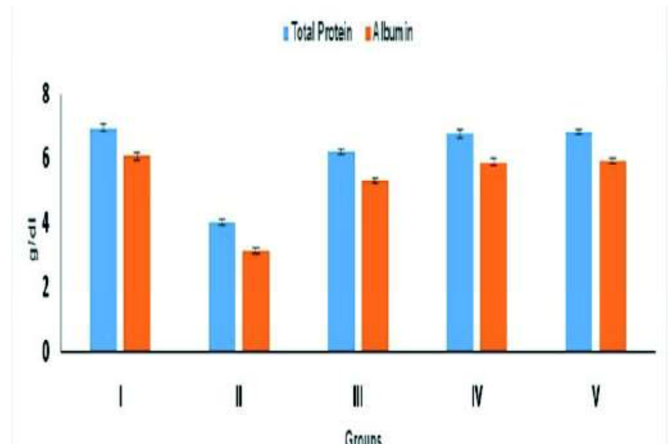


Fig. 2
Effect of daily oral administration of cow urine distillate on total protein and serum albumin in immunosuppressed mice.

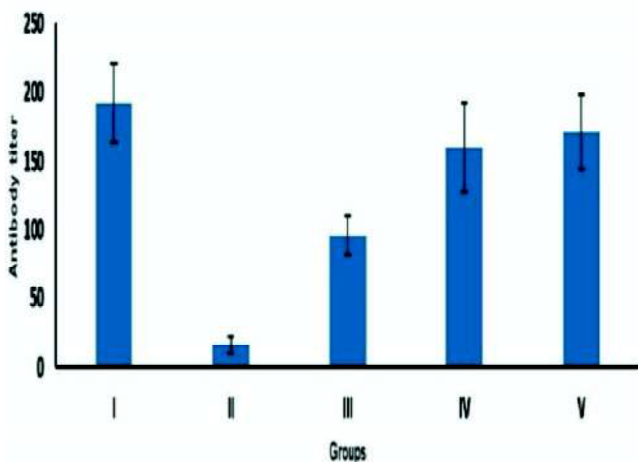


Fig. 3
Effect of daily oral administration of Cow urine distillate on antibody titer in immunosuppressed mice.

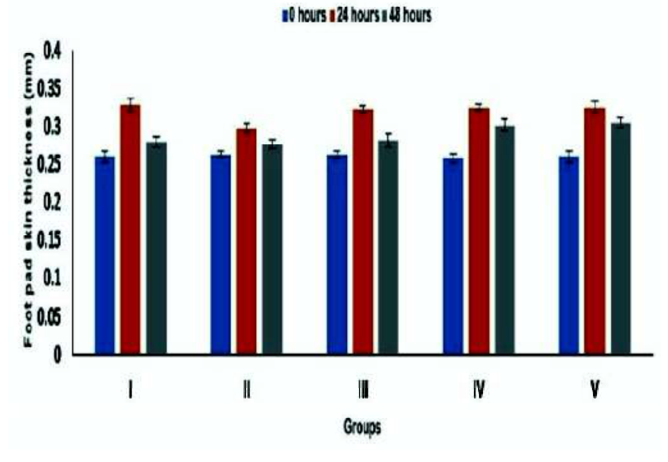
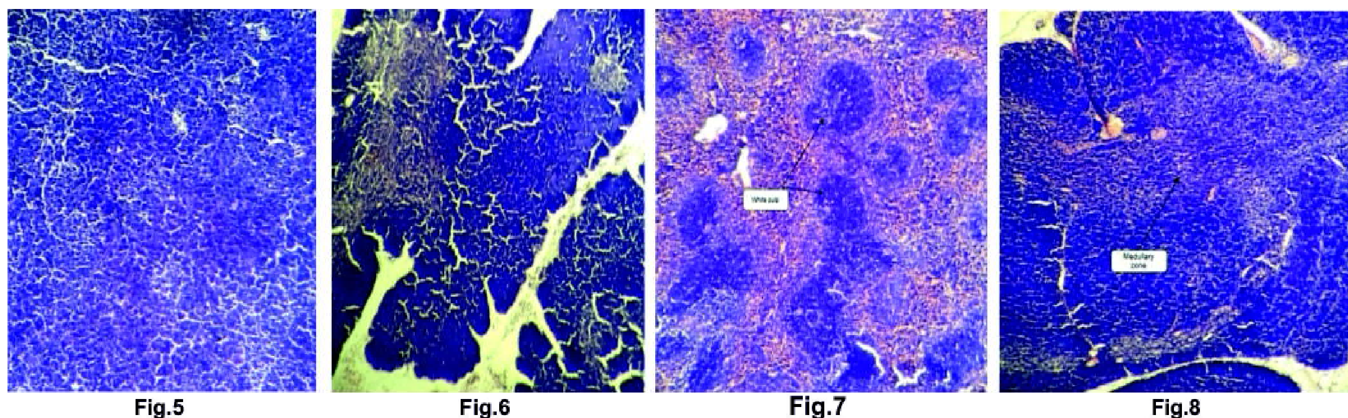


Fig. 4
Effect of daily oral administration of Cow urine distillate on foot pad skin thickness in immunosuppressed mice.



Figs 5-8: Spleen showing general disorganization of splenic tissue and loss in distinction between red pulp and white pulp in **Fig 5** and thymus showing marked disruption of organization and massive depletion of cellularity from immunosuppressed control group in **Fig 6** . From cow urine distillate treated immunosuppressed group V, spleen showing distinction between white pulp and red pulp in **Fig 7** and thymus showing distinction between cortical and medullary zone in **Fig 8**.

(0.325 ± 0.008 mm) as compared to immunosuppressed control group II at 24 h. The foot pad skin thickness was decrease in all groups at 48 h as compared to 24 h. However, there was significant difference in foot pad skin thickness in Cow urine distillate treated immunosuppressed group IV (0.302 ± 0.004 mm) and group V (0.305 ± 0.006 mm) as compared to immunosuppressed control group II (0.277 ± 0.006 mm) which is presented in Figure 4.

Histopathological examination

Gross examination revealed congestion of spleen and atrophy of thymus in immunosuppressed control group. No observable gross lesions in spleen and thymus were observed in group IV and V. There was marked disruption in spleen and thymus structure and many signs of pathological alterations were observed in cyclophosphamide given immunosuppressed control group. These alterations include the marked loss in distinction between the white and red pulps in spleen (Figure 5). There was decrease in the lymphocyte population in thymus (Figure 6). Spleen and thymus sections of the immunosuppressed group V treated with Cow urine distillate showed marked improvement in the splenic (Figure 7) and thymus structure (Figure 8).

DISCUSSION

In present study, Immunomodulatory effect of Cow urine distillate was assessed by estimation of total leucocyte counts, lymphocyte counts, serum total protein, serum albumin level, humoral and cell mediated immunity along with histopathological examination.

The result of the present study showed that there was decrease in the total leucocyte counts, lymphocyte counts, serum total protein, serum albumin level, humoral immunity and cell mediated immunity in the immunosuppressed control group given cyclophosphamide. The result is consistent with the report showing the decrease in all these parameter in mice given cyclophosphamide (Vaghasiya *et al.,* 2010; Hussain *et al.,* 2013; Abdalla *et al.,* 2013). This was due to immunotoxic effect of cyclophosphamide.

The total leucocyte counts and lymphocyte counts were significantly higher in immunosuppressed groups treated with Cow urine distillate (2 ml, 4 ml and 6 ml per kg, orally for 28 days, respectively) in dose dependent manner as compared to immunosuppressed control group. Similar findings of the increase in total leucocyte counts and lymphocyte counts were observed in immunosuppressed mice treated with the Cow urine distillate (10.8 ml/kg) for 19 days, orally (Naseema *et al.,* 2014).

The study on total protein and albumin level suggested dose related increase in both the parameters as compared to immunosuppressed control group. These may be due to anabolic effect of Cow urine distillate on protein metabolism (Panicker *et al.,* 2012).

Result regarding the humoral immunity showed dose dependent increase in antibody titer in Cow urine distillate (4 ml and 6 ml per kg) treated groups as compared to immunosuppressed control group. Similarly increase in antibody titer by administration of Cow urine distillate (10.8 ml/kg for 19 days) in immunosuppressed mice was also reported (Naseema *et al.,* 2014). Panchgavya feeding (50 mg/ rat for 30 days) increase the humoral immune response against cyclophosphamide induced immunosuppression by estimating serum gamma globulin concentration (Paliwal *et al.,* 2012). Additionally, increase in antibody titer observed in vaccinated chicken given panchgavya (0.5, 0.6 and 0.7 % of basal diet) with basal diet as compared to basal diet given group (Sumithra *et al.,* 2013).

Result regarding the cell mediated immunity

showed dose dependent increase in cell mediated immunity in Cow urine distillate (4 ml and 6 ml per kg) treated groups as compared to immunosuppressed control group at 24 h and 48 h. Additionally, increase in Cell mediated immunity in immunosuppressed mice treated with Cow urine distillate (10.8 ml/kg) was reported (Naseema *et al.,* 2014). The T-lymphoblatogenesis of Cow urine treated group was significantly higher as compared to dimethoate induced immunotoxic avian model (Ambwami *et al.,* 2006). Panchgvaya feeding significantly increase the cell mediated immune response as compared to cyclophosphamide induced immunosuppressed control group in chicken (Paliwal *et al.,* 2012).

On microscopic examination of spleen and thymus showed moderate population of lymphocyte in immunosuppressed mice treated with high dose of Cow urine distillate. This shows immunomodulatory activity of Cow urine distillate. It is concluded that Cow urine distillate at the dose rate of 2 ml, 4 ml and 6 ml per kg body weight daily once orally for 28 days in immunosuppressed mice shows immunomodulatory effect.

ACKNOWLEDGEMENT

Authors are thankful to the Dean/Principal, College of Veterinary Science & Animal Husbandry, Anand Agricultural University, Anand for the financial support to carry out the research work. Authors also acknowledge Dr. Satish D. Patel, ZRC for providing mice for this research work, Dr. D.J. Ghodasara for carried out histopathological examination of tissues, Dr. K. N. Wadhwani for providing Laboratory Animal House facilities and Dr. S. V. Shah for providing Gir Cow urine for this research work.

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Received on : 21.12.2017
Accepted on : 26.12.2017

Research Article

EFFECTS OF *WITHANIA SOMNIFERA* ON HEMATOLOGICAL PARAMETERS IN CADMIUM INTOXICATED CHICKENS

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ABSTRACT

A total of 90 chicks of one day age were divided randomly into five groups of 18 birds each viz. group I (control), group II (100 ppm cadmium), group III (200 ppm cadmium), group IV (100 ppm cadmium + WSRP) and group V (200 ppm cadmium + WSRP). *Withania somnifera* root powder (WSRP) was administered orally in standard recommended feed at the rate of 1% concentration. Hematological parameters were studied from 5 birds from each group at 0, 15th , 30th , 45th and 60th days post treatment (DPT). In groups II and III, Hb, PCV, TEC, MCH, MCHC and lymphocytes decreased significantly ($P<0.01$) and there was significant($P<0.01$) increase in TLC and heterophils whereas no significant change was recorded in MCV, monocyte, eosinophil and basophil count. There was a significant ($P<0.01$) lesser decrease in Hb, PCV, TEC, MCH, MCHC, and lymphocytes in groups IV and V. There was a significant time and dose dependent decrease in values of Hb, PCV, total erythrocyte count (TEC), MCH and MCHC. In groups II and III, significant dose and time dependent increase in total leukocyte count and heterophil was recorded, along with significant decrease in lymphocyte observed.

Keywords: Cadmium, chickens, haematological parameters, *Withania somnifera*

INTRODUCTION

Among all heavy metals, cadmium is an important air, soil and water pollutant which comes into environment through different sources. It is very toxic metal and also an environmental and industrial pollutant. It is present in soil, water, air and food (Al-Kedheiry *et al.,* 2001; Cinar, 2003; Al-Attar, 2005; Eriyamremu *et al.,* 2006 and Kaplan *et al.,* 2011). Only a small proportion of absorbed cadmium (less than 10 percent in animal experiments) is eliminated, mainly in the urine and faeces (Friberg *et al.,* 1974). The heavy metals are toxic due to low rate of their elimination from the body. Poultry is highly susceptible to cadmium toxicity, as cadmium intoxication may occur through feed ingredients of plant origin and also from ingredients of animal origin like fish meal and shell grid. At the cellular level, cadmium induces oxidative stress in many organs (Bertin and Averbeck, 2006; Thevenod, 2009), which might result in physiological damage to the organs like kidneys, liver lung, pancreas, testes, placenta and bone (Jarup *et al.,* 1998; Nawrot *et al.,* 2008 and Jarup and Akesson, 2009). *Withania somnifera*, popularly known as ashwagandha, has been an important herb in the Ayurvedic and indigenous medical system for over 3000 years, and in the traditional system of medicine, this plant is most often described as adaptogen (Panossian, 2003). Historically, the plant has been used as an aphrodisiac, liver tonic, anti-inflammatory agent and astringent. This plant promotes physical and mental health, rejuvenate the body in debilitated conditions and increase longevity (Kulkarni and Dhir, 2008). Keeping in view the above facts, the present study was undertaken to study the ameliorative effects of *Withania somnifera* root powder (WSRP) administration on hematological parameters in cadmium treated chickens.

MATERIALS AND METHODS

Ninety day old broiler chicks were randomly divided into five groups of 18 birds each viz. group I (control), group II (100 ppm cadmium), group III (200 ppm cadmium), group IV (100 ppm cadmium + WSRP) and group V (200 ppm cadmium + WSRP). After two weeks of adaptation period, groups II and III were administered 100 ppm and 200 ppm cadmium as cadmium chloride monohydrate in triple glass distilled water respectively. Groups IV and V were administered 100 ppm and 200 ppm cadmium in triple glass distilled water along with *Withania somnifera* root powder orally in standard recommended feed at the rate of 1% concentration from 2 weeks of age of birds till 60 days post treatment (DPT). During the adaptation period of 2 weeks birds were given plain triple glass distilled water. In this study, hematological parameters viz. hemoglobin concentration, packed cell volume, total erythrocyte count, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, total leukocyte count and differential leukocyte count were studied at 0, 15th , 30th , 45th and 60th days post treatment (DPT). The blood of five birds from each group was collected using EDTA as anticoagulant at the above said intervals. The results obtained were analysed by SPSS 2007 software (SPSS,

2007).

RESULTS AND DISCUSSION

Mean values of various haematological parameters of different groups at 0, 15, 30, 45 and 60 days intervals have been depicted in Table 1. Average hemoglobin concentration of experimental birds showed a significant decrease in Hb, PCV and TEC at each time interval i.e. from 0 to 60th DPT. From 15th to 60th DPT, significant difference was recorded between all the groups at each time interval. In general, the values of Hb, PCV and TEC in group III were significantly lower followed by groups II, V and IV, in comparison to group I.

The values of MCH indicates that there was no significant change in the values from 0 to 60th DPT in group I. When these values were compared between different groups at different time interval, in general, the values in group III were significantly lower followed by groups II, V and IV, in comparison to group I. The values of MCHC in group I depicted no significant change from 0 to 60th DPT. In groups II and III, the mean values of MCHC significantly decreased from 0 DPT to 60th DPT. When these values were compared between different groups at varying time intervals, from 15th DPT to 60th DPT, groups II and III revealed significantly lowered values of MCHC as compared to groups I, IV and V, whereas group III had lowest values of MCHC as compared to group I followed by groups II, IV and V at each time intervals. However, no significant difference was recorded between groups I, IV and V at all above time intervals i.e. from 15th DPT to 60th DPT.

The values of TLC in group I did not show any significant variation from 0 to 60th DPT. In groups II and III, the mean values of TLC increased significantly from 0 DPT to 60th DPT. In group IV, mean values of TLC depicted a significant increase was recorded from 0 DPT to 30th DPT only. The TLC values in group V manifested a significant increase from 0 DPT to 60th DPT. When the values of TLC were compared between different groups at particular time intervals, at 15th DPT, groups II, III and V were significantly different as compared to groups I and IV whereas group III had highest values of TLC as compared to group I followed by groups II, V and IV. At 30th, 45th and 60th DPT the mean values of TLC were significantly different between all the groups where group III had highest values of TLC as compared to group I followed by groups II, V and IV.

In group I, the mean heterophil values showed no significant change from 0 DPT to 60th DPT. In groups II and III, mean values of heterophil indicated a significant increase from 0 to 60th DPT in both the groups. In group V, the heterophil values increased significantly till 60th DPT compared to 0 DPT. When the mean values of heterophil were compared between different groups at different time intervals, at 15th DPT, groups II and III had significantly higher values of heterophils as compared to groups I, IV and V, whereas group III showed highest heterophil value as compared to group I, followed by groups II, V and IV, but groups I, IV and V were insignificantly different from each other. From 30th DPT to 60th DPT, groups II, III and V had significantly higher values of heterophil as compared to groups I and IV, whereas group III revealed highest values of heterophils as compared to group I, followed by groups II, V and IV.

The mean percent lymphocyte values did not show any significant change. In groups II and III, the mean values of lymphocyte recorded a significant decrease was from 0 DPT to 60th DPT. In group IV, the lymphocyte values exhibited a significant decrease at 45th DPT as compared to 0 DPT. In group V, the mean lymphocyte values manifested a significant decrease from 0 DPT upto 60th DPT. When the mean values of lymphocyte between different groups were compared at different time intervals, at 15th DPT, groups II, III and V had significantly lowered values of lymphocytes as compared to groups I and IV. The group III revealed lowest value of lymphocytes as compared to group I followed by groups II, V and IV.

The values of monocytes, eosinophils and basophils in all the groups i.e. I, II, III, IV and V did not show any significant change from 0 to 60 DPT. When the mean values of these cells were compared between different groups, there was no significant difference at any time intervals i.e. from 0 to 60th DPT between any groups.

These results of various haematological values are in confirmation with those of El-Demerdash *et al.* (2004); Zhang *et al.* (2005) and Vasiljeva *et al.* (2011). The decrease in Hb, PCV, TEC, MCH, MCHC in the present study might have occurred due to reduced hemoglobin synthesis leading to reduced erythropoiesis (Neuwirt *et al.,* 1976). Decrease in the number of erythrocyte number may also be associated with a direct damaging effect of cadmium on RBCs. Cadmium can accumulate in erythrocytes and affects membrane structure by promoting lipid peroxidation and inhibiting enzymes involved in the removal of specific activated oxygen species (Vasiljeva *et al.,* 2011). The increase in TLC may be due to activation of the immune system (El-Demerdash *et al.,* 2004) as evident by heterophilia. Whereas, alteration in differential leucocyte count may be due to alteration of lymphoid: myeloid ratio (Karmaker *et al.,* 2000). In groups IV and V, lesser decrease in Hb, PCV, TEC, MCH, MCHC and lymphocyte values and significant increase in TLC and heterophils as compared to groups II and III, may be attributed to concurrent feeding of WSRP and its ameliorative effects. It has also been reported that *Withania somnifera* root powder as such or its extract improves hematological parameters in different toxic and normal conditions of animals (Ansari *et al.,* 2013).

It was concluded from the present studies that the administration of 1% WSRP in feed significantly

Table 1:

Hematlogical parameters (Mean±SE) in different groups at different time intervals in chickens.

Group\DPT	Group I	Group II	Group III	Group IV	Group V
Hemoglobin concentration (gm/dl)					
0th DPT	9.79 ±0.005^{Aa}	9.78 ± 0.004^{Aa}	9.78 ± 0.005^{Aa}	9.78 ± 0.006^{Aa}	9.78 ±0.004^{Aa}
15th DPT	9.80 ± 0.006^{Aa}	9.13 ± 0.012^{Bd}	7.87 ± 0.011^{Be}	9.75 ± 0.007^{Bb}	9.71 ±0.006^{Bc}
30th DPT	9.81 ± 0.004^{Aa}	8.22 ± 0.006^{Cd}	7.15 ± 0.008^{Ce}	9.73 ± 0.005^{Bb}	9.66 ±0.006^{Cc}
45th DPT	9.80 ± 0.004^{Aa}	7.15 ± 0.005^{Dd}	6.66 ± 0.006^{De}	9.74 ± 0.004^{Bb}	9.51 ±0.006^{Dc}
60th DPT	9.81 ± 0.004^{Aa}	6.68 ± 0.007^{Ed}	5.96 ± 0.007^{Ee}	9.74 ± 0.004^{Bb}	9.41 ±0.012^{Ec}
Packed cell volume (percentage)					
0th DPT	32.80±0.200^{Aa}	32.60±0.245^{Aa}	32.60±0.45^{Aa}	32.80±0.200^{Aa}	32.60±0.245^{Aa}
15th DPT	32.60±0.245^{Aa}	31.00±0.000^{Bc}	28.20±0.200^{Bd}	32.20±0.200^{Aab}	31.80±0.200^{Bb}
30th DPT	32.20±0.200^{Aa}	29.20±0.200^{Cb}	26.80±0.200^{Cc}	32.40±0.245^{Aa}	31.80±0.200^{Ba}
45th DPT	32.60±0.245^{Aa}	26.80±0.200^{Dc}	25.80±0.200^{Dd}	32.60±0.245^{Aa}	31.60±0.245^{Bb}
60th DPT	32.40±0.245^{Aa}	25.80±0.200^{Ec}	24.20±0.200^{Ed}	32.60±00.245^{Aa}	31.60±0.245^{Bb}
Total erythrocyte count (million/µl blood)					
0th DPT	3.34±0.022^{Aa}	3.33±0.011^{Aa}	3.33±0.011^{Aa}	3.34±0.022^{Aa}	3.38±0.021^{Aa}
15th DPT	3.31±0.007^{Aa}	3.16±0.009^{Bc}	2.89±0.018^{Bd}	3.28±0.026^{Aa}	3.23±0.010^{Bb}
30th DPT	3.31±0.014^{Aa}	2.98±0.028^{Cc}	2.73±0.007^{Cd}	3.31±0.022^{Aa}	3.21±0.017^{Bb}
45th DPT	3.34±0.010^{Aa}	2.74±0.004^{Dc}	2.62±0.007^{Dd}	3.30±0.022^{Aa}	3.19±0.018^{Bb}
60th DPT	3.33±0.009^{Aa}	2.65±0.004^{Ec}	2.45±0.022^{Ed}	3.34±0.018^{Aa}	3.20±0.015^{Bb}
Mean corpuscular hemoglobin (MCH) (pictogram/pg;)					
0th DPT	29.32±0.181^{Aa}	29.34±0.094^{Aa}	29.34±0.094^{Aa}	29.32±0.181^{Aa}	29.32±0.181^{Ca}
15th DPT	29.68±0.110^{Ab}	28.89±0.060^{Bc}	27.23±0.187^{Bd}	29.67±0.214^{Ab}	30.17±0.094^{Aa}
30th DPT	29.64±0.126^{Aab}	27.59±0.256^{Cc}	26.13±0.063^{Cd}	29.37±0.193^{Ab}	29.93±0.068^{ABa}
45th DPT	29.44±0.079^{Aa}	26.13±0.025^{Db}	25.38±0.186^{Dc}	29.49±0.186^{Aa}	29.63±0.172^{BCa}
60th DPT	29.48±0.071^{Aa}	25.17±0.152^{Eb}	24.35±0.207^{Ec}	29.14±0.152^{Aa}	29.43±0.136^{Ca}
Mean corpuscular hemoglobin concentration (MCHC) (g/dl)					
0th DPT	29.84±0.173^{Aa}	30.00±0.226^{Aa}	30.00±0.226^{Aa}	29.84±0.173^{Aa}	29.84±0.173^{Ba}
15th DPT	30.07±0.219^{Aa}	29.43±0.037^{Bb}	27.92±0.192^{Bc}	30.27±0.168^{Aa}	30.38±0.029^{Aa}
30th DPT	30.47±0.186^{Aa}	28.14±0.204^{Cb}	26.68±0.225^{Cc}	30.04±0.225^{Aa}	30.18±0.019^{ABa}
45th DPT	30.09±0.230^{Aa}	26.70±0.191^{Db}	25.79±0.183^{Dc}	29.89±0.220^{Aa}	30.09±0.239^{ABa}
60th DPT	30.27±0.218^{Aa}	25.77±0.108^{Eb}	24.62±0.196^{Ec}	29.87±0.228^{Aa}	29.79±0.232^{Ba}
Total leucocyte count (thousands/µl blood)					
0th DPT	24.20±0.030^{Aa}	24.26±0.096^{Ea}	24.18±0.019^{Ea}	24.19±0.018^{Da}	24.19±0.015^{Ea}
15th DPT	24.62±0.088^{Ad}	26.67±0.084^{Db}	28.56±0.052^{Da}	24.63±0.049^{Cd}	25.07±0.208^{Dc}
30th DPT	24.39±0.011^{Ae}	30.47±0.140^{Cb}	34.43±0.027^{Ca}	25.22±0.039^{Ad}	27.09±0.523^{Cc}
45th DPT	24.34±0.009^{Ae}	34.560±0.034^{Bb}	37.67±0.063^{Ba}	25.23±0.050^{Ad}	29.89±0.398^{Bc}
60th DPT	24.36±0.022^{Ae}	38.52±0.153^{Ab}	40.27±0.139^{Aa}	25.26±0.024^{Ad}	31.17±0.115^{Ac}
Heterophil (percentage)					
0th DPT	25.40±0.245^{Aa}	25.20±0.200^{Ea}	25.40±0.245^{Ea}	25.40±0.245^{Aa}	25.40±0.245^{Ca}
15th DPT	25.80±0.200^{Ac}	29.40±0.245^{Db}	32.60±0.245^{Da}	26.00±0.316^{Ac}	26.00±0.316^{Cc}
30th DPT	25.80±0.200^{Ad}	33.40±0.245^{Cb}	35.60±0.245^{Ca}	26.20±0.200^{Ad}	27.60±0.245^{Bc}
45th DPT	25.60±0.245^{Ad}	35.80±0.200^{Bb}	38.20±0.374^{Ba}	26.20±0.374^{Ad}	28.20±0.374^{ABc}
60th DPT	25.40±0.245^{Ad}	38.40±0.245^{Ab}	41.00±0.316^{Aa}	26.20±0.374^{Ad}	29.00±0.316^{Ac}
Lymphocyte (percentage)					
0th DPT	59.40±0.245^{Aa}	59.20±0.374^{Aa}	59.40±0.245^{Aa}	59.00±0.316^{Aa}	59.20±0.374^{Aa}
15th DPT	59.20±0.374^{Aa}	53.60±0.245^{Bc}	51.40±0.245^{Bd}	58.40±0.245^{ABab}	57.80±0.374^{Bb}
30th DPT	59.00±0.316^{Aa}	50.60±0.245^{Cc}	48.40±0.245^{Cd}	58.40±0.245^{ABa}	54.80±0.374^{Cb}
45th DPT	59.00±0.447^{Aa}	48.40±0.245^{Dd}	45.60±0.316^{De}	58.00±0.316^{Bb}	53.80±0.374^{CDc}
60th DPT	59.00±0.316^{Aa}	45.20±0.374^{Ed}	43.00±0.316^{Ee}	57.80±0.374^{Bb}	53.40±0.510^{Dc}

Different small letters (a, b, c, d and e) indicate significant (P<0.05) difference between groups on a particular day, whereas different capital letters (A, B, C, D and E) indicate significant (P<0.05) difference between days within a particular group.
DPT= Days post treatment

reduced the adverse effects of cadmium administered at the rate of 100 ppm and 200 ppm on hematological parameters in chickens.

ACKNOWLEDGEMENT

Authors are thankful to Dean, College of Veterinary and Animal Sciences, GB Pant University of Agriculture and Technology, Pantnagar for providing financial help to carry out this research work.

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Received on : 27.06.2017
Accepted on : 27.08.2017

Research Article

PROPHYLACTIC EFFICACY OF *PODOPHYLLUM HEXANDRUM* AGAINST HEAT AND COLD STRESS IN RATS

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ABSTRACT

In the present study, ameliorating potential of *Podophyllum hexandrum* against cold and heat stress was investigated in rats. Following exposure to heat and cold stress, animals were found to be dull, depressed and lethargic. Heat exposure produced significant decrease in mean values of haemoglobin, PCV, TLC and TEC, while cold stress caused significant increase in haematological parameters. There was significant decrease in serum total protein level and increase in cholesterol and triglycerides levels in heat and cold stress. The activities of serum ALT and AST in heat and cold stressed rats were significantly increased in comparison to control. The activity of ALP was also significantly decreased. Pre-treatment with *P. hexandrum* rhizome extract @ 25 mg/kg/day for 28 days showed significant reversal in haematological parameters with respect to cold and heat stressed rats.

Key words: Heat and cold stress, hematology, liver function, *Podophyllum hexandrum*, rats.

INTRODUCTION

The "thermoneutral zone" or "zone of comfort" is a particular range of environmental temperature over which an animal does not show any sign of discomfort and uses a minimum amount of metabolic energy to maintain normal body temperature. Within this zone, energetic efficiency is maximized due to minimum energy requirement for maintaining routine body physiology. An external temperature above or below the thermally neutral zone causes heat or cold stress, respectively, and results in increased corticosteroid secretion (Brown and Nester, 1973). Activation of stress system leads to production of glucocorticoids and some by-products and if these by-products are not dealt within a productive way, then they may result in physiological reactions or illness and diseases (Chrousos, 2000) including hypertension (Lawler *et al.,* 1981), behavioural changes (Mercier *et al*, 2003) and immunosuppression (Olson *et al.,* 1997).

Podophyllum hexandrum (family Berberidaceae) thrives in the Himalayan region at 3000-4000 meters altitude. Rhizomes of this plant have been used in Ayurveda against various ailments like nervous disorders, viral and bacterial infections, genital warts, leukemia, Hodgkin's and non-Hodgkin's lymphoma, constipation, common cold etc (Arora *et al.,* 2005). *Podophyllum hexandrum* has several molecules such as quercetin and flavanoids as its phytoconstituents which are well known inhibitors of oxidative stress (Georgetti *et al.,* 2003) and inflammatory response. In view of medicinal properties of the plant, the present study was undertaken to explore its prophylactic efficacy in heat and cold stress in rats.

MATERIALS AND METHODS

Rhizomes of *Podophyllum hexandrum* were collected from the upper hilly areas of Uttarakhand and authenticated by the Department of Biological Sciences G.B.Pant University of Agriculture & Technology, Pantnagar. The dry rhizomes were washed, air dried and coarsely powdered. The powder was soaked in absolute alcohol for 48 hrs with intermittent stirring at 40°C with the help of magnetic stirrer. The infusion was filtered through muslin cloth and centrifuged at 3000 rpm for 15 minutes to get the supernatant. The supernatant was dried to get the alcoholic extract (AL). The left over material was soaked in distilled water for 48 hours with intermittent stirring; supernatant was separated and dried to get the final aqueous extract (AQ). The extracts thus obtained were stored at -20°C.

Sixty albino rats (Sprague Dawley) of 2 to 2.5 months of age, weighing between 150 to 200 gm, were procured from Laboratory Animal Resource Centre, IVRI, Izatnagar and acclimatized for two weeks before the commencement of the experiment. The animals were kept in plastic cages in the experimental laboratory animal shed of the Department of Pharmacology & Toxicology, College of Veterinary and Animal Sciences, Pantnagar, under standard managemental conditions. The experimental protocol was approved by the Institutional Animal Ethics Committee.

Rats were randomly divided into ten groups of 6 rats each- I-Control, II- Aqueous extract (AQ), III- Alcoholic extract (AL), IV- Vitamin C (V), V- Heat stress (H), VI- Aqueous extract + heat stress (AQH), VII- Alcoholic + heat stress (ALH), VIII- Cold stress (C), IX- Aqueous + cold stress (AQC), X- Alcoholic + cold stress (ALC). Dose of *Podophyllum hexandrum* (25 mg/kg orally) was selected based on previous study (Rahal *et al.,* 2009)

Thermal and cold stress were induced in the experimental rats from 21st day to 28th day by exposing them to 42°C and 8˚C, respectively for 8 hours

(Dhanalakshmi *et al.,* 2006). All the rats were sacrificed on 29th day and blood was collected to evaluate haematological and biochemical parameters like total protein, albumin, globulin in plasma along with aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), cholesterol, triglycerides and creatinine estimation in serum using specific diagnostic kits. Data obtained in the present study were statistically compared by analysis of variance.

RESULTS AND DISCUSSION

The rats subjected to heat and cold stress in the present study were dull, depressed and lethargic. Haematological parameters viz., total erythrocyte count, haemoglobin, packed cell volume and total leukocyte counts were found to be elevated significantly in cold stressed rats and decreased significantly in heat stressed rats as compared to control (Table 1).

A significant ($p<0.05$) decrease in the TEC, TLC, Hb and PCV levels was observed in heat stressed rats which is in accordance with previous findings (Okab *et al.,* 2008; Elagib *et al.,* 2008). Lower levels of haemoglobin and other blood parameters in the present study can be attributed to the effect of higher temperature on changes in distribution of iron in the organisms, which can influence the haemopoietic processes (Jamadar and Jalnapurkar, 1995).

Effect of cold is manifested in the form of heat conservation. Heat loss from the body surface is reduced initially by vasoconstriction in the skin vessels and then followed by shivering and nonshivering thermogenesis. There was a significant increase in the TEC, TLC, Hb and PCV levels in cold stressed rats. Several previously reported studies showed that cold exposure can lead to haemoconcentration with increase in haemoglobin, PCV, RBC and WBC which is similar to the present findings in our study (Bokenes *et al.* , 2000).

Both aqueous and alcoholic extracts of *P. hexandrum* rhizome significantly prevented alterations in the hematological profile after seven days of heat and cold stress exposure. These findings suggest that *P. hexandrum* rhizome extracts showed protective effect on the haemopoietic system against heat and cold stress. This increased haemoglobin might be due to the hepato-stimulatory and hepato-protective effects of *P. hexandrum* resulting in the formation of more haemoglobin by the bone marrow which is under the control of erythropoietic factors released by hepatic cells.

Table 1:
Effect of *Podophyllum hexandrum* on hematology and serum enzymatic profile of heat and cold stressed rats

Groups	Dose(mg/kg)	Hb (g/dl)	PCV (%)	TEC (10^6/µl)	ALT(U/L)	AST(U/L)	ALP(U/L)	TLC (10^3/µl)
I	—	12.45±0.35	38.49±1.41	7.64±0.23	27.23±2.53	56.25±4.17	29.46±0.45	9.75±0.24
II	25	12.81±0.54	39.07±1.34	7.48±0.14	26.50±2.25	52.75±1.85	30.43±0.63	9.82±0.26
III	25	13.23±0.28	39.40±0.68	7.53±0.42	27.75±1.63	54.50±2.52	29.07±1.10	9.30±0.34
IV	15	12.67±0.28	38.83±1.58	7.57±0.35	26.20±1.54	50.25±1.74	32.92±1.42	9.27±0.15
V	—	10.56±0.32[a]	31.21±1.62[a]	6.22±0.28[a]	46.24±0.58[a]	86.33±2.13[a]	23.27±0.68[a]	7.75±0.42[a]
VI	25	11.18±0.45	34.18±0.38	6.94±0.39	38.57±1.68	70.75±2.24[b]	27.35±1.35	8.58±0.35
VII	25	11.74±0.36	35.16±1.26	7.14±0.26	32.64±1.25[b]	76.33±3.43	28.83±1.43[b]	8.38±0.37
VIII	—	14.36±0.45[a]	43.82±0.58[a]	9.06±0.19[a]	42.61±2.39[a]	79.75±2.62[a]	24.67±1.32	11.63±0.11[a]
IX	25	13.52±0.37	41.47±0.93	8.42±0.29	36.43±2.15	65.25±1.41[c]	27.62±1..35	10.75±0.17
X	25	13.25±0.47	39.24±0.41	8.29±0.23	32.42±1.09[c]	69.00±4.17	29.26±0.96[c]	10.14±0.22[c]

Values in table are Mean ± SE (n=6), [a]P<0.05vs group I within same column, [b]P<0.05vs group V within same column, [c]P<0.05vs group VIII within same column
Groups (I-Control, II-Aqueous extract, III-Alcoholic extract, IV-Vitamin C, V-Heat stress, VI-Aqueous + heat stress, VII-Alcoholic + heat stress, VIII-Cold stress, IX-Aqueous + cold stress, X-Alcoholic + cold stress)

Table 2:
Effect of *Podophyllum hexandrum* on biochemical parameters of heat and cold stressed rats

Groups	Dose (mg/kg)	Albumin (g/dl)	Globulin (g/dl)	A/G	Total Protein (g/dl)	Cholesterol (mg/dl)	Creatinine (mg/dl)	Triglycerides (mg/dl)
I	—	3.95±0.13	3.23±0.28	1.22±0.21	7.18±0.23	64.62±3.41	0.35±0.07	48.92±1.34
II	25	3.85±0.21	3.27±0.17	1.18±0.14	7.11±0.20	58.18±2.25	0.32±0.01	47.15±1.26
III	25	3.97±0.16	3.15±0.25	1.26±0.13	7.12±0.18	62.86±1.15	0.37±0.06	49.52±2.54
IV	15	3.98±0.22	3.26±0.16	1.22±0.09	7.25±0.19	59.46±2.15	0.38±0.07	43.92±1.17
V	—	3.12±0.28	2.92±0.18	1.06±0.12	6.04±0.24[a]	85.42±2.26[a]	0.42±0.03	76.52±3.62[a]
VI	25	3.37±0.17[b]	3.18±0.24	1.06±0.14	6.55±0.21	70.46±2.63	0.37±0.04	65.27±4.17
VII	25	3.42±0.29	3.21±0.12	1.07±0.25	6.62±0.18	72.17±3.84	0.39±0.05	61.42±4.26[b]
VIII	—	2.97±0.18[a]	3.02±0.18	0.98±0.14	6.00±0.16[a]	92.56±4.79[a]	0.40±0.02	67.71±2.86[a]
IX	25	3.47±0.17	3.14±0.12	1.13±0.07	6.61±0.16	76.53±4.97[c]	0.35±0.02	58.67±1.95
X	25	3.55±0.07	3.17±0.24	1.09±0.08	6.64±0.15	78.64±3.69	0.37±0.03	56.89±2.58

Values in Table are Mean ±SE (n=6); [a]P<0.05 vs group I within same column; [b]P<0.05 vs group V within same column
[c]P<0.05 vs group VIII within same column, Groups (I-Control, II-Aqueous extract, III-Alcoholic extract, IV-Vitamin C, V-Heat stress, VI-Aqueous + heat stress, VII-Alcoholic + heat stress, VIII-Cold stress, IX-Aqueous + cold stress, X-Alcoholic + cold stress)

The present study throws some light on the several biochemical alterations that occur during the process of heat and cold stress in mammals, taking rat as a representative animal. Heat or cold stress is observed when there is an imbalance between heat generation and dissipation within the body.

A significant increase in the activity of AST, ALT and decrease in ALP was recorded in cold and heat stress groups as compared to control (Table 1). Administration of aqueous and alcoholic extract of *P. hexandrum* was able to counteract the effects of stress partially as depicted in the behaviour of the rats. Vitamin C was most effective as the rats of this group were healthy.

The rise in serum levels of AST and ALT in heat and cold stressed rats has been attributed to the loss of structural integrity of hepatocytes. These enzymes are located into the cytoplasm and are released into circulation after cellular damage (Ahmed and Khater, 2001). The increase in enzymatic transaminase activities might also be caused by rise in gluconeogenesis load. Marai *et. al.* (1995) also recorded the increase in transaminase enzyme activities in high ambient summer temperatures. *P. hexandrum* seems to preserve the structural integrity of hepatocellular membrane as evident from the significant reduction in these enzymes in heat and cold stressed rats.

Loss of ALP activity in heat and cold stress is the consequence of changes in the permeability of plasma membrane in addition to changes in the balance between synthesis and degradation of enzyme protein (Hardonk & Koudstaal, 1976), thus lowering the enzyme activity. The hepatoprotective potential of higher dose of *P. hexandrum* rhizome extract was supported by the significant decrease in the level of ALT and AST after 7 days of heat and cold stressed rats. Moreover, *P. hexandrum* rhizome extract also brought the activity of serum ALP near to control. These findings suggest the protective potential of *P. hexandrum* against heat and cold stress.

In the present study, a significant decrease in the level of serum total protein was observed in heat and cold stressed rats, which was restored back to almost normal value by *P. hexandrum* rhizome extract (Table 2). Similar findings have been recorded earlier by Selamoglu and Yurekli (2005). An acute heat stress, results in protein catabolism which leads to decrease in total proteins and increase in non-protein nitrogen and creatinine in blood serum (Vercoe, 1974). This is similar to our findings with increase in creatinine level which indicates protein catabolism.

The reduced serum total protein in cold exposed rats may be due to decrease in secretion of insulin, because insulin has a global effect on protein metabolism (Okab *et al.,* 2008). High adrenergic stimulation generated by exposure of cold for homeothermic animals reduces insulin secretion through α_2-adrenergic receptor which leads to decreased serum protein. It was accompanied by increased concentrations of energy nutrients in sera like glucose, cholesterol, triglycerides and free fatty acids which substantiated the significance of corticosterone in meeting the energy crisis during stress. In the present study, there was significant increase in cholesterol and triglycerides level in both heat and cold stressed rats. Similar results were recorded by Selamoglu and Yurekli (2005). Increased concentrations of glucose, AST and ALT indicated stimulation of gluconeogenetic process (Borges *et al.,* 2004). Decreased levels of total serum proteins during hot ambience (Khan *et al.,* 2002) along with increased creatinine, urea and uric acid levels also confirmed the role of corticosterone as proteolytic hormone. Increased serum lipase could result due to elevated levels of corticosterone as in mammals (Brobst, 1999).

In the present study, cold stress increased the cholesterol level in blood of rats when compared to control groups probably due to an increase in thyroid activity and epinephrine level. Increase in the concentration of independent fatty acids causes accumulation of triglyceride in liver (Gorski *et al.,* 1988) leading to raised triglycerides levels and increased lipid peroxidation as observed in the present study. There was significant reduction in the level of serum cholesterol and triglyceride in the heat and cold stressed rats treated with *P. hexandrum* rhizome extract. In case of albumin and total protein, *P. hexandrum* rhizome extract reduces the negative effect of heat and cold stress but the alteration was not significant as compared to control. All these observations are suggestive of the protective role of *P. hexandrum* on stress induced alterations in the function of liver related to lipid metabolism.

Therefore, it can be concluded from the study that *P. hexandrum* at the dose rate of 25 mg/kg body weight orally prevents heat and cold stress induced haemato-biochemical alterations in rats.

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Received on : 16.08.2017
Accepted on : 19.09.2017

Research Article

PHARMACOKINETICS OF CEFQUINOME IN COW CALVES FOLLOWING SINGLE INTRAMUSCULAR ADMINISTRATION

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ABSTRACT

The disposition kinetics of cefquinome after a single intramuscular administration at dosage level of 2 mg.kg^{-1}b.wt. was investigated in healthy cow calves and an appropriate dosage regimen was calculated. Blood samples were collected at different time intervals and the concentration of drug in plasma was estimated by microbiological assay technique. Pharmacokinetic data after intramuscular administration was best described by one-compartment open model. The peak plasma concentration of cefquinome at 1 hwas 8.19 ± 0.03 ìg.ml^{-1}, which decreased to 0.19 ± 0.001 ìg.ml^{-1} at 18 h. The mean values of absorption rate constant (Ka) and absorption half-life ($t_{1/2Ka}$) were 4.22 ± 0.26 h^{-1}, 0.17 ± 0.01 h, respectively. The mean values of area under curve (AUC), apparent volume of distribution (Vd_{area}),mean residence time (MRT) and total duration of pharmacological effect (td) were 23.64 ± 0.39 ìg.ml^{-1}.h, 0.28 ± 0.01 L.kg^{-1}, 3.61 ± 0.04 h and 6.96 ± 0.09 h, respectively. The mean values of elimination half-life ($t_{1/2â}$) and total body clearance were 2.33 ± 0.03 h and 0.084 ± 0.001 L.kg^{-1}.h^{-1}, respectively. The systemic bioavailability (F) following i.m. administration of cefquinome in buffalo calves was 63.90 ± 1.48 per cent. To maintain a minimum therapeutic concentration of cefquinome as 0.25 µg.ml^{-1}, a satisfactory dosage regimen of cefquinome should be 2.52 mg.kg^{-1}followed by 2.45 mg.kg^{-1}repeated at 12 h intervals.

Keywords: Cefquinome, cow calves, dosage, pharmacokinetics, intramuscular.

INTRODUCTION

Cefquinome, an aminothiazolyl fourth generation cephalosporin, has been developed solely for veterinary use. The advantages of cefquinome over the earlier cephalosporins include extended spectrum â-lactam activity, penetration ability into the periplasmic space of Gram-negative bacteria, enhanced binding with penicillin-binding proteins (Bryskier, 1997) stability against β-lactamases (Limbert *et al.,* 1991) and enhanced bioavailability and potency. It has a broad spectrum of activity and 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. The disposition kinetics of cefquinome has also been investigated in other species of animal (Tiwari *et al.,* 2015; Rana *et al.,* 2015 and El-Sayed*et al.*, 2015). Since, there is absolutely no information available on pharmacokinetics of cefquinomein cow calves, the present study was undertaken to determine the pharmacokinetics of cefquinome in cow calves following a single intramuscular administration.

MATERIALS AND METHODS

Experimental animals

The study was conducted on six male cow calves of age between 6 - 12 months and weighing 70 - 120 kg. The animals were dewormed and kept under observation for 2 weeks of acclimatization before the commencement of the experiment. During the experimental period, the animals were maintained on concentrate and free grazing. Water was provided *ad libitum*. The average day temperature in the shed was about 25^{0}C during the experimental period. The experimental protocol followed the ethical guidelines on the proper care and use of animals and has been approved by the institutional animal's ethics committee.

Bioassay of the drug

The concentration of cefquinome in the plasma was determined by a standard microbiological bioassay technique (Arret *et al.,*1971) using *Escherichia coli* (MTCC739) as the test organism. A solution of cefquinome (0.25 µg.ml^{-1}) was used as standard . These assay plates were incubated at 32°C for 6h. At the end of incubation, the diameter of the zone of inhibition of each well was measured with a Fisher Lilly Antibiotic Zone Reader (Fisher Scientific, New Jersey, USA). For each sample, 9 replicates

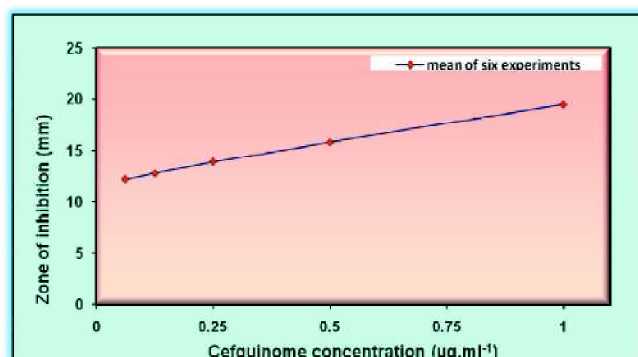

Fig 1
Standard curve of cefquinome in plasma of cow calves. Each point represent the mean of results from 12 assays.

were analyzed. This assay could detect a minimum of 0.05 μg.ml^{-1} of cefquinome. The standard curve of cefquinome was prepared by adding different concentrations of the drug from 0.0625 - 1 μg.ml^{-1}in plasma of cow calves (Fig 1).

Pharmacokinetic variables and dosage regimen

Pharmacokinetic parameters were calculated by the computed least-square regression technique (Gibaldi and Perrier, 1982). Different estimates of the volume of distribution were obtained from the following equation:

$$Vd_{area} = \frac{\text{Dose (mg.kg}^{-1})}{\beta \times AUC}$$

$$Vd_{B} = \frac{\text{Dose (mg.kg}^{-1})}{B}$$

$$Vd_{ss} = \frac{\text{Dose (mg.kg}^{-1}) \times AUMC}{AUC^2}$$

The priming (D) and Maintanance doses (D$'$) of cefquinome, at various dosage intervals, for maintaining differentMICs was calculated from the following equations:

$$D = C_{p(min)}^{\infty} . Vd_{area} (e^{\beta\tau})$$

$$D' = C_{p(min)}^{\infty} . Vd_{area} (e^{\beta\tau} - 1)$$

Where β is the elimination rate constant and τ is the dosage interval.

RESULTS AND DISCUSSION

The plasma concentration of cefquinome at different time intervals following its i.m. administration at the dosage level of 2 mg.kg.$^{-1}$b.wt. was observed (Fig. 2). In the present study, i.m. injection of cefquinome resulted into appreciable plasma concentration of drug (0.24± 0.002 μg.ml^{-1}) at 0.017 h, which gradually increased and peak plasma concentration (C_{max}) of cefquinome (8.19 ± 0.03 μg.ml^{-1}) was reported at 1 h (t_{max}) and the drug level above MIC was detected in plasma upto 10 h. The rapid appearance of drug in plasma indicated fast systemic absorption following i.m. injection. The peak plasma concentration of 7.16 μg.ml^{-1} has been observed in goats (Tiwari *et al.,* 2015) following i.m. administration of cefquinome. The peak plasma cefquinome concentration (8.19 ± 0.03 μg.ml^{-1}) observed in cow calves after i.m. administration was higher than the peak plasma cefquinome concentration observed in sheep (Rana *et al.,* 2015), goat (Dumka*et al.,* 2013) and buffalo calf (Dinakaran*et al.,* 2013).

The plasma concentrations of cefquinome at different time intervals following its i.m. administration at the dosage level of 2 mg.kg^{-1}b.wt. was observed and a semilogarithmic plot of mean plasma concentration-time profile was drawn. The evaluation of the results on observed plasma levels of cefquinome indicated that following i.m. administration, the plasma concentration versus time data can be best fitted to one-compartment open model.

The various pharmacokinetic parameters

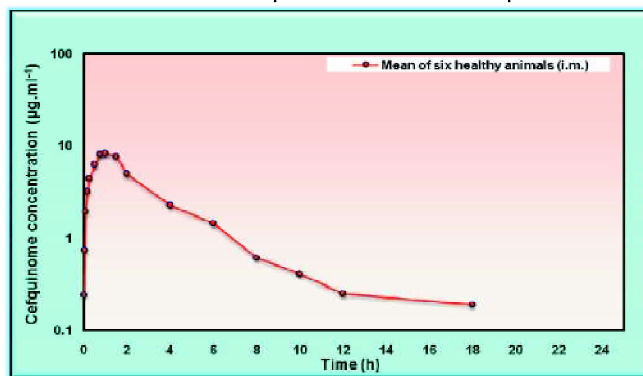

Fig 2
Semilogarithmic plot of cefquinome concentration in plasma versus time following single intramuscular administration at the dose rate of 2 mg.kg^{-1} body weight in healthy cow calves.

Table 1:
Pharmacokinetic parameters of cefquinome after single intramuscular administration at the dose rate of 2 mg.kg^{-1} body weight in healthy cow calves.

Pharmaco-kinetic	Unit	Cow calves number						Mean ± SE
Parameter[a]		C_1	C_2	C_3	C_4	C_5	C_6	
A'	μg.ml^{-1}	8.55	7.88	7.67	8.35	7.50	7.95	7.98 ± 0.16
Ka	h^{-1}	3.06	4.26	4.70	4.50	3.97	4.80	4.22 ± 0.26
$t_{½Ka}$	h	0.23	0.16	0.15	0.15	0.18	0.14	0.17 ± 0.01
B	μg.ml^{-1}	8.44	7.48	7.15	7.84	7.31	7.58	7.63 ± 0.19
β	h^{-1}	0.31	0.30	0.29	0.29	0.29	0.31	0.30 ± 0.004
$t_{½\beta}$	h	2.24	2.31	2.39	2.39	2.39	2.24	2.33 ± 0.03
AUC	μg.ml^{-1}.h	24.43	23.08	23.02	25.18	23.32	22.79	23.64 ± 0.39
AUMC	μg.ml^{-1}.h^2	86.91	82.68	84.67	92.81	86.45	78.53	85.34 ± 1.94
Vd_{area}	L.kg^{-1}	0.26	0.29	0.29	0.27	0.29	0.28	0.28 ± 0.01
Cl_B	L.kg^{-1}.h^{-1}	0.082	0.087	0.086	0.080	0.086	0.088	0.084 ± 0.001
MRT	h	3.36	3.58	3.68	3.69	3.71	3.45	3.61 ± 0.04
td	h	6.70	6.92	7.16	7.16	7.16	6.70	6.96 ± 0.09
C_{max}	μg.ml^{-1}	8.10	8.23	8.16	8.20	8.28	8.28	8.19 ± 0.03
t_{max}	h	1	1	1	1	1	1	1
C_{max}/t_{max}	ratio	8.10	8.23	8.16	8.20	8.28	8.28	8.19 ± 0.03
T/P	ratio	1.24	1.22	1.22	1.22	1.19	1.19	1.21 ± 0.01
AUC/MIC	ratio	97.72	92.08	92.32	100.7	93.28	91.16	94.53 ± 1.55
F	%	67.32	65.40	59.07	67.91	60.43	63.29	63.90 ± 1.48

Table 2:
Intramuscular priming (D)and maintenance (D/) doses of cefquinomein healthy cow calves at various dosage intervals formicroorganisms of different susceptibility.

Microorganisms	Dosage regimen of cefquinome ($mg.kg^{-1}$)				
susceptibility (MIC)[a]	Dose	Dosage interval (h)			
		8	10	12	16
0.0625	D	0.19	0.35	0.63	2.08
	D/	0.17	0.23	0.61	2.06
0.125	D	0.38	0.69	1.26	4.17
	D/	0.34	0.65	1.23	4.13
0.25	D	0.76	1.39	2.52	8.34
	D/	0.69	1.32	2.45	8.27
0.5	D	1.52	2.77	5.04	16.68
	D/	1.38	2.63	4.90	16.54
1	D	3.04	5.54	10.08	33.37
	D/	2.76	5.26	9.8	33.09

[a] Values given are expressed as $\mu g.ml^{-1}$.

ofcefquinome, which describes its absorption and elimination after i.m. administration in cow calves, were calculated on the basis of drug levels in plasma. The mean values of absorption rate constant and absorption half-life were 4.22± 0.26 $h^{-1}$and 0.17± 0.01 h, respectively. The high value of absorption rate constant in the present study, further confirmed that after i.m. administration, the absorption of cefquinomewas very quick. In accordance to the present findings, the rapid absorption of cefquinome following i.m. injection has also been reported in goats and beagle dogs. The absorption rate constant and absorption half-life of cefquinome were 4.57 h^{-1} and 0.15 h in goats (Tiwari *et al.,* 2015) and 5.69 h^{-1} and 0.14 h in beagle dogs (Zhou *et al.,* 2015). In contrast to present findings, lower value of absorption rate constant and high value of absorption half-life of cefquinome have been reported in goats (Rana *et al.,* 2015).

The high values of AUC (23.64 ± 0.39 $\mu g.ml^{-1}.h$) and AUMC (85.34 ± 1.94 $\mu g.ml^{-1}.h^2$) obtained after i.m. administration of cefquinomein cow calves indicated that the vast area of body covered under drug concentration. The value of AUC(23.64 ± 0.39 $\mu g.ml^{-1}.h$) and AUMC (85.34 ± 1.94 $\mu g.ml^{-1}.h^2$) of cefquinomeafter i.m. administrationwas significantly (P<0.01) lower than the value of AUC (37.04 ± 0.60 $\mu g.ml^{-1}.h$)and AUMC (151.28 ± 3.95 $\mu g.ml^{-1}.h^2$) of cefquinome after i.v. injection in cow calves.In agreement to the present findings, same value of AUC (23.78 $\mu g.ml^{-1}.h$) were reportred by other workers (Dumka*et al.,* 2013). In contrast to the present findings, slightly low value of AUC but high value of AUMC was reported in sheep and goats which were 16.65 $\mu g.ml^{-1}.h$ and 157.05 $\mu g.ml^{-1}.h^2$, respectively, in sheep (Rana*et al.,* 2015) and 19.82 $\mu g.ml.^{-1}.h$and 155.85 $\mu g.ml^{-1}.h^2$, respectively, in goats (Dumka *et al.,* 2013). The value of AUC and AUMC obtained in the present study was slightly higher than the AUC (17.16 $\mu g.ml^{-1}.h$) and AUMC (41.89 $\mu g.ml^{-1}.h^2$) reported in goats (Tiwari *et al.,* 2015) following i.m. administration of cefquinome. Whereas, these values of AUC and AUMC obtained in the present study, were much lower than the corresponding values of AUC (52 $\mu g.ml^{-1}.h$) and AUMC (542 $\mu g.ml^{-1}.h^2$) reported in goats (Hamed*et al.,* 2016) following i.m. administration of cefquinome.

The volume of distribution on the basis of area under plasma concentration-time curve (Vd_{area}) was 0.28± 0.01L.kg^{-1}after i.m. injection ofcefquinome. This value ofVd_{area} was significantly (P<0.01) higher than the $Vd_{area}$of cefquinomeafter i.v. injection in cow calves (0.23± 0.01L.kg^{-1}). In agreement to the present observation, low value of Vd_{area}(0.31 L.kg^{-1}) in buffalo calves (Dinakaran*et al.,* 2013). In contrast to the present findings, high value of Vd_{area}(2.07 L.kg^{-1})in sheep (Rana *et al.,*2015) and (2.58 L.kg^{-1}) in goats (Tiwari *et al.,* 2015) was reported following i.m. administration of cefquinome.

The elimination of cefquinomewas slow with elimination half-life ($t_{1/2\beta}$) 2.33± 0.03 h following i.m. administration in cow calves. This value was significantly (P<0.01) lower than the elimination half-life of cefquinomeafter i.v. injection in cow calves (2.89 0.05 h). Similar to our findings, low values of elimination half-life of cefquinome were observed in goats (Tiwari *et al.,* 2015). Whereas, a long elimination half-life has been reported in sheep (Rana*et al.,* 2015).

The total body clearance (Cl_B) of cefquinomein the present study was 0.084± 0.001L.$kg^{-1}.h^{-1}$.This value was significantly (P<0.01) higher than the total body clearance of cefquinomeafter i.v. injection in cow calves (0.054± 0.001L.$kg^{-1}.h^{-1}$). The value of $Cl_B$of cefquinome after i.m. administration obtained in the present study, was lower than the value of Cl_B(0.12L.$kg^{-1}.h^{-1}$) in sheep (Rana *et al.,* 2015), whereas, higher than the values of Cl_B in goats(Tiwari *et al.*, 2015).

The value of MRT of cefquinomein cow calves was 3.61± 0.04 h following its i.m. administration. This value was significantly (P<0.01) lower than the MRT of cefquinome after i.v. injection in cow calves (4.08± 0.07 h). In contrast to the present findings, slightly lower values of MRT have been observed as 2.44 h in goats (Tiwari *et al.*, 2015) and higher values of MRT in sheep (Rana *et al.,* 2015).

Among various pharmacokinetic parameters, bioavailability plays an important role in therapeutic efficacy of a drug. Bioavailability refers to the percent of drug absorbed from an extravascular site of administration into the central compartment for pharmacological action.This parameter determines the relationship between drug dosage and intensity of action. To calculate bioavailability, equal doses of drug are administered by the intravenous and extravascular routes and then the area under the two curves are compared. Cefquinomewas well absorbed following intramuscular administration with systemic bioavailability (F) of 63.90± 1.48 per cent. Similar to our findings, the systemic bioavailability has been reported to

be 57.39 per cent in goats by Dumka *et al.*(2013).

Lack of any significant adverse effect, rapid absorption, good bioavailability, high value of AUC and maintenance of therapeutic plasma concentration for long period of time (up to 10 h) following i.m. injection, suggests that cefquinome is most suitable for i.m. administration for the treatment of mild to moderate bacterial infections in cow calves. However, in severe infections where initially high diffusion gradient is desired, the importance of first injection by i.v. route cannot be ruled out.

The loading and maintenance doses of cefquinometo be repeated at different time intervals by i.m. route, to maintain the minimum therapeutic plasma levels of 0.0625, 0.125, 0.25, 0.50, and 1.0 $\mu g.ml^{-1}$ were calculated in the present study. Thus the dosage regimen of cefquinometo maintain the MIC of 0.25 ìg.ml^{-1} in cow calves, at the dosage interval of 12 h are 2.52 and 2.45mg.kg^{-1}b.wt., respectively, as loading and maintenance dose. These doses are significantly (P< 0.01) higher than the priming and maintenance doses of cefquinome(1.01 and 0.95mg.kg^{-1}b.wt.) in cow calves at 12 h interval by i.v. route. It was revealed that to maintain minimum therapeutic plasma levels, higher dosage is required for i.m. injection as compared to the dosage required for i.v. injection. No other studies have been conducted to determine the dosage regimen of cefquinomefor i.m. injection in cow calves as well as in other animal species.

$A^{/}$ and B, are zero-time plasma concentration intercepts of regression lines of absorption and elimination phases, respectively; Ka and β, absorption and elimination rate constant, respectively, in the mono-exponential equation that describes the plasma concentration-versus-time data; $t_{1/2Ka}$ and $t_{1/2â}$, half-lives of absorption and elimination phases, respectively; AUC, area under the plasma concentration-time-curve; AUMC, area under the first moment of the plasma concentration-time-curve; Vd_{area}, volume of distribution from AUC; Cl_{B}, total body clearance of the drug; MRT, mean residence time of drug in body; td, total duration of pharmacological effect; C_{max}, maximum plasma concentration; t_{max}, time required to attain peak plasma level; T/P, ratio of the drug present in the peripheral to central compartment; and F, per cent of drug availability in the central compartment after extravascular administration.

ACKNOWLEDGMENT

The authors are thankful to the Dean, College of Veterinary Science & Animal Husbandry, Mhow, Nanaji Deshmukh Veterinary Science University,Jabalpur for providing necessary facilities.

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Received on : 06.06.2017
Accepted on : 28.06.2017

Research Article

ASSESSMENT OF LUTEOLIN PRETREATMENT ON SERUM BIOMARKERS AND HISTOPATHOLOGICAL PARAMETERS IN MICE MODEL OF CLP-INDUCED SEPSIS

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ABSTRACT

The present investigation was undertaken to assess the effect of luteolin pretreatment in CLP-induced sepsis in mice. Luteolin was administered intraperitoneally one hour before caecal ligation and puncture (CLP) surgery in mice. Histopathological effects were assessed in heart, kidney, liver and aortic tissues and estimation of serum SGOT and SGPT levels were also done which are indicators of tissue damage. Histopathology of tissues showed reduction in infiltration of inflammatory cells and degenerative changes of tissue with the luteolin pretreatment in septic mice. Level of enzymes SGPT and SGOT was also attenuated in luteolin pretreated septic mice which increases in tissue damage.

Keywords: Histopathology, luteolin, mice, mice, Sepsis.

INTRODUCTION

Sepsis is one of the leading causes of death worldwide in non coronary intensive care units with high incidence and mortality. It is associated with tremendous burden for healthcare system with significant morbidity and mortality due to sepsis-associated multi-organ failure (Gill *et al.,* 2014). It exhibits as a systemic inflammatory response syndrome (SIRS) in the presence of a known or suspected infection. The inability of the circulatory system to provide adequate blood supply to the tissues is a main contributor to the progressive organ failure and resultant high level of mortality (Romand *et al.,* 1994; Zardi *et al.,* 2007). Despite the advances in early care, treatment and management with antibiotics, steroid hormones, fluid resuscitation and vasopressors (Deitch, 1992; Hinshaw, 1996) (Dombrovskiy *et al.,* 2007; Gaieski *et al.,* 2013), the high incidence and mortality rates associated with it underscores the need for further research of effective treatment (Dejager *et al.,* 2011).

There has been considerable attention in recent years on phytochemical drugs of plant origin due to their diverse therapeutic properties. Flavonoids are one of the most effective chemical classes reported to possess wide range of health-promoting activities and pharmacological effects, such as antioxidant, anti-inflammatory, anticancer, neuroprotective, and cardioprotective effects (Daglia *et al.,* 2014; Xue *et al.,* 2014; Nabavi *et al.,* 2015). Luteolin is a common flavonoid found in many edible foods including green pepper, broccoli, olive oil, celery, parsley and dandelion (Kanai *et al.,* 2016). This flavone, luteolin is reported to exhibit various pharmacological activities such as anti-inflammatory (Chen *et al.,* 2014; Kanai *et al.,* 2016), cardioprotective, antidiabetic (Zang *et al.,* 2016) and antioxidant (Xiong *et al.,* 2017). Our study has therefore selected luteolin, a flavonoid to investigate the preventive effect of luteolin against the CLP-induced sepsis. The present study was designed to evaluate the effect of luteolin in CLP-induced sepsis in mice by assessing enzyme activity such as SGPT and SGOT and the histopathological changes in liver, kidney and heart of septic mice.

MATERIALS AND METHODS

Experimental Animals

Apparently healthy adult male Swiss Albino mice (25-30 g) were procured from the Laboratory Animal Resource Section, ICAR-Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh, India. Mice were housed in polypropylene cages with free access to feed and water in the animal house of the Division. All the experimental protocols and procedures were approved by Institute Animal Ethics Committee (IAEC) of the ICAR-Indian Veterinary Research Institute, Izatnagar.

CLP-induction

Mice were fasted overnight before the induction of CLP-induced sepsis but allowed water *ad libitum*. Caecal ligation and puncture to induce sepsis was according to method described by Wichterman and co-workers (1980). CLP surgery was done by using suitable anaesthesia. A ventral midline incision (2-cm) was performed, further caecum was exposed and ligated with 3-0 silk just distal to the ileocecal valve and punctured twice with a 21-gauge needle. The cecum was then returned to the abdomen and the abdominal incision was closed in the layers. All operated mice were given isotonic sodium chloride solution (1 ml/mouse) subcutaneously to prevent dehydration.

Sham-operated mice had undergone the same surgical procedure except CLP. All the surgical maneuvers were carried out according to the procedures laid down by the Institutional Animal Ethics Committee (IAEC).

Dose and treatment schedule

The mice were divided into four groups: Group-I, Sham operated; Group-II, Luteolin+Sham (Lut+Sham); Group- III, sepsis (CLP); Group-IV, Luteolin+sepsis (Lut+CLP). Luteolin was administered intraperitoneally one hour before CLP surgery @ 0.2 mg/kg body weight in Group-IV based on previous study (Kotanidou *et al.,* 2002). Luteolin (0.2 mg/kg BW) was also provided to the Sham animal (Group-II).

Biochemical estimation

Blood samples were collected in BD vacutainer SST for serum separation. Serum was used to estimate enzymes aspartate amino transferase (AST/SGOT) and alanine amino transferase (ALT/SGPT) by diagnostic kits (Coral, Clinical systems).

Histopathological examination of liver, heart, kidney and aortic tissue

Liver, heart, kidney and aortic tissues were collected in 10% neutral buffered formalin. The tissues were processed to obtain 4-5 ìm thick paraffin embedded sagittal sections. The sections were stained by hematoxylin and eosin (H&E) technique (Luna, 1972) for microscopic evaluation of lesions.

Drugs and chemicals

Luteolin was purchased from Cayman Chemicals, USA. Luteolin stock was prepared in alcohol and further diluted with isotonic normal saline. All other chemicals were of analytical grade.

Statistical analysis

Results of the different experiments are expressed as mean±SEM. The mean values from different groups were analyzed by one way ANOVA followed by Tukey's Post-hoc Test using Graph Pad Prism Version 5.0. software.

RESULTS

Effect of luteolin on serum alanine amino transferase (ALT/SGPT) level

Figure 1 shows a significant (p<0.01) increase in SGPT level in septic mice (37.83±3.08 U/L; n=3) in comparison with sham animal (20.95±2.01 U/L; n=3). The luteolin pretreated septic mice showed significant (p<0.05) decrease (26.19±2.01 U/L; n=3; p<0.05) in serum SGPT level in comparison with septic mice (37.83±3.08 U/L; n=3). There was no significant change in luteolin pretreated sham mice SGPT level (21.53±1.54 U/L; n=3) compared to sham mice.

Effect of luteolin on serum aspartate amino transferase (AST/SGOT) level

A significant (p<0.001) increase was observed in SGOT level of CLP-induced septic mice (324.8±5.23 U/L; n=3) in comparison to sham operated mice (86.72±5.91 U/L; n=3). Luteolin pretreated septic mice showed significant (p<0.01) attenuation in serum SGOT level (202.5±35.05 U/L; n=3) in comparison with septic mice. Luteolin pretreated sham mice (99.52±8.61 U/L; n=3) did not show any significant change in enzyme level compared to sham mice (Figure 2).

Effect of luteolin on histopathology of heart tissue

Histopathology of heart section in sham-operated mice and luteolin pretreated sham operated showed intact normal architecture of heart. Sepsis showed marked myocarditis characterized by marked infiltration of mononuclear cells between myofibres along with myocardial necrosis as compared to the sham, Lut+Sham

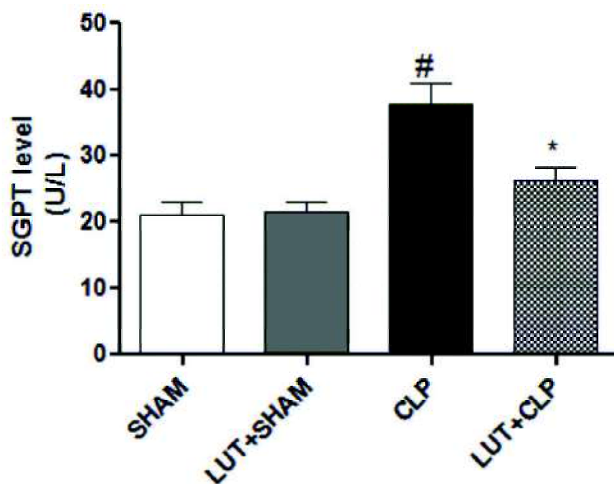

Figure 1:
Effect of luteolin on serum SGPT level in different groups of mice. Data were analysed by one-way ANOVA followed by Tukey's multiple comparison test. $^{\#}p$<0.01 in comparison with sham. $^{*}p$<0.05 in comparison with sepsis (CLP) group.

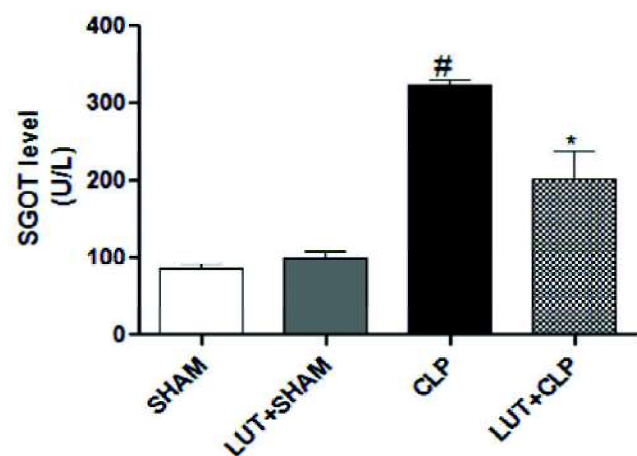

Figure 2:
Effect of luteolin on serum SGOT level in different groups of mice. Data were analysed by one-way ANOVA followed by Tukey's multiple comparison test. $^{\#}p$<0.001 in comparison with sham. $^{*}p$<0.01 in comparison with sepsis (CLP) group.

and Lut+sepsis group. Heart section of luteolin pretreated septic mice showed less infiltration of inflammatory cells between myofibres compared to the septic group of mice (Figure 3A-D).

Effect of luteolin on histopathological parameters of kidney tissue

The kidney section of septic mice showed marked infiltration of inflammatory cells around the bowman's capsule, reduced glomerular content along with the degenerative changes in the tubules as compared to sham operated mice which showed normal architecture of kidney. Luteolin pretreated mice showed improved histoarchitecture of kidney shown by the intact bowman's capsule with glomerulus and reduced infiltration of inflammatory cells in the tubules compared to septic mice (Figure 4A-D).

Effect of luteolin on histopathology of liver tissue

Histopathology of liver section of the luteolin pretreated sham operated mice showed normal histoarchitecture of liver similar to sham mice. However, sepsis group of mice showed acute hepatitis with marked infiltration of inflammatory cells in hepatic parenchyma along with degenerative changes. The luteolin pretreated mice showed reduced infiltration of inflammatory cells in hepatic parenchyma (Figure 5A-D).

Effect of luteolin on histopathology of aortic tissue

In aortic tissue section, septic group of mice showed aortic inflammation characterized by marked infiltration of inflammatory cells in the tunica adventitia and tunica media of aorta as compared to the normal architecture of sham group. Luteolin pre-treated mice showed reduced infiltration of inflammatory cells as compared to septic mice group (Figure 6A-D).

DISCUSSION

Increased in level of SGPT is a sensitive indicator of acute liver damage and elevation of this enzyme may

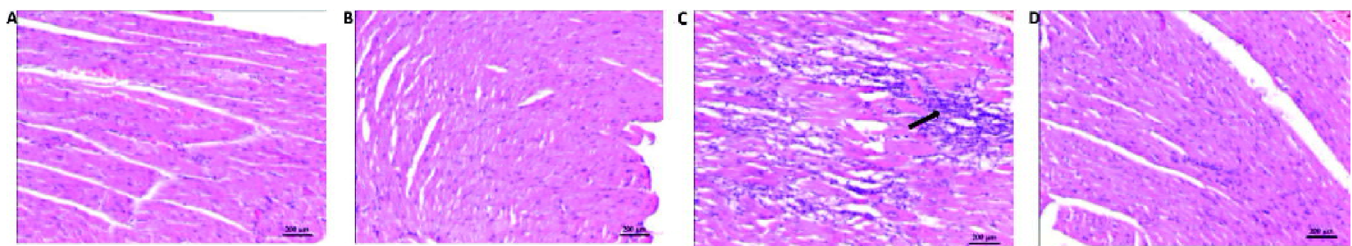

Figure 3

(A) SHAM group of mice showing normal histology of heart. X100 H&E. (B) LUT+SHAM group of mice showing intact normal architecture of heart, X100 H&E. (C) CLP group of mice showing myocarditis characterized by marked infiltration of mononuclear cells between myofibres (thin arrow) along with myocardial necrosis. X100, H&E. (D) LUT pretreated mice showing less infiltration of inflammatory cells between myofibres . X100, H&E.

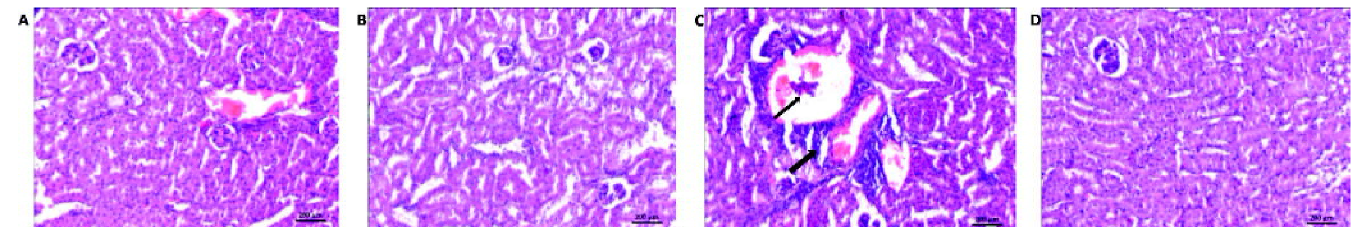

Figure 4

(A) SHAM group of mice showing normal architecture of kidney, X100,H&E. (B) LUT+SHAM showing the intact architecture of kidney, X100, H&E. (C) CLP group of mice showing marked infiltration of inflammatory cells around the Bowman's capsule (thick arrow),reduced glomerular content (thin arrow) along with the degenerative changes in the tubules. X100 H&E. (D) LUT pretreated mice showing improved histoarchitecture of kidney shown by the intact Bowman's capsule with glomerulus and reduced infiltration of inflammatory cells in the tubules. X100 H&E.

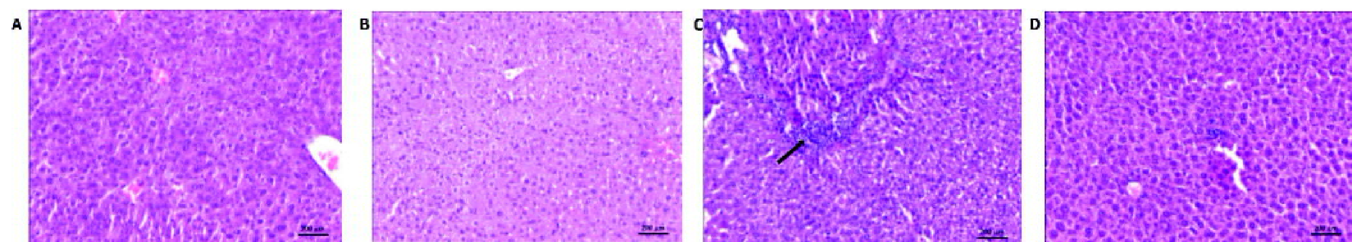

Figure 5

(A) SHAM group of mice showing normal histoarchitecture of liver, X100, H&E. (B) LUT+SHAM show normal architecture of liver, X100, H&E. (C) CLP group of mice showing acute hepatitis with marked infiltration of inflammatory cells in hepatic parenchyma along with degenerative changes (arrow) X100 H&E. (D) LUT treated mice showing reduced infiltration of inflammatory cells in hepatic parenchyma, X100 H&E.

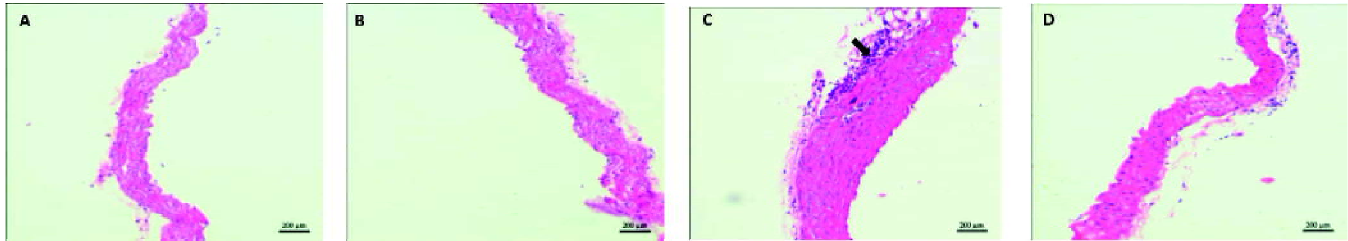

Figure 6
(A) SHAM group of mice show normal architecture of the aorta. H&E, X100. (B) LUT+SHAM group of mice showing normal histoarchitecture of aorta without any appreciable gross lesions. H&E, X100. (C) CLP group of mice showing marked infiltration of inflammatory cells in the tunica adventitia region of aorta (as indicated by arrow). H&E, X100. (D) LUT pre-treated mice showing reduced infiltration of inflammatory cells as compared to sepsis group with improved histoarchitecture. H&E, X100.

due to damage of the tissue producing acute hepatic necrosis (Bigoniya and Singh, 2013). Thus, estimation of ALT/SGPT which is predominantly found in hepatocytes and other tissues is a relied biomarker of hepatotoxicity and liver abnormalities (Geelani *et al.,* 2015). Aspartate amino transferase (AST/SGOT) is a cytoplasmic enzyme which catalyzes the reductive transfer of an amino group from aspartate to á-ketoglutarate to yield an oxaloacetate and glutamate. SGOT is present in liver, also kidney and cardiac muscle. Therefore, elevated levels of AST signify injury to tissues like liver and also heart, brain and kidney (Dufour *et al.,* 2001). In agreement to our result, serum levels of liver enzymes SGOT and SGPT have been reported to increase significantly during early sepsis (Cameron *et al.,* 1981). Wang and co-workers (1993) also reported increase in SGPT and SGOT level at 10 hr, and the levels increased further at 20 hr post-CLP. Luteolin treatment in CCl_4 hepatic damage decreased the elevated levels of SGPT, SGOT which may be a consequence of the stabilization of plasma membrane as well as repair of hepatic tissue damage caused by CCl_4 (Thabew *et al.,* 1987). In accordance to previous report, luteolin pretreatment in septic mice showed significant change in enzyme activity of SGPT and SGOT.

Many cytokines are released by leukocytes and renal tubular cells in damaged kidney and are important components of both initiation and extension of inflammation in acute kidney injury. Histopathological analysis of kidney in CLP mice reported a widespread damage of tubules, epithelial necrosis and vacuolation of cytoplasm with inclusions and casts (Lingaraju *et al.,* 2015). In this study, luteolin pretreated mice showed intact bowman's capsule with glomerulus and reduced infiltration of inflammatory cells in the tubules compared to septic mice.

Present study suggested that luteolin pretreatment significantly reduced the inflammatory cells in the liver tissue. Previously, it has been reported that luteolin pretreatment normalized the CCl_4-induced changes having only focal coagulative necrosis, zonal necrosis and slight fatty changes with normal hepatocyte (Bigoniya and Singh, 2013). The luteolin pretreated mice in the present study also showed reduced infiltration of inflammatory cells in hepatic parenchyma.

Heart section of luteolin pretreated septic mice in our study showed less infiltration of inflammatory cells between myofibres compared to the septic mice. Histopathological evaluation in different experimental models of myocarditis showed significant reduction in infiltration of inflammatory cells/infiltration in myocardium by other flavonoid apigenin (Zhang *et al.,* 2016) and quercetin treatment (Marina *et al.,* 2010).

Histopathology of tissues showed reduction in infiltration of mononuclear cell and degenerative changes of tissue with the luteolin pretreatment in septic mice. The observation of the present study taken together, suggests that CLP resulted in pronounced infiltration of inflammatory cells leading to tissue damage with increase activity of marker enzymes SGPT and SGOT implying tissue damage and luteolin pretreatment in sepsis attenuated the mononuclear infiltration.

ACKNOWLEDGEMENT

Authors are thankful to the Director, ICAR-Indian Veterinary Research Institute, Izatnagar for providing the necessary facilities for the completion of this work.

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Received on : 26.07.2017
Accepted on : 29.09.2017

Research Article

EVALUATION OF HEPATOPROTECTIVE EFFECT OF AQUEOUS EXTRACT OF *MURRAYA KOENIGII* IN HEPATOTOXIC RAT MODEL

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ABSTRACT

The study was planned to evaluate hepatoprotective effect of aqueous extracts of *Murraya koenigii* following repeated oral administration for 28 days in carbon tetrachloride induced hepatotoxic rats by determining haematological and biochemical parameters and Histopathological examination. Thirty six male albino Wistar rats divided equally into six groups. Group I served as vehicle control and received normal saline solution, Group II served as hepatotoxic control and received 50 % carbon tetrachloride in olive oil at the dose rate of 1 ml/kg body weight, i.p. twice in a week throughout the study period. Rats of group III, IV, V and VI also received 50 % carbon tetrachloride in olive oil at the dose rate of 1 ml/kg body weight, i.p., twice in a week throughout the study period for induction of hepatotoxicity. Group III received standard drug silymarin @ 50 mg/kg of body weight (p.o.) daily once for 28 days of dosing period. Group IV, V and VI received aqueous extracts of *M. koenigii* @ 100, 200 and 400 mg/kg, daily once for 28 days of dosing period. *M. koenigii* at all dose levels showed significant (P<0.05) reduction in serum ALT, AST, GGT, ALP, bilirubin, creatinine kinase and serum creatinine and increase in serum albumin, globulin and total protein level and restored histological structure as compared to CCl_4 treated rats in dose dependent manner. The findings of present study suggested that aqueous extracts of *M. koenigii* revealed hepatoprotective effect at dose dependent manner in a 28 days study in rats.

Key words: Carbon tetrachloride, hepatoprotective effect, *Murraya koenigii,* rats.

INTRODUCTION

Liver is the main organ where exogenous chemicals are metabolized and eventually excreted and also it involves in many other biochemical and physiological processes. As a consequence, liver cells are exposed to significant concentrations of chemicals or many toxicants, which can result in liver dysfunction, cell injury and even organ failure. Liver cell injury caused by various toxicants such as certain chemotherapeutic agents, carbon tetrachloride, thioacetamide, chronic alcohol consumption and microbes is well-studied. Numerous studies noted that CCl_4 is widely used to induce liver damage because it is metabolized in hepatocytes by cytochrome P_{450} generating a highly reactive carbon-centered trichloromethyl free radical, leading to initiating a chain of lipid peroxidation and thereby causing liver fibrosis (Fang *et al.,* 2008). Large many number of reports are found with regard to use of CCl_4 induced hepatotoxic rat model for hepatotoxicity studies. Silymarin is most commonly used standard hepatoprotective drug in experimental animals. In the last few decades there has been an exponential growth in the field of herbal medicine. Plant based medicine is getting popularized in developing and developed countries owing to its natural origin and lesser side effects and used in India since time immemorial. *Murraya koenigii* (English- Curry Leaf Tree; Hindi- Mitta Neem; Gujarati: Mitho Limdo) belongs to the family Rutaceae, and is extensively used in food preparation like for flavouring curries, chutneys, soups and pickles in India and several Asian countries (Birari *et al.,* 2010). The present study is intended to explore whether the aqueous extracts of leaves of *M. koenigii* could have protective effect on hepatocytes in albino Wistar rats which may contribute to the development of new formulation for the treatment of liver disease in animals.

MATERIALS AND METHODS

Experimental Animals

The study was conducted on adult healthy male albino Wistar rats. Rats of 8-12 weeks of age were procured from Zydus Research Centre (ZRC), Moraiya, Ahmedabad, Gujarat. Protocol for this study was designed as per CPCSEA guidelines and was approved by IAEC vide Ref. No. VPT/198/2015.

Preparation of plant extracts

Leaves of *M. koenigii* were collected from the local area of Anand district (Gujarat) after authentication by Botanist, Department of Genetics and Plant Breeding, B.A. College of Agriculture, AAU, Anand. Leaves were washed thoroughly, chopped and air dried under shade for 7 days and then crushed to pulverize in an electric grinder. The dried leaves were extracted in Soxhlet extractor with water separately for 8 hours. The extract was then reduced to a dark colored molten mass by using rotary evaporator under reduced pressure.

Evaluation of acute oral toxicity

The acute oral toxicity studies of aqueous extracts of *M. koenigii* was carried out as per OECD guideline No. 423. Albino Wistar rats were taken for the study and dosed once with 2000 mg/kg, orally. The treated rats were

monitored for sign of toxicity and mortality for the first critical 4 hours and thereafter daily up to 14 days for general clinical signs and symptoms, as well as mortality. Aqueous extract of *M. koenigii* did not reveal any toxic effect on rats even at 2000 mg/kg doses, respectively. On this basis, the dose were selected for this study.

Experimental design

Carbon tetrachloride induced hepatotoxicity in rats was used to study hepatoprotective effects of aqueous extract of *M. koenigii*. After 1 hour fasting, all the animals except vehicle control group were administered 1 ml/kg (i.p.) carbon tetrachloride in olive oil (1:1) twice in a week, throughout the study period (28 days) and then after 1 hour all those rats were administered test compounds (Yadav *et al.,* 2016). Silymarin used as a positive control, was prepared in 0.9 % saline solution and administered at 50 mg/kg of body weight (p.o.) to animals of group III. Aqueous extracts of *M. koenigii* were dispersed in water using the same amount of saline solution and administered to animals of group IV, V and VI at dose of 100, 200 and 400 mg/kg (p.o.), respectively, for 28 days of dosing period. Vehicle control group rats were administered normal saline solution (NSS) orally, once daily for 28 days. On 29th day, blood samples were collected from the retro-orbital puncture for hematological and serum was separated for biochemical analysis.

Hematological and biochemical parameters

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

Histopathology

On 29th day of study, all the rats from each group were sacrificed by cervical dislocation and subjected to post mortem examination for gross and histopathological lesions. Liver and kidneys were collected and examined after opening the body of sacrificed rats. For histopathological examinations, tissues from liver and kidneys were collected in 10 % formalin and preserved for processing. The formalin fixed tissues were processed by paraffin wax embedding method of tissue sectioning. Sections were cut at 5–6 µ thickness with automatic section cutting machine (Leica, Automatic Microtome Machine, Germany), and were stained with hematoxylin and eosin (H and E) for microscopic examination.

Statistical analysis

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

RESULTS

No mortality has been observed during experimental period. No behavioural changes were observed in any treatment group and vehicle control group till end of experimental period. Rats of hepatotoxic control group demonstrated dull, depressed and anorectic changes along with reduced body weight gain and sluggishness in fourth week of experiment. There was significant decrease in TEC, Hb, PCV, MCV, MCH, MCHC, TLC and platelets in hepatotoxic control group II as compared to vehicle control group I. There was significant ($P<0.05$) increase in all these parameters in silymarin treated group III and *M. koenigii* extract treated group VI (400 mg/kg) as compared to hepatotoxic control group II (Table-1). There was significant ($P<0.05$) increase in ALT, AST, GGT, ALP, CK, bilirubin and creatinine level in group II as compared to vehicle control group I. There was significant decrease in these biochemical parameters level in treatment control group III and *M. koenigii aqueous* extract treated group IV (100 mg/kg), group V (200 mg/kg) and group VI (400 mg/kg) as compared to group II in dose dependent manner (Table-2). There was significant decrease in serum albumin, globulin, total protein in group II as compared to group I. There was significant ($P<0.05$) increase in these serum biochemical parameters in treatment groups III, IV, V and VI as compared to group II group in dose dependent manner (Table-2). Liver collected from rats of vehicle control group had normal colour whereas rats of hepatotoxic control group II showed paleness and diffuse necrotic foci. Liver sections from the rats treated with CCl_4 alone (Group II) showed massive changes throughout the lobules, with sinusoidal dilatation, cellular vacuolization necrosis, distortion of the central venules and ballooning of hepatocytes (Figure 1). Sylimarin treated group (Group-III) showed normal hepatocytes without any damage. The treatment of rats with *M. koenigii* (100 mg/kg) revealed more or less intact central vein but still widened sinusoidal spaces. The treatment of rats with *M. koenigii* (200 mg/kg) showed fatty degeneration, less necrotic foci and sinusoidal dilatation, whereas, treatment of rats with *M. koenigii* (400 mg/kg) showed moderate dilatation of sinusoidal spaces (Figure 2) and restored normal histoarchitecture of liver section. Microscopic changes in

Table 1:
Effect of *M. koenigii* on hematological parameters (Mean ± SE) in different experimental groups (n=6)

Groups	TEC (10^6/µl)	Hb (g/dl)	PCV (%)	MCV (fl)	MCH (pg)	MCHC (g/dl)	TLC (10^3/µl)	PLT (10^5/µl)
I	8.08 ±0.37^{e}	15.56±0.72^{g}	43.87±1.02^{g}	54.69 ±1.66^{h}	19.28 ± 0.36^{f}	42.35±0.70^{f}	8.88±0.27^{e}	9.30 ± 0.04^{j}
II	6.15 ±0.24^{a}	8.62 ± 0.70^{a}	26.57 ±1.33^{a}	29.77 ±1.02^{a}	12.92 ±0.36^{a}	27.61 ±0.69^{a}	4.62 ±0.41^{a}	5.43 ± 0.05^{a}
III	7.70±0.09^{de}	14.45 ±0.49^{fg}	41.75 ±0.32^{g}	51.77±2.49^{gh}	18.32±0.17^{ef}	41.09 ± 0.69^{f}	8.72 ±0.22^{e}	8.60 ± 0.02^{i}
IV	6.22 ± 0.24^{a}	9.89 ± 0.10^{ab}	28 ± 1.73^{abc}	38.13 ± 2.91^{bc}	13.7 ±1.12^{ab}	32 ± 0.73^{c}	5.33 ± 0.17^{ab}	6.59 ± 0.03^{d}
V	6.93±0.21^{abcd}	11.07±0.36^{bcd}	29.18±0.35^{abcd}	37.82 ± 1.10^{bc}	13.78 ± 0.55^{ab}	35.13 ± 0.67^{d}	6.43 ± 0.19^{c}	6.48 ± 0.04^{c}
VI	7.6 ± 0.23^{de}	11.6 ± 0.50^{cd}	30.95±0.56^{cde}	33.83 ± 1.17^{ab}	14 ± 0.27^{ab}	37.83 ± 39^{e}	7.3 ± 0.14^{d}	6.39 ± 0.02^{bc}

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

Table 2:
Effect of *M. koenigii* serum biochemical parameters (Mean ± SE) in different experimental groups (n=6)

Groups	ALT (U/l)	AST (U/l)	GGT (U/l)	Albumin(g/dl)	Globulin (g/dl)	Total Protein (g/dl)	ALP (U/l)	CK (U/l)	Serum bilirubin (mg/dl)	Serum creatinine (mg/dl)
I	66.47±1.88^{a}	152.17±3.13^{a}	24.76±1.41^{a}	4.47 ±0.10^{b}	3.53 ±0.14^{f}	8.00 ± 0.23^{f}	168.98 ± 5.69^{a}	673.02 ± 6.63^{a}	0.21 ± 0.07^{a}	0.35 ± 0.03^{a}
II	481.6±2.91^{h}	338.83±9.13^{i}	85.91±3.57^{g}	3.58 ±0.11^{a}	2.14 ±0.13^{a}	5.72 ± 0.11^{a}	433.64 ± 4.48^{h}	1036.33 ± 4.19^{g}	1.51 ± 0.14^{e}	1.29 ± 0.08^{f}
III	92.90±1.45^{b}	165.67±1.48^{b}	37.71±1.76^{b}	4.44 ±0.10^{b}	3.14±0.06^{ef}	7.59 ± 0.05^{ef}	190.65 ± 4.83^{b}	676.62 ± 6.58^{a}	0.27 ± 0.03^{ab}	0.51 ± 0.02^{ab}
IV	269.46±4.13^{g}	247.5±3.15^{gh}	61.38± 0.70^{e}	4.28± 0.13^{b}	2.38±0.13^{abc}	6.67 ± 0.13^{bc}	330.88 ± 4.55^{g}	938.22 ± 4.39^{f}	0.71 ± 0.06^{cd}	0.79 ± 0.03^{de}
V	195.62±2.89^{e}	234.33±1.76^{ef}	50.71± 1.79^{d}	4.39± 0.09^{b}	2.6±0.06^{bcd}	6.99 ± 0.11^{cd}	254.7 ± 3.91^{d}	751.33 ± 4.21^{c}	0.60 ± 0.04^{bcd}	0.67 ± 0.04^{bcd}
VI	176.75±4.58^{d}	207.67±3.42^{d}	44.07± 1.45^{c}	4.35± 0.11^{b}	2.93±0.19^{de}	7.29 ± 0.29^{de}	207.24 ± 2.99^{c}	730.5 ± 4.34^{b}	0.42 ± 0.16^{abc}	0.63 ± 0.02^{bcd}

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

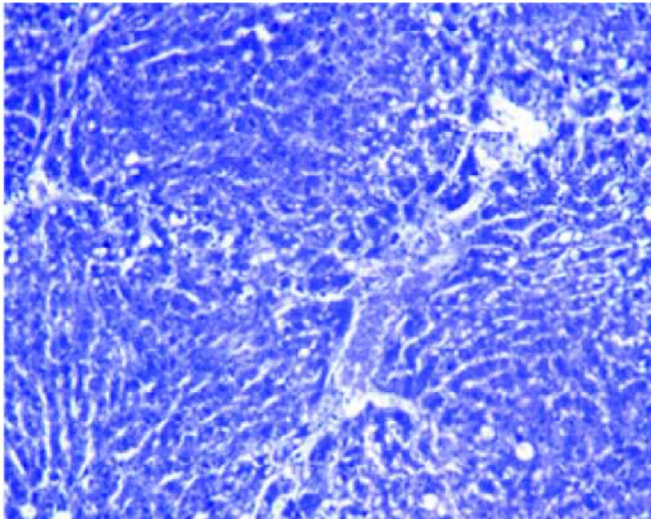

Fig.1:
Section of liver of rat from hepatotoxic control rats (group II) showing vacuolation in hepatocytes (H& E stain X 120)

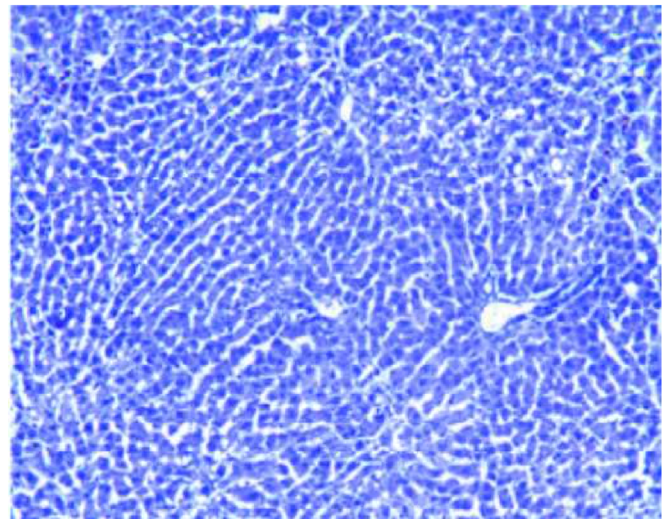

Fig.2:
Section of liver from *M. koenigii* treated group (group VI) showing moderate dilatation of sinusoidal spaces, mild distortion of central veinule and ballooning of hepatocytes (H & E stain X 120).

kidneys of rats from group II revealed congestion with degeneration and necrosis of renal tubular epithelium, congestion with cloudy swelling in tubular cells. Silymarin treated group (III) showing almost normal structure of renal tubules. Kidneys of rats of other groups revealed moderate to mild congestion and degenerative changes in dose dependent manner in histological structure. Section of spleen of rats of hepatotoxic control group II showed lesions like mild congestion and hemorrhage with multifocal area of necrosis and mild lymphoid depletion whereas rats of groups III, IV, V and VI did not reveal any distinct microscopic changes in spleen.

DISCUSSION

At the end of study, there was significant decrease in RBCs, Hb and PCV of rats of hepatotoxic control group as compared to vehicle control group. There was significant increase in RBCs, Hb and PCV of hepatotoxic rats receiving aqueous extracts of *M. koenigii* @ 400 mg/kg and silymarin @ 50 mg/kg body weight as compared to rats of hepatotoxic control group. Similarly, Ponmozhi and Ramya (2015) found similar results on anti-anemic activity of

alcoholic extract of *M. koenigii* leaves (dose @ 500 mg/kg of b. wt., p.o. for day 2 to 15) on phenylhydrazine induced anemia in rats. Choudhury and Sinha, (2015) also found similar results on effect of aqueous extract of *M. koenigii* (dose @ 250 mg/kg and 500 mg/kg b. wt. once daily orally for 7 days) on hematological parameter in albino rats. Likewise, there was significant decrease in MCV, MCH and MCHC of rats belonging to hepatotoxic control group (29.77 ± 1.02 fl, 12.92 ± 0.36 pg and 27.61 ± 0.69 g/dl) as compared to vehicle control (54.69 ± 1.66 fl, 19.28 ± 0.36 pg and 42.35 ± 0.70 g/dl) rats. There was significant increase in MCV and MCHC of hepatotoxic rats receiving aqueous extracts of *M. koenigii* and silymarin @ 50 mg/kg body weight as compared to rats of hepatotoxic control group. Similarly, significant increased MCV and MCHC were also reported by Ponmozhi and Ramya (2015) and Choudhury and Sinha (2015) for *M. koenigii* treated group. Likewise, there was significant decrease in TLC and platelets of rats belonging to hepatotoxic control group (4.62 ± 0.41 × 10^3/µl and 542.5 ± 4.88 × 10^3/µl, respectively) as compared to vehicle control rats (8.88 ± 0.27 × 10^3/µl and 930.83 ± 3.61 × 10^3/µl, respectively). There was significant increase in TLC and platelets of hepatotoxic rats receiving aqueous extracts of *M. koenigii* (200 and 400 mg/kg) and silymarin at dose rate of 50 mg/kg body weight as compared to rats of hepatotoxic control group. Similarly, Ramchandran *et al*. (2015) reported a significant dose related increase in the total WBC count in methanolic extract of *M. koenigii* leaves (250, 500 and 750 mg/kg, p.o.) treated group as compare to control group.

In the present study, there was significant increase in ALT, AST and GGT level in carbon tetrachloride induced hepatotoxic control group (481.6 ± 2.91 U/l, 338.83 ± 9.13 U/l and 85.91 ± 3.57 U/l, respectively) as compared to vehicle control group (66.47 ± 1.88 U/l, 152.17 ± 3.13 U/l and 24.76 ± 1.41 U/l, respectively). This was due to the release of these enzymes from hepatic parenchymal cells, which were indicating a considerable hepatocellular injury mainly free radical injury of hepatocytes caused by CCl_4 (Bishayee *et al.,* 1995). In present study all extract treated group and treatment control group (silymarin 50 mg/kg) showed significant decrease ALT, AST and GGT as compared to hepatotoxic control group. Similarly, Ghosh *et al.* (2012) reported aqueous leaf extract of *M. koenigi* treated group showed significantly increased ALT and AST compared to control group in dose dependent manner. Sathaye *et al.* (2012) also reported that administration of *M. koenigi* (1 g/kg and 2 g/kg) in ethanol induced hepatotoxic rats showed significant decrease in ALT compared to hepatotoxic control group. Naidu *et al.* (2014) also found similar results showed that CCl_4 caused substantial raise in levels of ALT and AST which was reversed by administration of methanolic extract of *M. koenigii* in dose dependent manner. Shaikh *et al.* (2015) also reported significant reduction in levels of the ALT and AST in methanolic extract of *M. koenigii* treated group compared to carbon tetrachloride induced hepatotoxic rats. Similarly, Pande *et al*. (2009) studied acetone extract of *M. koenigii* bark significantly reduced the ALT and AST level as compared to hepatotoxic control group.

In the present study, there was significant decrease in serum albumin, globulin and total protein level in carbon tetrachloride induced hepatotoxic control group (3.58 ± 0.11 g/dl, 2.14 ± 0.13 g/dl and 5.72 ± 0.11 g/dl, respectively) as compared to vehicle control group (4.47 ± 0.10 g/dl, 3.53 ± 0.14 g/dl and 8.00 ± 0.23 g/dl, respectively). Similarly, Shaikh *et al.* (2015) reported a significant increase in the serum albumin and total protein level in methanolic extract of *M. koenigii* treated group compared to hepatotoxic control group. Present study indicated that the group of rats given standard reference compound silymarin at 50 mg/kg body weight significantly increased serum albumin, globulin and total protein level in hepatotoxic rats. Hepatotoxic rats receiving aqueous extracts of *M. koenigii* at doses of 100, 200 and 400 mg/kg body weight for 28 days showed significant increase in serum albumin, globulin and total protein level as compared to rats of hepatotoxic control group except *M. koenigii* (100 mg/kg) for globulin level only. In the present study, there were significant increase in alkaline phosphatase (ALP) and creatinine kinase (CK) in carbon tetrachloride induced hepatotoxic group (433.64 ± 4.48 U/l and 1036.33 ± 4.19 U/l, respectively) as compared to vehicle control group (168.98 ± 5.69 U/l and 673.02 ± 6.63 U/l, respectively). Sathaye *et al.* (2012) showed that significant increase in ALP value in hepatotoxic group as compared to vehicle control group also reported aqueous extract of *M. koenigii* revealed hepatoprotective activity.

Histological examination of sections of liver of rat from hepatotoxic control group showed massive changes throughout the lobules, with sinusoidal dilatation, cellular vacuolization, necrosis and distortion of the central venules, ballooning of hepatocytes. Similarly, Shaikh *et al.* (2015) found that liver sections from the rats treated with CCl_4 (2 ml/kg, i.p.) showed massive changes throughout the lobules, with mononuclear infiltration around portal triad with sinusoidal dilatation, cellular vacuolization, necrosis and inflammatory infiltrations of the portal triads and distortion of the central venules. Sathaye *et al.* (2012) reported fatty changes in few cells in case of 6% ethanol induced liver toxicity model in rats whereas administration of aqueous extract of *M. koenigii* showed hepatoprotective activity. Naidu *et al.* (2014) found that carbon tetrachloride treated liver sections showed disruptions in central vein, distended sinusoidal spaces, fatty degeneration, necrosis and vacuole formation. They found that administration of *M. koenigii* could reduce disruption of central vein, score of fatty degeneration, necrosis and hepatic injury in dose

dependent manner. Findings of present study agree with observations of Shaikh *et al.* (2015) for *M. koenigii* (50, 100 and 200 mg/kg of b. wt. orally for 14 days) against carbon tetrachloride induced hepatotoxicity in rats. They found that treatment of rats with *M. koenigii* (50 mg/kg) revealed lymphocytic collection in sinusoidal space. The treatment of animals with *M. koenigii* (100 mg/kg) showed near normal hepatocytes and occasional cell with nuclear condensation. Whereas, treatment with *M. koenigii* (200 mg/kg) and sylimarin showed normal hepatocytes without any damage (Shaikh *et al.*, 2015).

In the present study it is proved that aqueous extract of *M. koenigii* has hepatoprotective activity in dose dependent manner and it may be due to presence of various phytocomponents. In fact, further investigation is needed to know the hepatoprotective effects of particular phytocomponents of *M. koenigii* and its mechanism of action.

ACKNOWLEDGEMENT

Authors are thankful to the Dean/Principal, College of Veterinary Science & Animal Husbandry, Anand Agricultural University, Anand for the financial support to carry out this research work. Authors also acknowledge Dr. D.J. Ghodasara for support in histopathological examination of tissues.

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Received on : 25.10.2017
Accepted on : 28.11.2017

Research Article

ISOLATION, IDENTIFICATION AND ANTIBACTERIAL ACTIVITY OF ENDOPHYTIC BACTERIA ISOLATED FROM BABOOL LEAVES

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ABSTRACT

Natural products are naturally derived metabolites or by-products from microorganisms, plants and animals. These products have been exploited for human use for thousands of years and plants have been the chief source of compounds used for medicines. Endophytes are microorganisms that colonize internal tissue of plant. Interactions between plants and endophytic bacteria are mutualistic. Plants provide nutrient for bacteria and bacteria will protect plant from pathogens, helps in phytohormone synthesis, nitrogen fixation and also increase absorption of minerals. The aim of the study was to isolate, identify and to assess antibacterial activity of endophytic bacteria isolated from babool (*Acacia nilotica*) leaves. 18 endophytic bacteria were isolated and identified by sequencing of 16S rRNA gene. The isolated endophytic bacteria were identified as *Staphylococcus arlettae*. Considerable antibacterial activities against a panel of human pathogenic microorganisms (*Staphylococcus aureus*, *Streptococcus pyogenes*, *Bacillus cereus*, *Klebsiella pneumoniae, Salmonella Typhimurium* and *Escherichia coli*) were recorded in this study.

Keywords: Acacia nilotica, endophytic bacteria, 16S rRNA gene

INTRODUCTION

Plants are one of the most vital sources of medicines. Currently, large numbers of drugs in use are derived from plants. Medicinal plants are the chief source of secondary metabolites used as drugs and essential oils of therapeutic importance. The important advantages of medicinal plants for therapeutic uses in various ailments are their safety and also being inexpensive, effective, and their easy accessibility. Endophytic bacteria living in plant tissues deprived of doing substantive harm or gain benefit other than residency.The term endophyte (Gr. endon, within; phyton, plant) was first coined in 1866 by De Bary . An endophyte can be defined as a microorganism such as fungi or bacteria that expends either the complete or part of its lifecycle within the healthy tissues of a living plant, typically causing no symptoms of disease. Endophytic microorganisms that reside in the tissues of living plants are promising, less explored and useful sources of novel natural products for exploitation in medicine, agriculture, and industry (Anjum and Chandra, 2015).

The importance of endophytes had been demonstrated over a long period as a source of pharmaceutical bioactive compounds, as many of endophytes were exposed to produce novel bioactive metabolites such as antibacterial, antifungal, antiviral, antitumor, antioxidant, anti-inflammatory, immuno-suppressive drugs and many related compounds. Endophytes are well known for the production of various classes of natural products and have been reported to exhibit a broad range of biological activity and are grouped into various categories, which include alkaloids, terpenoids, steroids, lactones, phenolic compounds, quinones, lignans, etc. Importantly, secondary metabolites produced by endophytes provide a variety of fitness enhancements and exert several beneficial effects on host plants, such as stimulation of plant growth, nitrogen fixation and induce resistance to drought, herbivorous, parasitism etc. and as sources of novel biologically active secondary metabolites. Endophytes enter the plant tissue chiefly through the root zone; on the other hand, above ground portions of plants, may also be used for entry, such as cotyledons, stems, and flowers (Rosales et al., 2017).

Acacia nilotica (*Fabaceae* family) is one of the medicinal important species of the genus Acacia that grows naturally in North Africa and naturalized in all drier parts of India. *Acacia nilotica* is a pioneer species that is relatively fast growing on arid sites. Useful parts such as root, bark, leaves, flower, gum, pods etc. are used in medicines. Different parts of the plant like leaves and fruit contain tannin, flower contains stearic acid, isoquercetin, pod contains tannin and polyphenolic compounds, gum contains Arabic acid combined with calcium, magnesium and potassium. In traditional medicine, it is used for bleeding diseases, prolapsed, leucorrhoea, antibacterial, antifungal, antioxidant etc (Kaushal, 2017). Various investigators reported endophytic microbes from various plant exists in different ecosystems. Consequently, the opportunity to find new and interesting microorganism among myriads of plants in different ecosystems is great. Previously, numerous reports showed on diversity of endophytic bacteria, fungi in medicinal plants (Strobel *et al.,* 2004), but from available literature there is no report on endophytic bacteria from *Acacia nilotica* particularly in

India.

MATERIALS AND METHODS

Collection and Sterilization of babool leaves

Fresh leaves of babool (*Acacia nilotica*) were procured from Department of Botany, Jawaharlal Nehru Krishi Vishwavidyalaya, Jabalpur, M.P. Mature healthy plant leaves were collected. Eighteen leaves samples were taken and separated for further isolation of endophytic bacteria. Samples were immediately brought to laboratory and were used within 24 hrs and finally processed for isolation of endophytic bacteria. The leaves were surface sterilized with 0.1% sodium hypochlorite for 5 minutes, 0.01% Bavistin, 0.05% streptomycin, 70% ethanol, followed by five times rinses in double distilled water for 5 minutes. Leaves were dried in laminar flow (Mahajan *et al*., 2014).

Sterility check

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 plated on to nutrient agar media and incubated at 37°C for 24 hrs. Bacterial growth were observed after 24 hrs.

Preparation and sterilization of media

King's B (KB) media (HiMedia), Mueller Hinton media (HiMedia), Blood agar media (HiMedia) and BHI broth (HiMedia) 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 and isolation of endophytic bacteria

The media were 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 Gram's staining. After that a single colony was transferred into BHI broth and incubated at 37°C for 24 hours.

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 colony was transferred into BHI broth and then incubated at 37°C for 24 hrs and purity was checked by Gram's staining and stored for further work.

Characterization of endophytic bacteria

The characterization was done on the basis of staining, morphological, biochemical and molecular methods.

Staining

Gram's staining was done where thin smears of obtained cultures were prepared on separate glass slides, air dried and heat fixed. Each smear was covered with crystal violet and allowed to stand for 1 minute followed by washing with distilled water. Further Gram's iodine was added and allowed to stand for 1 minute. Decolourized with 95% ethyl alcohol and the slide was rinsed with distilled water. Slide was flooded with safranin to counterstain and allowed to stand for 1 minute. Smear was rinsed with distilled water and dried with absorbent paper. Slides were then observed at 100X.

Morphological characterization

Form, elevation, margin, surface, opacity and chromogenesis of isolated endophytic bacterial colonies were noted for morphological characterization.

Biochemical and molecular characterization

Various biochemical tests like catalase (Slide method), coagulase (Tube method), Voges-Proskauer, ONPG, urease, arginine utilization and sugar fermentation tests (Hi media Identification test kit) were performed.

DNA isolation– An overnight grown isolated endophytic bacterial colony were picked and resuspended it in 1 ml of autoclaved water in a microfuge tube. Later centrifuged for one minute at 10,000-12,000 rpm. Supernatant was removed and 200 µl of Instagene matrix was added to the pellet and incubated at 56°C for 15-30 minutes. Centrifuged pellet was vortexed for 10 seconds and then tube was placed in a 100°C boiling water bath for 8 minutes. Vortexed at high speed for 10 seconds. Spinned at 10,000-12,000 rpm for 2-3 minutes. Finally isolated DNA was stored at -20°C till further use.

To perform molecular identification of bacteria, marker gene for 16S rRNA was used. The PCR used universal primers 8F (5´AGAGTTTGATCCTGGCTCAG3´) and 1541R (5´AAGGAGGTGATCCAGCCGCA3´) to amplify approximately 1500 bp of 16S rRNA gene (Edward *et al*., 1989). PCR results were then visualized by electrophoresis and purified using Montage PCR clean up kit (Millipore). Data sequencing was done at Triyat Genomics, Nagpur, Maharashtra. Sequencing results were compared with existing sequences using Basic Local Alignment Search Tool program on National Centre for Biotechnology Information site (www.ncbi.nlm.nih.gov) to obtain the homology. The program MUSCLE 3.7 was used for multiple alignments of sequences. Finally, the program PhyML 3.0 aLRT was used for phylogeny analysis and HKY85 as substitution model.

Antibacterial assay

In vitro antibacterial activity of endophytic bacteria isolated from *Acacia nilotica* were studied. Various known pathogenic bacterial cultures viz. *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 700603), *Salmonella typhimurium* (ATCC 13311), *Bacillus cereus* (ATCC 11778), *Staphylococcus aureus* (ATCC 6538) and *Streptococcus pyogenes* (ATCC 12386) were procured from Hi media. Mc-Farlands nephlometer was used for the

determination of concentration of known culture as described by Henric *et al*. (1956). 1 ml of known culture containing 3.0× 10^9 cfu/ml as determined by Mc-Farlands nephlometer was used for antibacterial activity of endophytic bacteria. For determination of antibacterial activity of endophytic bacteria, broths were centrifuged at 4°C at 12000 rpm for 30 minutes. Supernatant of each of these broths were taken, sterile disc 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 discs were used as control drug to compare the effect of treatment during *in vitro* study. 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. Result were recorded as positive (growth) or negative (no growth) and zone of inhibition of growth exerted by these impregnated discs.

RESULTS

Isolation of endophytic bacteria

18 isolates of endophytic bacteria were obtained and were characterized using gram's staining (Table 1).

Gram's staining

Endophytic bacteria isolated from *Acacia nilotica* from showed that 100 per cent isolates shown gram positive reaction and were found to be cocci.

Morphological characterization

Growth characteristics of endophytic bacteria isolated from *Acacia nilotica* showed that 67 per cent were circular in shape while 33 per cent irregular in shape, 84 per cent were raised elevation on petriplate while 16 per cent were flat elevation, margin of 67 per cent colonies were entire while 33 per cent were undulated, the surface of the growth was smooth for the entire colonies and 96 per cent growth were opaque and white in colour (Table 2).

Biochemical and molecular characterization

The endophytic bacteria isolated from *Acacia nilotica* had shown positive reaction to catalase, ONPG, urease and sugar fermentation tests and negative reaction to coagulase, VP and arginine utilization test (Table 2 and Table 3).

Endophytic bacteria were identified by comparing 16S rRNA gene sequences obtained from bacterial isolates and those deposited in (NCBI) GenBank database using BLAST tool to obtain the sequences that displayed maximum similarity. Results of this analysis showed that one given sequence was actually similar to those

Table 2
Catalase and Coagulase reaction of endophytic bacteria isolated from *Acacia nilotica*

Sl.No.	Isolate No.	Catalase test	Coagulase test
1	JN-1a	Positive	Negative
2	JN-1b	Positive	Negative
3	JN-1c	Positive	Negative
4	JN-1d	Positive	Negative
5	JN-1e	Positive	Negative
6	JN-1f	Positive	Negative
7	JN-2a	Positive	Negative
8	JN-2b	Positive	Negative
9	JN-2c	Positive	Negative
10	JN-2d	Positive	Negative
11	JN-2e	Positive	Negative
12	JN-2f	Positive	Negative
13	JN-3a	Positive	Negative
14	JN-3b	Positive	Negative
15	JN-3c	Positive	Negative
16	JN-3d	Positive	Negative
17	JN-3e	Positive	Negative
18	JN-3f	Positive	Negative

Table 1
Growth of endophytic bacteria isolated from *Acacia nilotica* on Kings B media

S.No.	Isolate No.	Form	Elevation	Margin	Surface	Opacity	Chromo-genesis
1	JN-1a	Circular	Raised	Entire	Smooth	Opaque	Absent
2	JN-1b	Circular	Raised	Undulated	Smooth	Opaque	Absent
3	JN-1c	Irregular	Raised	Entire	Smooth	Opaque	Absent
4	JN-1d	Circular	Raised	Entire	Smooth	Opaque	Absent
5	JN-1e	Irregular	Flat	Undulated	Smooth	Opaque	Absent
6	JN-1f	Circular	Raised	Entire	Smooth	Opaque	Absent
7	JN-2a	Circular	Raised	Entire	Smooth	Opaque	Absent
8	JN-2b	Circular	Raised	Undulated	Smooth	Opaque	Absent
9	JN-2c	Irregular	Raised	Entire	Smooth	Opaque	Absent
10	JN-2d	Circular	Raised	Entire	Smooth	Opaque	Absent
11	JN-2e	Irregular	Flat	Undulated	Smooth	Opaque	Absent
12	JN-2f	Circular	Raised	Entire	Smooth	Opaque	Absent
13	JN-3a	Circular	Raised	Entire	Smooth	Opaque	Absent
14	JN-3b	Circular	Raised	Undulated	Smooth	Glistening	Absent
15	JN-3c	Irregular	Raised	Entire	Smooth	Opaque	Absent
16	JN-3d	Circular	Raised	Entire	Smooth	Opaque	Absent
17	JN-3e	Irregular	Flat	Undulated	Smooth	Glistening	Absent
18	JN-3f	Circular	Raised	Entire	Smooth	Opaque	Absent

Table 3
Other biochemical tests of endophytic bacteria isolated from *Acacia nilotica*

Sl.No	Isolate No.	VP test	ONPG test	Urease test	Arginine utilization test	Sugar utilization test		
						Sucrose	Maltose	Lactose
1	JN-1a	Negative	Positive	Positive	Negative	Positive	Positive	Positive
2	JN-1b	Negative	Positive	Positive	Negative	Positive	Positive	Positive
3	JN-1c	Negative	Positive	Positive	Negative	Positive	Positive	Positive
4	JN-1d	Negative	Positive	Positive	Negative	Positive	Positive	Positive
5	JN-1e	Negative	Positive	Positive	Negative	Positive	Positive	Positive
6	JN-1f	Negative	Positive	Positive	Negative	Positive	Positive	Positive
7	JN-2a	Negative	Positive	Positive	Negative	Positive	Positive	Positive
8	JN-2b	Negative	Positive	Positive	Negative	Positive	Positive	Positive
9	JN-2c	Negative	Positive	Positive	Negative	Positive	Positive	Positive
10	JN-2d	Negative	Positive	Positive	Negative	Positive	Positive	Positive
11	JN-2e	Negative	Positive	Positive	Negative	Positive	Positive	Positive
12	JN-2f	Negative	Positive	Positive	Negative	Positive	Positive	Positive
13	JN-3a	Negative	Positive	Positive	Negative	Positive	Positive	Positive
14	JN-3b	Negative	Positive	Positive	Negative	Positive	Positive	Positive
15	JN-3c	Negative	Positive	Positive	Negative	Positive	Positive	Positive
16	JN-3d	Negative	Positive	Positive	Negative	Positive	Positive	Positive
17	JN-3e	Negative	Positive	Positive	Negative	Positive	Positive	Positive
18	JN-3f	Negative	Positive	Positive	Negative	Positive	Positive	Positive

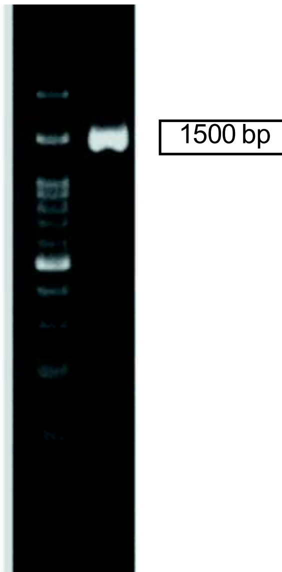

Fig 1
Agarose gel electrophoresis of 16S rRNA gene Polymerase Chain Reaction product of endophytic bacteria isolated from *Acacia nilotica* leaves
Lane L : 100 bp ladder
Lane S : PCR product

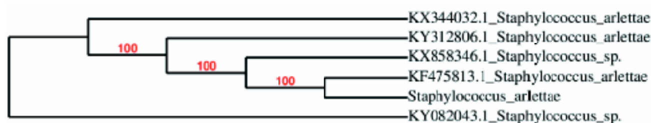

Fig 2
Construction of Phylogenetic tree

sequences of more than one species. The amplified PCR products were run on agarose gel electrophoresis using 2.0 per cent agarose gel in 1x Tris Acetate EDTA Buffer, where the fragments with 1500 bp were observed. Based on 16S rRNA sequence analysis, endophytic bacteria isolated from *Acacia nilotica* were identified as *Staphylococcus arlettae* (Fig 1 and 2).

***In vitro* antibacterial activity of isolated endophytic bacteria**

The endophytic bacteria isolated from *Acacia nilotica* shown antibacterial activity as 70 per cent isolates inhibited growth of *Staphylococcus aureus.*

DISCUSSION

The isolation of endophytic bacteria was in agreement with the findings of Baghat *et al*. (2014). They reported that the 90 per cent of isolated endophytic bacteria from *Capparis sinaica* were gram positive in nature. The biochemical characterization of endophytic bacterial isolates showed that all the endophytic bacterial isolates from *Acacia nilotica* were catalase positive and coagulase negative. Isolates which were coagulase negative were suggestive that isolates were of non-pathogenic in nature. Based on 16S rRNA sequence analysis which had revealed that the endophytic bacterial isolates from *Acacia nilotica* were found to be *Staphylococcus arlettae*. The result in the study indicated that distribution of endophytic bacteria mainly depended on environmental conditions such as temperature, humidity, UV irradiation and nutrients in the apoplast and not only on host (Sun *et al*., 2013). Out of 18 isolates, 12 were effective against *Staphylococcus aureus*. This antibacterial effect was seen in our experimentation which may be due to presence of metabolites which suggested that these metabolites of endophytic bacteria might had diffused in the culture medium and suppressed the growth of pathogenic bacteria. Sunkar and Nachiyar (2013) had found antibacterial activity of endophytic bacteria isolated from *Brassica oleracea* and the isolated endophyte was showing significant antimicrobial activity against *Klebsiella pneumoniae* NCIM 2883 (20 mm),

Staphylococcus aureus ATCC 25923 (25 mm), *Escherichia coli* ATCC 25922 (13 mm) and *Salmonella typhi* ATCC 6539 (12 mm). Thus, endophytic becteria isolated from babool revealed anti bacterial activity against certain human pathogenic organisims in this study.

ACKNOWLEDGEMENT

The authors are thankful to the Dean, College of Veterinary and Animal Sciences and Director of Research Services , N.D.V.S.U, Jabalpur for providing necessary facilities.

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Received on : 12.12.2017
Accepted on : 28.12.2017

INFLUENCE OF EXPERIMENTALLY-INDUCED ENDOMETRITIS ON REDOX STATE AND COMPARATIVE MODULATORY EFFICACY OF *EUCALYPTUS ROBUSTA* LEAVES CRUDE EXTRACT AND CEFIXIME IN RATS

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ABSTRACT

The present study was undertaken to evaluate modulatory effect of *Eucalyptus robusta* leaves crude extract and cefixime on oxidative stress induced after experimentally-induced endometritis in female wistar rats. Isolates obtained from clinical cases of endometritis and maintained in laboratory by subculturing the isolates were inoculated as 1 x 10^6 cfu/ml of *E. coli* and 1 x 10^8 cfu/ml of *Staphylococcus aureus* and were used for inducing uterine infection and inflammation i.e. induction of endometritis. Results of the present study did not show any alterations on absolute and organ weights of liver, kidney, spleen and brain and hematological parameters, however absolute and relative weights of uteri of endometritis group were significantly higher. Data revealed significantly higher levels of malondialdehyde in endometritic rats in all tissue, levels of reduced glutathione was significantly reduced in endometritis in liver and brain while the levels in kidneys and spleen were not altered. Activities of antioxidant enzymes (SOD and catalase) found to be significantly higher in all the organs/ tissues of rats of endometritis group while catalase activity was significantly higher in spleen and brain and non-significantly higher in liver and kidneys. Treatment with *Eucalyptus robusta* leaves extract and cefixime revealed that crude extract was more effective in maintaining the redox-state of tissues as compared to cefixime.

Key words: Wistar rats, Oxidative stress, endometritis, *Eucalyptus robusta*, cefixime

INTRODUCTION

Uterine diseases, such as metritis and endometritis, are highly prevalent in high-producing dairy cows and lead to economic losses because of decreased milk yield and fertility (Sheldon *et al.,* 2009). Clinical endometritis is very well characterized by purulent or mucopurulent uterine discharge, whereas Subclinical endometritis is a condition characterized with more than 18% polymorphonuclear (PMN) cells in uterine discharge. Bacterial infections play an important role in the complex etiology of these diseases. The most frequently isolated bacterial strains are *Escherichia coli* and *Trueperella pyogenes*, along with anaerobic bacteria, such as *Fusobacterium necrophorum* and *Prevotella melaninogenica* (Santos *et al.*, 2010). Besides these, *Streptococcus bovis* I*, Streptococcus bovis* II, *Streptococcus equines, Bacillus licheniformis,* β-*haemolytica* are responsible for endometritis, but *Escherichia coli* is considered to be the most predominant one. Although more than 70% of cows clear the uterine bacteria via innate immune responses, 17% to 37% of cows develop clinical endometritis, whereas 14% to 53% develop subclinical endometritis (Gilbert *et al.*, 2005).

Dynamic analysis of total oxidant capacity with subclinical endometritis causes alteration of endogenous antioxidants such as vitamin E and vitamin C, total oxidant capacity (TOC) and nitric oxide (NO) (Song *et al.* 2015). The production of reactive oxygen species, during the process called oxidative burst, plays a critical role in bacteria killing. Oxidant/antioxidant markers in the blood of buffalo-cows having uterine infection showed increased malondialdehyde (MDA) and decreased catalase (CAT), ascorbic acid (ASCA), reduced glutathione (R-GSH) and total antioxidant capacity (TAC) (Hanafi *et al.* 2008). Heidarpour *et al.* (2013) reported oxidative stress and trace elements before and after treatment in dairy cows with clinical and subclinical endometritis and suggested that decreases in antioxidant capacity and in trace elements (copper and zinc) could be associated with subsequent oxidative stress and increased susceptibility to endometritis and that endometritis resolution was related to the antioxidant and copper status and to decrease in oxidative stress.

Dairy cows seemed to have more oxidative stress and low antioxidant defense during early lactation or just after parturition than advanced pregnant cows causing uterinal disorders (Sharma *et al.*, 2011).

Some of the commonly used antibacterials in veterinary practice for treatment of endometritis are oxytetracycline, penicillins, aminoglycocides, quinolones and cephalosporins. These antimicrobial therapies have varying degrees of success, inconsistent recovery rate,

high cost of treatment, milk disposal problem after treatment, and emergence of microbial resistance (Santos *et al.*, 2010). Many plans have been reported to have antimicrobial properties (Dixit *et al.,* 2013; Watcho *et al.,* 2011). Eucalyptus plant extract has been reported to possess antimicrobial property against *Bacillus subtilis*, *Salmonella typhi*, *Staphylococcus aureus*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, *E. coli*, *Enterococcus faecalis* and *Proteus mirabilis* (Hossam *et al.*, 2008). Apparently, not much of information is available on use of medicinal plants based drugs against endometritis in animals including experimental animals.

Therefore, studies on antibacterial efficacy of plants and their antioxidant potential are necessary for developing novel diagnostic tools and therapeutic targets to address the oxidative stress induced during endometritis.

MATERIALS AND METHODS

Experimental animals

Healthy adult female Wistar rats weighing 180-250 gm were procured from Laboratory Animal House, Department of Veterinary Pharmacology & Toxicology, DUVASU, Mathura. Before start of the experiment, animals were kept in laboratory conditions for a period of seven days for acclimatization and maintained under standard managemental conditions with daily 12 hours of light-dark cycle along with *ad libitum* balanced pelletted feed (Ashirwad feed industries, Chandigarh) and clean drinking water. All the animals were kept under constant observation during entire period of study. Study was taken up after the approval of IAEC no. 97/IAEC/15 in compliance of CPCSEA guidelines.

Plant material

Leaves of *Eucalyptus robusta* Smith (Myrtaceae), locally known as Safeda, were collected from Veterinary University Campus, Mathura and identified taxonomically by Dr. Tariq Husain, Head and Scientist, Plant Diversity, Systematic and Herbarium Division at Council of Scientific and Industrial Research-National Botanical Research Institute, Lucknow, India (Specimen Voucher No. LWG-73) on the basis of the taxonomic features of the plant material.

Preparation of hot methanolic extract of leaves of Eucalyptus robusta

Hot methanolic extract of shade dried and coarsely powdered leaves of *Eucalyptus robusta* was prepared using soxhlet apparatus by hot percolation method. About 40-45 grams coarsely ground powder of leaves were placed in a porous cellulose thimble in soxhlet apparatus (capacity 500 ml). Thimble was placed in extraction chamber above the flask containing methanol. Flask was heated and the solvent was allowed to evaporate. Extraction process was allowed to continue for several hours (approximately 48 hrs), involving 20-22 cycles till the solvent siphon tube of extractor became almost colourless. Thereafter, the extract obtained was concentrated in a rotatory vacuum evaporator under reduced pressure to obtain semisolid mass and per cent yield was calculated. The extract was transferred into air tight containers and stored in refrigerator till used.

Isolation

Twenty samples of uterine discharge were collected in sterile swabs in test tubes from clinical cases of endometritis from cows and buffaloes presented in Teaching Veterinary Clinical Complex for isolation, identification and characterization of different bacteria involved in clinical endometritis in bovines.

Samples were transferred to sterilized 1ml nutrient broth and kept in incubator at 37ºC for 24 hours. After incubation period, broth was transferred on nutrient agar media and again kept for incubation at 37ºC for 24 h. Single colonies developed on nutrient agar media were sub-cultured in selective media (Eosine methylene blue agar media for *E. coli* and Mannitol salt agar media for *Staphylococcus aureus*) and kept again for incubation at 37ºC for 24 h.

Identification of bacteria

For identification of Gram- positive and Gram-negative bacteria Gram's staining was done as per standard procedure. After that specific biochemical tests (Indole, Methyl-Red, Voges-Prousker and Citrate) were also performed to identify and characterize the bacteria involved in clinical endometritis.

Experimental endometritis rat model

Stages of estrus cycle in rats were determined examining the vaginal smear. Rats in diestrus stage of cycle were selected for inducing endometritis as per the method of Nishikawa *et al.* (1985). Most common bacterial isolates (*E. coli* and *S. aureus*) from clinical bovine endometritic cases were used for induction of uterine infection in rats. Isolates were maintained in laboratory by subculturing the isolates. Suitable load of bacterial isolates (1×10^6 cfu/ml of *E. coli* and 1×10^8 cfu/ml of *Staphylococcus aureus*) in sterilized normal saline were used for inducing uterine infection and inflammation i.e. induction of endometritis in rats after undertaking pilot studies using first single bacterial culture and thereafter mixed bacterial infection.

After determining the diestrus stage of estrous cycle, rats were anesthetized using a combination of xylazine (@10 mg/kg body weight) and ketamine (@ 80 mg/kg body weight). A longitudinal incision in lower abdomen was given and uterus was exposed under sterilized conditions. Uterine horns were ligated at the cervical end to prevent possible leakage of leukocytes and bacteria through the cervical canal. 100μl of bacterial

suspension (load 1 x 10^6 cfu of *E. coli* and 1 x 10^8 cfu of *S. aureus*) was inoculated into uterine lumen of both the horns near the utero-tubal junction using a tuberculin syringe having 27-gauge needle. Abdomen was closed using proper surgical procedure and animals were allowed to recover from anaesthesia. Surgical procedure in sham-operated animals was same except for inoculation of bacterial suspension.

Experimental design

Thirty adult healthy female Wistar rats weighing 150-200 g were randomly divided into five groups of six animals each and subjected to different treatments groups viz. Group I (Control: Healthy, no treatment), Group II (Sham operated: Animals were operated as in endometritis group but not infected with bacterial culture and no treatment), Group III (Endometritis: Animals were operated and bacterial culture was inoculated in uteri to induce endometritis but no treatment), Group IV (Endometritis + Eucalyptus leaves extract: Animals were operated and bacterial culture was inoculated in uteri to induce endometritis and administered Eucalyptus leaves extract @ 25 mg/kg body weight by oral gavage) and Group V (Endometritis + cefixime: Animals were operated and bacterial culture was inoculated in uteri to induce endometritis and administered cefixime @ 15 mg/kg body weight by oral gavage).

During the entire study period animals were closely observed for any apparent clinical signs or symptoms. Body weight, body weight gain, organ weight (relative and absolute) was assessed after the experimental period.

Hematological parameters

Total leukocyte (TLC) and differential leukocytes counts (DLC), total erythrocytes count (TEC) and haemoglobin (Hb) were determined in whole blood using hematology autoanalyzer (Mindray).

Oxidative stress markers in liver, kidney, spleen and brain

Preparation of tissue homogenate

200 mg of tissue sample was weighed and taken in 2 ml of ice cold saline. 200 mg of sample weighed separately and taken in 2 ml of 0.02 M EDTA for GSH estimation. The homogenates were prepared by using tissue homogenizer under cold condition and were centrifuged for 10 min at 3000 rpm. The supernatant was used for estimation of different oxidative stress related parameters.

GSH was determined by estimating free –SH groups, using DTNB method of Sedlak and Lindsay (1968). For determining reduced glutathione, 10 % tissue homogenate was prepared in 0.02 M EDTA. The extent of lipid peroxidation was evaluated in terms of malonaldehyde (MDA) production, determined by method as described by Shafiq-ur- Rehman (1984). Superoxide dismutase was estimated as per the method described by Madesh and Balasubramanian (1998). Catalase was estimated and calculated by the method of Bergmeyer (1983). Total protein was estimated by method of Lowry *et al.* (1951).

Statistical analysis

Results are expressed as mean ± SEM. Statistical analysis of data was performed using SPSS 16 by one way ANOVA followed by Tukey's B multiple comparison post hoc test. A value of ($p<0.05$) was considered as statistically significant.

RESULTS

Data on body weight of rats of different treatment groups are presented in Table 1 which did not reveal any significant differences between the body weights of different groups including treatment groups before and after induction of endometritis. Data of absolute and relative weights of liver, kidney, spleen, brain and uterus of rats of different groups are presented in Table 2 and 3. Absolute and relative weights of uteri of rats of endometritis group (III) were significantly higher as compared to control and sham operated groups. Similarly, secretion index was also found to be significantly ($p<0.05$) higher in rats of endometritis compared to rest of the groups. Although secretion index was found to be markedly increased in rats of sham-operated group (II) as compared to control (I) and treatment groups (IV and V) but was not statistically significant. It was noticeable that both the secretion index, absolute and relative weights of uteri were significantly reduced in rats of treatment groups (leaves extract and cefixime) compared to those in control and sham operated group.

Hematological profile

Data of total leucocytes count and differential leucocytes count in rats of different groups are presented in Table 4. Although total leucocytes count was found to increase in endometritis group but it was not statistically significant. Unexpectedly higher values of TLC were also observed in blood of *Eucalyptus robusta* treated rats group compared to those in cefixime treated group or control group.

Lymphocytes count (%) in rats of control and treatment groups was almost comparable but rats of endometritis group revealed comparatively lower lymphocytes count, but it was not statistically significant. Difference in monocytes percent was also non significant but neutrophils count was found to be significantly higher ($p<0.05$) in endometritis group. Following treatment with plant extract and cefixime, neutrophils count was significantly reduced to almost normal values as observed in control groups. Data on total erythrocyte count (TEC) and haemoglobin (Hb) values in rats of different groups (Table 5) revealed that both these hematological indices did not significantly differ between healthy control (group I), sham operated (group II), endometritis (group III) and

endometritis groups treated with Eucalyptus (group IV) or cefixime (group V).

Oxidative stress markers

The levels of malonaldehyde (MDA) as a result of lipid peroxidation and anti oxidant biomarkers (reduced glutathione, superoxide dismutase and catalase) in liver, kidneys, spleen and brain of rats of different groups including treatment with Eucalyptus leaves extract @ 25 mg/kg body weight and cefixime @ 15 mg/kg body weight are summarized in Fig. 1. Data revealed significantly higher ($p<0.05$) levels in endometritic rats in all the tissue compared to the rats of control and sham operated group except for in kidneys in sham operated group. It is further evident from the data both Eucalyptus leaves extract and Cefixime treatment significantly reduced the levels of MDA in the tissues and these values in different tissues were almost comparable to those observed in healthy control rats of group I.

Data presented in Fig. 2 on levels of reduced glutathione, an endogenous antioxidant molecule in different organs/tissues of rats of different groups revealed that compared to control and sham operated groups GSH level was significantly reduced in endometritis (group III) in liver and brain while the levels in kidneys and spleen were not altered. Activities of antioxidant enzymes (SOD and catalase) are summarized in Fig. 3 and 4, respectively. Activities of SOD were found to be significantly higher in all the organs/ tissues of rats of endometritis group while catalase activity was significantly higher in spleen and brain and non significantly higher in liver and kidneys of rats of group III compared to those in rats of group I. Compared to the findings of oxidative stress biomarkers in rats of endometritic group, rats of groups IV and V treated with Eucalyptus leaves extract and cefixime respectively showed marked to significant alterations in MDA levels and activities of superoxide dismutase, reduced glutathione and catalase in different organs/tissues of rats and these values were almost comparable to those observed in control group rats.

Total proteins levels in liver, kidneys, spleen and brain of rats of different treatment groups are summarized in Table 6. Total proteins levels were found to be significantly higher in liver, kidneys and spleen compared to those in control and sham operated group of rats. Rats of group IV and V treated with Eucalyptus leaves extract and cefixime respectively showed remarkable to significant decrease in total protein content and these values were almost comparable to those in control and sham operated groups. However, level of total proteins in brain among different groups were comparable and did not differ significantly even in endometritis group.

DISCUSSION

Rat endometritis model in the present study was developed using a mixture of *E. coli* (1x 10^6 cfu/ml) and *Staphylococcus aureus* (1 x 10^8 cfu/ml) inoculated into uterus of rats during diestrus phase of estrus cycle. Induction of endometritis and consistency of model was based on presence of leucocytes, epithelial cells and some nonucleated cornified cells in vaginal smear as per the method of Nishikawa *et al.* (1985). Similar murine endometritis model has been used by Nishikawa *et al.* (1985), Nishikawa and Baba (1985) and Mikamo *et al.* (1999) for evaluating efficacy of certain drugs including plant extracts against endometritis. Animals of all the treatment groups were observed during study period and found apparently normal and healthy. Texture of hair coat and appearance of animals was also normal. General health parameters (body weight, relative and absolute organs weight of liver, kidney, spleen and brain) did not differ significantly between different treatment groups whether endometritis or Eucalyptus leaves extract and/or cefixime treated groups when compared to the control and sham-operated groups. Also, appearance of the experimental animals to be apparently healthy might be due to localized bacterial infection which was limited to uteri only and of very short duration. However, significant increase in the absolute and relative weights of uteri of rats of endometritic group is suggestive of severe inflammation and purulent endometritis and uteri filled with oedematous fluid. Significant decrease in absolute and relative weight of uteri in extract and cefixime treated rats groups as compared to endometritis group and these values being close to control group values are suggestive of marked efficacy and great potential of *E. robusta* leaves extract against bacterial endometritis in animals.

Leukocytes are involved in uterine defence mechanisms against bacterial infections. Migration of neutrophils from peripheral blood is initiated by chemotactic factors which are released at the site of inflammation which in turn results in significant increase in their number in uterine discharge within few hours of infection and remain elevated for next few days (Troedsson *et al.,* 1990). Similar possibilities about movement and infiltration of neutrophils in uterus and its milieu in our study too cannot be ruled

Table 1:
Effect of experimental endometritis and treatment with methanolic extract of *Eucalyptus robusta* leaves @ 25 mg/kg body weight and cefixime @ 25 mg/kg body weight on body weights of rats of different groups

Groups	Body weight (g)	
	Before endometritis	After endometritis
Control	220.33 ± 16.65 [a]	225.46 ± 16.65 [a]
Sham-operated	225.16 ± 8.83 [a]	234.00 ± 5.02 [a]
Endometritis control	221.66 ± 11.86 [a]	225.33 ± 8.46 [a]
Endometritis + *E. robusta*	221.16 ± 10.35 [a]	228.33 ± 10.01 [a]
Endometritis + Cefixime	220.50 ± 8.94 [a]	224.00 ± 10.87 [a]

Values (Mean ± SEM; n=6) bearing same superscripts in the same column did not differ significantly ($p<0.05$)

Table 2:
Effect of treatment with methanolic extract of *Eucalyptus robusta* leaves @ 25 mg/kg body weight and cefixime @ 25 mg/kg body weight on absolute weight and relative weight of liver, kidney and spleen of rats of different groups

Groups	Liver wt (g)		Kidney wt (g)		Spleen wt (g)	
	Absolute	Relative	Absolute	Relative	Absolute	Relative
Control	8.05 ± 0.70[a]	4.05 ± 0.16[a]	1.55 ± 0.13[a]	0.78 ± 0.04[a]	0.63 ± 0.04[a]	0.32 ± 0.02[a]
Sham operated	8.43 ± 0.40[a]	3.62 ± 0.20[a]	1.81 ± 0.09[a]	0.77 ± 0.04[a]	0.72 ± 0.03[a]	0.31 ± 0.01[a]
Endometritis control	9.14 ± 0.53[a]	4.05 ± 0.04[a]	1.84 ± 0.11[a]	0.82 ± 0.05[a]	0.81 ± 0.05[a]	0.36 ± 0.02[a]
Endometritis + *E. robusta*	9.43 ± 0.58[a]	4.15 ± 0.27[a]	1.92 ± 0.08[a]	0.84 ± 0.02[a]	0.83 ± 0.12[a]	0.39 ± 0.06[a]
Endometritis + Cefixime	8.42 ± 0.64[a]	3.74 ± 0.16[a]	1.90 ± 0.05[a]	0.85 ± 0.03[a]	0.65 ± 0.09[a]	0.29 ± 0.03[a]

Values (Mean ± SEM; n=6) bearing same superscripts in the same column did not differ significantly ($p<0.05$)

Table 3:
Effect of treatment with methanolic extract of *Eucalyptus robusta* leaves @ 25 mg/kg body weight and cefixime @ 25 mg/kg body weight on absolute weight and relative weight of brain and uterus of rats of different groups

Groups	Brain wt (g)		Uterus wt (g)	
	Absolute	Relative	Absolute	Relative
Control	1.51 ± 0.10[a]	0.76 ± 0.04[a]	0.63 ± 0.04[a]	0.32 ± 0.02[a]
Sham operated	1.74 ± 0.02[a]	0.74 ± 0.02[a]	0.93 ± 0.53[a]	0.39 ± 0.02[a]
Endometritis control	0.93 ± 0.86[a]	0.53 ± 0.06[a]	2.29 ± 0.26[b]	1.01 ± 0.11[b]
Endometritis + *E. robusta*	1.37 ± 0.13[a]	0.61 ± 0.08[a]	1.02 ± 0.17[a]	0.45 ± 0.07[a]
Endometritis + Cefixime	1.55 ± 0.16[a]	0.68 ± 0.05[a]	0.88 ± 0.10[a]	0.39 ± 0.03[a]

Values (Mean ± SEM; n=6) bearing same superscripts in the same column did not differ significantly ($p<0.05$)

Table 4:
Effect of treatment with methanolic extract of *Eucalyptus robusta* leaves @ 25 mg/kg body weight and cefixime @ 25 mg/kg body weight on total leukocyte count (TLC) and differential leukocyte count (DLC) of rats of different groups

Groups	Total leukocytes count (10^3/ìl)	Lymphocytes (%)	Monocytes (%)	Granulocytes (%)
Control	12.6 ± 2.11[a]	68.21 ± 1.25[a]	3.11 ± 0.46[a]	28.5 ± 1.44[a]
Sham operated	10.98 ± 2.04[a]	70.5 ± 1.88[a]	3.51 ± 0.48[a]	25.98 ± 1.78[a]
Endometritis control	16.25 ± 2.49[a]	58.05 ± 3.83[a]	4.11 ± 0.40[a]	40.40 ± 3.50[b]
Endometritis + *E. robusta*	23.76 ± 6.23[a]	68.68 ± 4.79[a]	4.18 ± 0.19[a]	24.20 ± 3.10[a]
Endometritis + Cefixime	12.75 ± 2.57[a]	68.93 ± 1.08[a]	4.03 ± 0.11[a]	27.03 ± 1.08[a]

Values (Mean ± SEM; n=6) bearing same superscripts in the same column did not differ significantly ($p<0.05$)

Table 5:
Effect of treatment with methanolic extract of *Eucalyptus robusta* leaves @ 25 mg/kg body weight and cefixime @ 25 mg/kg body weight on total erythrocyte count (TEC) and hemoglobin (Hb) of rats of different groups

Groups	Total erythrocyte count (10^6/ìl)	Hb (g/dl)
Control	7.64 ± 0.09[a]	11.06 ± 0.19[a]
Sham operated	7.92 ± 0.21[a]	12.10 ± 0.28[a]
Endometritis control	7.98 ± 0.08[a]	12.20 ± 0.27[a]
Endometritis + *E. robusta*	6.78 ± 0.71[a]	10.65 ± 1.13[a]
Endometritis + Cefixime	6.24 ± 0.97[a]	9.36 ± 1.47[a]

Values (Mean ± SEM; n=6) bearing same superscripts in the same column did not differ significantly ($p<0.05$)

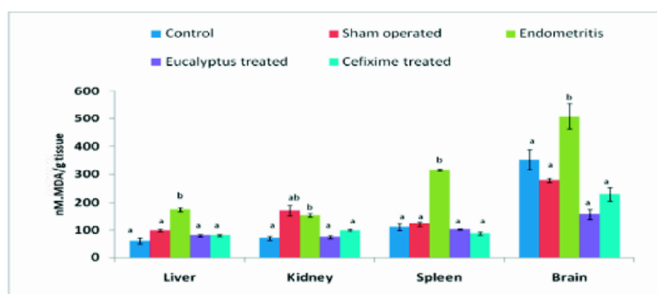

Fig. 1:
Effect of treatment on malonaldehyde (MDA) in different organ tissues of rats of different groups.

Table 6:
Effect of treatment with methanolic extract of *Eucalyptus robusta* leaves @ 25 mg/kg body weight and cefixime @ 25 mg/kg body weight on total protein in different organs/ tissues of rats of different groups

Groups	Liver	Kidney	Spleen	Brain
Control	38.36 ± 0.79[a]	37.22 ± 0.83[ab]	37.32 ± 0.64[ab]	36.86 ± 1.35[a]
Sham operated	33.41 ± 1.17[a]	31.93 ± 1.32[a]	30.57 ± 0.55[a]	32.87 ± 0.89[a]
Endometritis control	60.05 ± 4.20[b]	54.82 ± 3.62[c]	51.82 ± 4.54[c]	37.88 ± 1.46[a]
Endometritis + *E. robusta*	42.54 ± 2.65[a]	37.02 ± 1.83[ab]	36.16 ± 14.82[ab]	34.21 ± 2.10[a]
Endometritis + Cefixime	43.28 ± 2.05[a]	39.85 ± 0.51[b]	40.45 ± 0.77[b]	35.11 ± 1.18[a]

Values (Mean ± SEM; n=6) bearing same superscripts in the same column did not differ significantly ($p<0.05$)

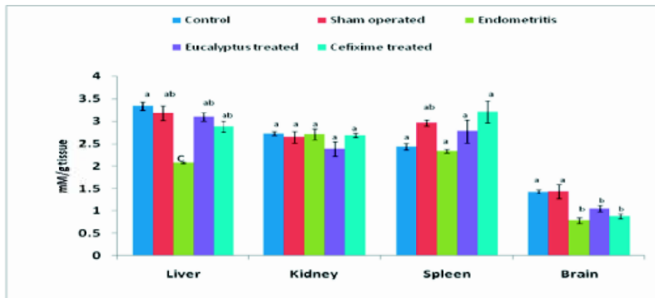

Fig. 2:
Effect of treatment on reduced glutathione (GSH) in different organs/ tissues of rats of different groups.

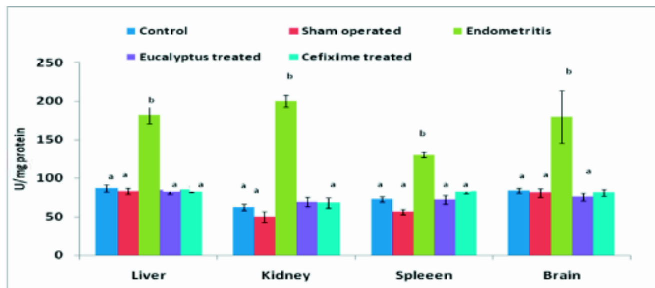

Fig. 3:
Effect of treatment on SOD in different organs/ tissues of rats of different groups.

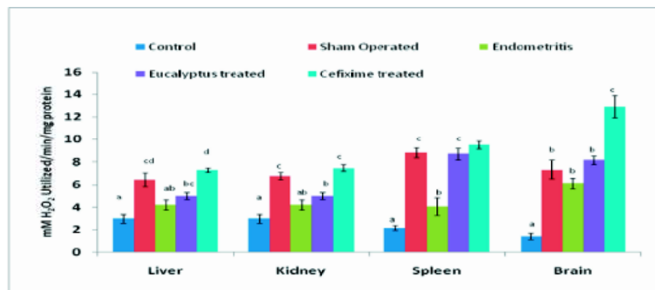

Fig. 4:
Effect of treatment on catalase in different organs/ tissues of rats of different groups.

out. Results of haematological studies (total leucocytes count and differential leucocytes count) also substantiate validity of establishment of rat's endometritis model in our study as there was significant increase in per cent neutrophils counts and significant decrease in per cent lymphocytes count in rats of endometritis group (III) compared to those in healthy control group (I) and sham-operated group (II). Significant increase in neutrophils count in rats of endometritis group might be due to stress produced by endometritis whereas in the treatment groups (Eucalyptus leaves extract and cefixime), the neutrophils count was significantly reduced towards control group values is indicative of a curative potential of Eucalyptus leaves extract with an ability to reduce stress. Our findings are in agreement with the findings of Richmond and Mackley (2000) who have reported that Eucalyptus alleviates stress in birds by enhancing general health status and immunity. The resultant effect leads to reduction in heterophills count and increase in lymphocytes. This protective effect of eucalyptus may be attributed to higher content of flavanoids present in it that scavenge free radicals (Bello, 2015). But, total erythrocytes count and haemoglobin levels in rats of endometritis group did not show any change as compared to those in rats of control group, primarily may be due to localized infection.

The group of rats suffering with endometritis revealed disturbed oxidative-antioxidant milieu as there was increased malonaldehyde (MDA) concentration, increase in activity of superoxide dismutase and decrease in activity of reduced glutathione (GSH) and catalase activity as compared to healthy control and sham operated animals. It is very well known that inflammatory diseases are associated with enhanced oxidative reactions and reduced antioxidant defense capacities. Lipid peroxidation is a well-established mechanism of oxidative damage caused by ROS and the measurement of the MDA provides a convenient index of lipid peroxidation (Nielsen *et al.*, 1997). The findings of present study are in agreement with Behiman *et al.* (2001). Oxidative stress resulting from imbalance between oxygen free radicals and antioxidants have negative effect on animal health and production (Ahmed, 2007) and it initiates tissue damage (Bedwal and Bahugma, 1994) and plays a significant role in female biology.

Many studies have reported oxidative stress markers and antioxidants status in patients with endometritis. Induction of inflammation causes activation and recruitment of mononuclear phagocytes and activated macrophages which leads to oxidative stress. Induction of oxidative stress markers results in increased concentration of cytokines, growth factors and other co-inflammatory mediators. However, many studies reported inconsistent results with endometritis induced oxidative stress (Gupta *et al.*, 2006). Therefore, it is virtually impossible to postulate a definite conclusion regarding role of oxidative stress in endometritis. However, Ota *et al.* (1999) suggested that increased oxidative stress biomarker concentration is due to xanthine oxidase concentration in patients suffering with endometriosis and xanthine oxidase is recognised as reactiving oxygen species generating enzyme. Ota *et al.* (1998) have also documented that the patients suffering with endometritis/ endometriosis have elevated manganese, copper and zinc superoxide dismutase along with glutathione peroxidase antioxidant enzyme that prevent the detrimental effects of oxidative stress.

Increased SOD and decreased GSH activity shows that both these enzymes scavenge reactive oxygen species and inhibit production of hydroxyl level. On the other hand, depression in GSH levels in different tissues

clearly demonstrates that it is actively involved in neutralizing and detoxification of free radicals generated during oxidative stress. Decreased concentration of GSH might also be due to its role as a powerful cellular antioxidant and used as a substrate for different enzymes that removes excess of reactive oxygen species. Decreased activity of catalase is suggestive of its utilization in conversion of harmful free radicals to harmless molecules like conversion of hydrogen peroxide which is formed during dismutase reaction to water molecules. Superoxide dismutase catalyzes the dismutation of superoxide radicals into hydrogen peroxide and molecular oxygen. Hydrogen peroxide degrades further to water by other antioxidant enzymes, such as glutathione peroxide and catalase. Glutathione peroxidase proteins catalyze the reduction of organic hydroperoxides, lipid peroxides, and hydrogen peroxide, using glutathione as the reducing agent, thereby also protecting cells from oxidative damage resulting from normal oxidative metabolism.

Considerable increase in oxygen requirements at the time of increased metabolic demands results in augmented production of ROS. During the periparturient period, antioxidants are required to reduce ROS accumulation. An imbalance between increased production of ROS and availability of antioxidants may expose cows to increased oxidative stress. The possibility that oxidative stress during the transition period may be a major underlying cause of inflammatory and immune dysfunction in dairy cows is supported by several studies (Bernabucci *et al.*, 2005; Bliznetsova *et al.*, 2008). Significant decrease in reduced glutathione levels (GSH), increase in malonaldehyde (LPO), decreased catalase and increased superoxide dismutase (SOD) activities were observed in endometritis group. These findings are in agreement with the findings of Hanafi *et al.* (2008) who have reported that buffalo cows having endometritis revealed disturbed oxidative status with increased MDA, decreased NO, CAT, GSH-R and as compared to healthy animals. Inflammatory diseases are associated with enhanced oxidative reactions and reduced antioxidant defence capabilities.

Treatment of endometritic rats with Eucalyptus leaves extract resulted in reduction in lipid peroxidation and restoration of antioxidant molecule and enzymes towards values found in control group rats. These observations are suggestive of the potent antioxidant property of Eucalyptus citriodora leaves extract. It is reported that the phenolic extract of Eucalyptus possess a powerful antioxidant property and is more potent than vitamin C, vitamin E and even carotenoids (Dai and Mumper, 2010) due to their polyphenolic as well as flavanoid content present in leaves (Dezzi *et al.*, 2015).

ACKNOWLEDGEMENT

The authors are thankful to Principal Investigator of ICAR funded Network Project on “Outreach Programme on Ethnoveterinary Medicine” for financial support and Hon’ble Vice Chancellor of the University for necessary facilities.

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Received on : 12.12.2017
Accepted on : 28.12.2017

A Report

OPIOID CRISIS IN UNITED STATES OF AMERICA

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ABSTRACT

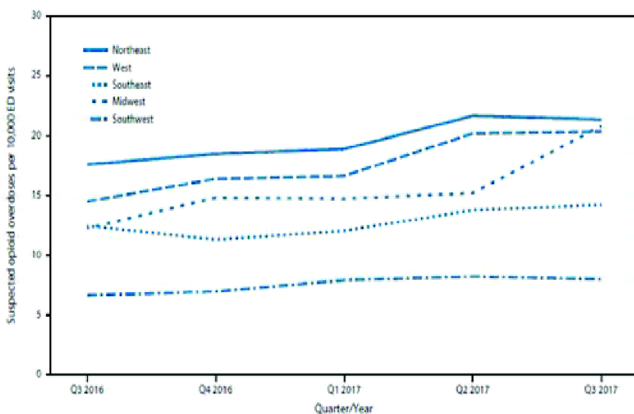
One of the leading causes of drug overdose is Opioid. In the US, more than 2 million people struggle with opioid use disorders. Opioids, often prescribed as main medications for chronic pain. But scientists are identifying ways to help combat the epidemic, which include getting treatment faster, developing safer opioids and helping patients choose appropriate treatment. Since 2010, overdose deaths involving predominantly illicit opioids (heroin, synthetic nonmethadone opioids, or both) have increased by 200%. Scientist are trying to find a way to combat the epidemic which include getting people treatment faster, developing safer opioids and helping patients choose appropriate treatment . safer prescription opioids could ultimately decrease the number of deaths caused by abusing prescription opioids. buprenorphine and naltrexone-representing pharmacologically and conceptually opposite approaches-are available for office-based treatment.

INTRODUCTION

Everyday more than 115 Americans die after overdosing on opioids (https://wonder.cdc.gov., 2017). The center of disease control and prevention estimates that the total "economic burden" of prescription opioid misuse alone in the united states is $78.5 billion a year, including the costs of healthcare, lost productivity, addiction treatment, and criminal justice involvement (Florence, 2013).

In the late 1990s pharmaceutical companies reassured the medical community that patients would not become addicted the prescription opioid pain relievers, and healthcare providers began to prescribe them at greater rates. This subsequently led to widespread diversion and misuse of these medications before it became clear that these medications could indeed be highly addictive (Morone, 2013). In 2015 more than 33,000 Americans died as a result of an opioid overdose including prescription opioids, heroin and illicitly manufactured fentanyl, a powerful synthetic opioid (CBHQ, 2015).

Opioid overdoses in large cities increase by 54 percent in 16 states.



Quarterly rate of suspected opioid overdose, by US region
Source: Centers for Disease Control and Prevention. (Vivolo-Kantor *et al.,* 2017) .

Underlying factors in drug overdose deaths

Drug overdose accounted for 52404 deaths in the United States in 2015, which are more death than for AIDS at its peak in 1995. Provisional data from the US centers for disease control and prevention indicate drug overdose deaths increased again from 2015 to 2016 by more than 20% (Dowell *et al,* 2017). Since 2010, overdose deaths involving predominantly illicit opioids (heroin, synthetic nonmethadone opioids, or both) have increased by 200%.

Fentanyl is approximately 50 times as potent as heroin. This provides strong economic incentives for drug dealers to mix Fentanyl with heroin and other drug dealers to mix fentanyl with heroin and other drugs because smaller volumes can provide equally powerful effects with lower costs and easier transport. It can be difficult to identify fentanyl, particularly in the white powder heroin typically sold in western states might be limiting the penetration of fentanyl into this market for now (Volkow *et al.,* 2017).

The role of science in the opioid crisis

Volkow and Collins addressed the opioid epidemic – a major public health crisis of our times 8. NIH(National Institute of Health) and NIDA(National Institute on Drug Abuse) also addressed this crisis.There is no evidence that opioids are effective for long-term control of chronic pain. There is not any randomized, controlled clinical trial that has shown that opioids are helpful in controlling chronic pain (https://prevention.nih.gov/docs/programs/p2p/ODPPainPanelStatement). Opioid misuse is a major problem for which innovative solutions are urgently needed. A recent systemic review showed that self-management programs are effective in reducing pain and improving function and mood (Kamper *et al.,* 2015). The initial assessment in pain-management program, 60% of patients were taking opioids. By the end of the program, the percentage was down to 10% and that rate was maintained at 12 months of follow-up (Nicholas *et al.,* 2014).

Research finds new ways to fight opioid crisis

Scientists are trying to find a way to combat the epidemic which include getting people treatment faster, developing safer opioids and helping patients choose appropriate treatment.

Interim therapy

As the opioid crisis continues to escalate, researchers have found an intervention for reducing these risks among opioid-dependent people who are stuck on waitlists seeking treatment who are very susceptible to infected with HIV or hepatitis, as well as dying from an overdose. The interim therapy could help protect patients from the potentially fatal dangers of illegal opioid use by safely and responsibly providing medication while they await more intensive treatment. [Presenter: Dr. Stacey Sigmon at the University of Vermont's College of Medicine]

Safer opioid drugs could save lives

Opioid drugs are the most widely prescribed and effective type of pain medication, but they are highly addictive and have some unpleasant and potentially deadly side effects. Researchers may have found a way to make opioids safer by separating the drugs' pain relieving effects from their most dangerous side effect such as respiratory suppression as opioid overdose deaths are mostly due to respiratory suppression, safer prescription opioids could ultimately decrease the number of deaths caused by abusing prescription opioids. [Presenter: Dr. Laura Bohn at The Scripps Research Institute.]

Trials can help people choose between treatments:

Two medications, buprenorphine and naltrexone — representing pharmacologically and conceptually opposite approaches — are available for office-based treatment. Yet until now, patients, families, and providers have had no data to help guide their choice of treatment. New findings from two trials (one in the US, one in Norway) comparing these approaches will help people choose between the two very different treatments. [Presenters: Dr. John Rotrosen at New York University School of Medicine and Dr. Lars Tanum at the University of Oslo and Akershus University Hospital in Norway].

The Social life of an opioid

According to Late Neuroscience pioneer Jaak Panksepp our body's naturally produced opioids (endorphins and enkephalins). They are critical to the nurturing bonds that develop between parents and offspring and also between monogamous mates in mammals. His work showed that blocking one opioid system in the brain increases distress calls of infants separated from their mothers.

To address the opioid crisis we should recognize the connections between bonding, stress and pain by generating more love to have less opioid use. Understanding the biology and commonalities between trusting social relationships and the opioid system can change the way we think about treatment.

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Received on : 05.11.2017
Accepted on : 19.11.2017

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