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Review Article

ANTI-INFLAMMATORY POTENTIAL OF SOME PLANTS : AN OVERVIEW

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ABSTRACT

Inflammation is a normal protective response to tissue injury caused by physical trauma, noxious chemical or microbial agents. It is the body response to inactivate or destroy the invading organisms, to remove the irritants and set the stage for tissue repair. It is triggered by the release of chemical mediators from injured tissue and migrating cells. The inflammatory response involves a complex array of enzyme activation, mediator release, fluid extravasation, cell migration, tissue breakdown and repair which are aimed at host defense and usually activated in most disease conditions. Drugs presently in use for the management of inflammation are associated with well known side and toxic effects. Some plants like *Alpinia galanga*, *Boswellia serrata* and *Commiphora mukul* have been mentioned in Ayurveda to possess anti-inflammatory activities. It has been suggested that Alpinia, Boswellia and Commiphora possess phytochemicals like flavonoids, terpenes, phytosterols etc. which have diverse medicinal properties. This review describes some of the aspects of anti-inflammatory properties of these medicinal herbs.

Key words: *Alpinia galanga*, *Boswellia serrata, Commiphora mukul* Curcuma longa, inflammation

INTRODUCTION

Anti-inflammatory drugs are agents that reduce inflammation. Non steroidal anti-inflammatory drugs (NSAIDs) represent one of the most common classes of medications used worldwide with an estimated usage of >30million per day for inflammation and related disorders (Bennet, 2005; Wallace and Ferraz, 2010). Unfortunately, besides the excellent anti-inflammatory potential of the NSAIDs, the severe side effects such as gastrointestinal (GI) ulceration, perforation, obstruction and bleeding have limited the therapeutic usage of NSAIDs. It has been found that conventional synthetic NSAIDs accelerate damage and erosion of joint cartilage, advancing the osteoarthritis process. These NSAIDs are also known to cause liver and kidney damage with long-term use (Shah *et al.,* 2016). Experimental research have shown that the use of proven natural anti-inflammatory herbal agents have not been shown to cause erosion injury to the intestinal tract, acceleration of cartilage destruction or production of liver and kidney toxicities. This enables practitioners to use these substances in a safe and responsible way (Apu *et al.,* 2012).

Current status of research and development in the subject

The core chemicals classes of anti-inflammatory agents have been reported from natural sources to engage a wide range of compounds. Such compounds are polyphenols, lignans, anthraquinones, flavonoids, alkaloids, terpenoids, saponins, polysaccharides and peptides. Several studies proved that naturally occurring coumarins and flavonoids act as dual inhibitors of cyclo-oxygenase and 5-lipoxygenase activities (Shaikh *et al.,* 2015). Several leads from plant sources, like curcumin, resveratrol, baicalein, betulinic acid, ursolic acid and oleanolic acid are being studied as possible drugs for the future against inflammation (Gautam and Jachak, 2009).

From the study done so far, it has been believed that flavonoids are major anti-inflammatory agents. Some of them act as phospholipase inhibitors and some have been reported as TNF-α inhibitors in different inflammatory conditions. Biochemical investigations have also shown that flavonoids are able to inhibit both cyclo-oxygenase and lipo-oxygenase pathways of arachidonic acid metabolism depending upon their chemical structures. Alkaloids containing pyridine ring system have also been reported to have striking anti-inflammatory activity. For example, Berberine from Berberis is traditional remedy to treat rheumatisms. Significantly terpenoids inhibit the development of chronic joint swelling. However, still many herbal plants have not undergone through scientific investigations for inflammation and related problems like rheumatism.

Previous studies have contributed much in the understanding of the compounds responsible for the known anti-inflammatory action, their mechanism of action and therapeutic values. Compounds such as bromelain act as anti-inflammatory agent due to its fibrinolytic and fibrinogenolytic effects. Xanthones are also implicated in the anti-inflammatory and analgesic effects. Unlike other anti-inflammatory, analgesic agents, xanthones were reported to have very less or no side effects such as ulcerogenicity and blood clotting. Terpene acids such as madecassic acid and asiatic acid from *Centella asiatica* have been reported to be an effective analgesic and anti-inflammatory compound. Curcumin is the most important ethnobotanical drug isolated from *Curcuma longa* and is

reported to have a variety of medical applications including anti-inflammatory activity. Yet another compound, salicin, from *Salix alba* was also found to be very effective anti-inflammatory and analgesic agent and was proved better than aspirin. Gingerol and its analogues in *Zingiber officinale* are potent antioxidant, antinociceptive and anti-inflammatory agents. Much of the current research trend is towards the isolation, purification, identification and characterization of active principles from crude extracts. However, there is a hidden fact that the different components present in the crude plant drugs may be more efficient and potent than any of the single purified compound which may help to nullify the toxic effects of individual constituents. Most of the commonly used modern medicines have originated from the plant sources. At the same time plants like *Alpinia galanga*, *Bosewellia serrata* and *Commiphora mukul* may yield such compounds free from side effects. The development of neutraceuticals from them could substitute the present generic market to a great extent (Rajagopal*et al*., 2013).

Work done so far

From ancient times Indian, Chinese, Egyptian, Greek, Roman and Syrian medicinal system documented the use of different plant based medicine for different diseases (Kamboj, 2000). According to WHO, nearly 75-80% of world population still depends on herbal medicine. Active constituents from plant sources directly used as therapeutic agent and phytoconstituents are also served as lead molecule for the synthesis of various drugs (Kamboj, 2000; Verma and Singh, 2006). Indigenous or herbal medicines confer considerable economic benefits to most rural and poor people. WHO noted that about 25% of modern medicines are descended from plants sources used traditionally and research on traditional medicinal herbal plant leads discovery of 75% of herbal drugs (Verma and Singh, 2006).

Alpinia galanga

Alpinia galanga is a perennial herb found commonly throughout the Western Ghats, Mysore, Goa, and Gujarat. Studies have shown the plant to possess anti-inflammatory, analgesic, antioxidant, antifungal, antibiotic, antibacterial, anti-ulcer, and anticancer properties (Thomas *et al*, 1996; Juntachote and Berghofer, 2005). The anti-inflammatory and analgesic effects of *Alpinia galanga* has been studied in a variety of rheumatologic conditions (Nagashekhar and Shivprasad, 2005; Altman and Marcussen, 2001). Yu *et al.* (2009) isolated *p*-coumaryl alcochol-γ-0- ether having phenyl-propanoid structure, which selectively and substantially suppress IFN-γ, production in CD4 T lymphocyte (T helper) cells. Isolated chavicol analogues, viz, acetoxychavicol acetate (ACA) and hydroxychavicol acetate (HCA), have been comparably examined, where ACA exhibited potent antioxidant activity, increased cell apoptosis, and decreased cytokine production by T helper cells, whereas HCA suppressed T-bet expression and might act as a beneficial therapeutics for treating inflammatory immune disorders caused by extravagant activation (Min *et al*, 2009).

During past several years, *Alpinia galanga* is gaining lot of interest according to researchers' point of view. It is commonly known as Rasna in Sanskrit, Kulanjan in Hindi, and Galangal in English. Recently many pharmacological studies have been conducted on *Alpinia galanga.* The extensive literature survey revealed that *Alpinia galanga* is important medicinal plant with diverse pharmacological spectrum. The plant shows the presence of many chemical constituents which are responsible for varied pharmacological and medicinal property. The evaluation needs to be carried out on *Alpinia galanga* in order to use the formulations of the plant in their practical clinical applications, which can be used for the welfare of mankind. (Shetty and Monisha, 2015). Evaluation of the anti-inflammatory potential of rhizome of *Alpinia galanga* (total alcoholic extract (TAE) and total aqueous extract (TAQ)) in acute (carrageenan-induced paw oedema) and sub-acute (cotton pellet-induced granuloma) rat models indicated significant effect (Satish and Dhananjayan, 2003; Rastogi and Mahrotra, 1995). The anti-inflammatory and analgesic activity of the topical preparation of *Alpinia galanga* from methanolic extract was reported. The significant anti-inflammatory activity was seen against carrageenan-induced oedema in rats and in a formalin test (Shetty and Monisha, 2015). Natural bioactive compounds and crude hydro-alcoholic fractions isolated from the Alpinia species like *A. galanga, A. zerumbet, A. officinarum*, etc. showed potential activities as anti-inflammatory and analgesic agent. Aqueous and hydro-alcoholic extracts from leaves and rhizomes of above species possesses key factors responsible for anti-nociceptive and anti-allergic properties (Ghosh and Ranjan, 2013).

The active principle 1'S'-1' acetoxychavicol acetate from rhizome of *Alpinia galanga* has been reported to possess various activities like anti-inflammatory, antimicrobial and antioxidative activities(Nakamura *et al.,* 1998). The rhizome also contains flavonoids which are responsible for anti-inflammatory effects of this indigenous plant. The flavonoids which have been isolated from galangal roots are galangin, alpinin and kaempferide (Cahrles*et al*., 1992). Galangin (3,5,7-trihydroxyflavone) is a flavonoid with other properties like antimicrobial and anticancer activities (Ciolino and Yeh, 1999). In a study *Alpinia galanga* was tested against the standard anti-inflammatory compound indomethacin. In the rat paw method the test compound showed significant inhibition of 52.5% against indomethacin with inhibition of 68.75% (Kameswari *et al.,* 2015).

Sharma *et al.*(2015) evaluated the anti-

inflammatory activity of *Alpinia galanga* by carrageenan induced edema in Wistar albino rats. It was found that the extract inhibits the paw edema significantly ($p<0.05$) at both 250 mg/kg and 500 mg/kg dose levels when compared with control. It was concluded that the phenolic compounds like flavonoids and phenolic acids are copiously found in this plant and the anti-inflammatory activity was probably due to inhibition of peripheral inflammatory mediators. Further investigations are anticipated to identify the active components and lead to their further clinical use. Unnisa and Parveen (2011) suggested the anti-inflammatory effect of *Alpinia galanga* in carrageenan induced rat paw edema model. Moreover it was concluded that the magnitude of inhibition, onset and the period of action suggest that the anti-inflammatory mechanism of the methanolic extract may be through inhibition of prostaglandin synthesis by reduced action of cyclo-oxygenase. The anti-inflammatory and analgesic effects of *Alpinia galanga* has been studied in a variety of rheumatologic conditions (Nagashekhar and Shivprasad, 2005; Altman and Marcussen, 2001). A herbal formulation containing *Alpinia galanga,* Joint Care B, has shown dose-dependent inhibition of carrageenan-induced paw inflammation and granuloma weight in croton oil-induced granuloma pouch model in rats (Venkataranganna *et al.,* 2000).

Boswellia serrata

Boswellia serrata is one of the medicinal plants of *Burseraceae* family. In the plant kingdom, *Burseraceae* family is characterized with 17 genera and 600 species wide spread in all tropical region. Genus *Boswellia* contains about 25 known species. Most of them occur in Arabia, north eastern coast of Africa and India. In India it is found in Western Himalaya, Rajasthan, Gujarat, Maharashtra, Madhya Pradesh, Bihar and Orissa (Sunnichan *et al.,* 1998; Upadhayay, 2006). Sharma *et al*. (2010) observed that the oral administration of different fractions of *B. serrata* showed suppression of inflammation and mechanism of action of extract might be linked to lipoxygenase and/or cycloxygenase. In a study, Ramakrishnan*et al*. (2011) concluded that commercial extract of *B. serrata* at the dose level 45 mg/kg and hexane extract of *B.Serrata* at the dose level 180 mg/kg showed significant anti-inflammatory effect in Wistar albino rats using carrageenan induced paw edema model. In cotton pellet granuloma model both the commercial and hexane extract of *B. serrata* at the dose of 45 mg/kg showed significant anti-inflammatory effect in Wistar albino rats. In recent study, *Boswellia serrata* gum resin boswellic acid has been reported to exhibit anti-inflammatory activity (Rajput *et al.,* 2015).

In *Boswellia serrata* gum resin, boswellic acid exhibits anti-inflammatory, anti-atherosclerotic and anti-arthritic activities. The gum is well known house hold fumigant and is used as an anti-inflammatory agent when applied externally. Boswellic acid and its derivatives are novel, specific, non-redox inhibitor of 5-lipoxygenase (5-LOX), an enzyme in neutrophils responsible for the conversion of arachidonic acid to 5- HETE and leukotrienes which causes vasoconstriction, bronchoconstriction, increase vascular permeability and chemotaxis (Safayhi *et al.,* 1992).

Commiphora mukul

Commiphora mukul commonly known as guggul possesses a vast ethno-medical history and represents a phytochemical reservoir of great medical value. It plays a very important role as a key ingredient of the treatment procedures. It contains a wide number of phytochemical constituents i.e., flavonoids, terpenes, phytosterols etc. which have different biological activities like antimicrobial, anti-inflammatory, anti-carcinogenic activities and various other important medicinal properties. There is a need to review this plant in order to provide scientific proof for its application in traditional medicinal system. Guggulsterone is a main active substance in gugulipid, an extract of *Commiphora mukul*, used to treat a variety of disorders in humans, including dyslipidemia, obesity and inflammation. The traditional uses of *Commiphora mukul* include as anti-inflammatory, antispasmodic, antibacterial, antifungal, carminative, emmenagogue, alterative, antiseptic, apertif, astringent, sedative, stomachic, diaphoretic, diuretic, antispasmodic, antisuppurative, aperient, expectorant, a thyroid stimulant (Vitamins-etc.com, 2001), anthelmintic, depurative, vulnerary, antiseptic, demulcent, aphrodisiac, stimulant, liver tonic, detergent, anti-spasmodic, hematinic, diuretic and lithonotriptic (Varier, 1994). Native to India, *Commiphora mukul* grows wild in the Indian states of Rajasthan, Gujarat, Karnataka, Assam and in Afghanistan, Arabia, and northeast Africa in rocky dry areas (AyuHerbal.com; Varier, 1994; Atal *et al.,* 1975). Oleogum resin (known as guggul) from the guggul tree, *Commiphora mukul* has been used to treat various diseases including hyper-cholesterolemia, atherosclerosis, rheumatism and obesity over several thousands years. Since the first study demonstrating the therapeutic effects of guggul in an animal model in 1966, numerous preclinical and clinical trials have been carried out. Guggulsterone isolated from guggul has been identified as the bioactive constituent responsible for guggul's therapeutic effects. Guggulsterone has been found to potently inhibit the activation of NF-κB, a critical regulator of inflammatory responses. Such repression of NF-κB activation by guggulsterone has been proposed as a mechanism of the anti-inflammatory effect of guggulsterone. Because of its safe, quick-acting and highly effective anti-inflammatory properties that also enhance circulation, guggul also offers a safe and effective pain relieving alternative to NSAIDs. It is ideal for those whose work involves a lot of back bending followed by stiffness and pain. For the zealous gardener, the yoga

practitioner or after a strenuous gym workout, guggul will effectively relieve the stiffness and pain usually within an hour or two that would usually take anywhere from one to several days to resolve (Chaturvedi and Singh,1965; Kakrani, 1981; Manjula *et al.,* 2006). Guggulsterone also appears to reduce circulatory levels of pro-inflammatory cytokines such as IL-2 and TNF-α (Manjula *et al*, 2006). It also reduces COX-2 mRNA levels and suppresses its TNF-α mediated induction (Shishodia and Aggarwal, 2004). Kimura *et al.* (2001) have observed that *Commiphora mukul* showed anti-inflammatory effects in adjuvant induced granuloma model in mice.

Curcuma longa

For centuries, extract of rhizome of turmeric (*Curcuma longa* Linn.) has been used as anti-inflammatory agents in Ayurvedic and Traditional Chinese Medicine (TCM). *In vivo* studies assessing turmeric's antiarthritic effects have revealed that turmeric extracts containing substances called curcuminoids prevent formation of osteoclasts (osteoclastogenesis) and consequent destruction of periarticular bone in a model of rheumatoid arthritis. Curcuminoids have been the subject of numerous scientific studies for exploring their health benefits. Available literature suggests that this group of compounds has great medicinal value in suppressing tumours, inflammation, tissue oxidation, etc. Curcumin is the yellow phenolic compound present naturally in various types of herbs, especially in turmeric. Curcumin acts as a natural antioxidant (Kuo *et al.,* 1996; Lim *et al.,* 2001), anti-microbial, anti-inflammatory (Sharma *et al.,* 2005; Sandur *et al.,* 2007), anti-Alzheimer (Hamaguchi *et al.,* 2010) and anti-cancer in preclinical as well as clinical studies (Sharma *et al.,* 2004; Liu and Hong, 2006). Moreover, it has been seen to possess antidiabetic, antirheumatic, nephroprotective, hepatoprotective, neuroprotective, hypoglycaemic and cardioprotective activities (Asai *et al.,* 2001; Sharma *et al.,* 2005; Aggarwal, 2010). It is also helpful in suppressing thrombosis and provides protection against myocardial infarction (Naksuriya *et al.,* 2014).

Brouet and Ohshima (1995) and Chan *et al.* (1998) conducted studies using 1-20 μM concentrations of curcumin and reported an *ex vivo* inhibition of induction of macrophage NOS (Nitric Oxide Synthase) activity. Since inhibition of iNOS activity can be correlated with intervention during carcinogenesis, considerable implications for cancer chemoprevention can be attributed to the use of curcumin. Effect of oral curcumin on inflammation and related disorders in humans has been the subject of number of studies. In post-operative patients treated with curcumin (@400 mg, three times daily for 5 days), a significant anti-inflammatory effect was observed. A significant improvement in the inflammatory symptoms without apparent toxicity was reported after treatment for 2 weeks in 18 rheumatoid arthritis patients were treated with oral curcumin (@300mg, four times daily). Similarly, in another study, ten patients with inflammatory bowel disease given pure curcumin (@0.55-1.65 g daily) for a period of 2 months showed encouraging clinical improvement (Satoskar *et al.,* 1986).

Lal *et al.* (1999) and Lal *et al.* (2000) evaluated the effects of oral curcumin on ophthalmological conditions and reported an improvement in patients having anterior uveitis after treatment with curcumin (@375 mg, three times daily, orally) for 12 weeks. In their second study, eight patients with idiopathic inflammatory orbital pseudotumors were given the same dose of curcumin for 6–22 months. After 2 years, complete responses were reportedly observed in half of the patients.

In a series of studies, *in vivo* efficiency of turmeric extracts was found in preventing or treating rheumatoid arthritis(RA). It was observed during the course of one study that if treatment was started before joint inflammation had set in; a fraction containing 41% curcuminoids could prevent joint inflammation (Funk *et al.,* 2006). Another study concluded that curcuminoids were the actual components of the extract that can prevent RA *in vivo*, while other constituents of the extract had inhibitory effects on the protective action. To explain the effects of curcuminoid extracts, it was suggested that curcuminoids prevent local activation of NF-kB and expression of genes subsequently regulated by NF-kB. These included the genes which mediate joint inflammation and destruction e.g. chemokines, RANKL and cyclooxygenase 2 (COX2) (Funk *et al.,* 2006).

Buhrmann *et al.* (2011) observed that curcumin inhibited IL-1-mediated inflammatory signalling and apoptosis *in vitro* using human tenocyte model. The study concluded, among other things, that curcumin has a potential role in treating tendinitis by modulating NF-kB signalling. In a study on 32 female Sprague-Dawley rats to know the role of curcumin in preventing bone loss after ovariectomy. The study concluded that curcumin, in case of post-menopausal osteoporosis, can be used as an alternative to oestrogen-therapy (Hussan *et al.,*2012). Heo *et al.* (2014) prepared β-cyclodextrin (CD) conjugated GNPs (CGNPs) which were allowed to form inclusion complexes with curcumin (CUR-CGNPs), and their effects on inhibition of RANKL-induced osteoclastogenesis were seen in cultures of bone-marrow derived macrophages (BMMs). A significant decrease in $TRAP^+$ (tartrate-resistant acid phosphatase positive) multinuclear cells in BMMs was observed, and there was no cytotoxicity. In addition to this, the group also observed decreased expression of osteoclast-specific genetic markers, which included c-Fos, NFATc1 (nuclear factor of activated T cells 1), TRAP and OSCAR in response to CUR-CGNPs. The study further strengthened the belief that curcumin can be used as a therapeutic agent in prevention and treatment of

osteoporosis.

Li *et al.* (2015) used a DXM-induced osteoporosis animal model system to investigate proximal tibial bone microarchitecture in GIOP, and the effects of curcumin in such a model. Treatment with DXM induced hypercalciuria in mice, which subsequently decreased when the mice were treated with curcumin. It was also observed that on treatment with curcumin, there was a decrease in DXM-induced bone resorption, increase in serum OCN and down regulation of CTX and TRAP-5b, both markers of bone resorption. Curcumin also reversed the deleterious effects of DXM-treatment and stimulated bone remodelling. There was a significant decrease in TRAP+ stained area (H&E staining), with decrease in OPG/RANKL/RANK signalling and MMM-9 expression on supplementation with curcumin (Li *et al.,* 2015). Another study involving the same animal model was conducted by Chen *et al. (*2016). Osteoporosis induction was followed by treatment with curcumin (@100 mg/kg/day) for another 60 days. It was observed that curcumin up regulated the expression of markers that support osteoblast differentiation. It was also observed that the ratio of OPG expression to RANKL expression also increased in curcumin-treated cells. On western blot analysis, it was observed that DXM inhibited Wnt signalling pathway, which was re-activated by curcumin. Taking all these observations into account, it was concluded that curcumin has a promising potential in the treatment of GC-induced osteoporosis (Chen *et al.,* 2016).

CONCLUSION

Plants have played a significant role in human health care since the ancient times. Traditional plants exert great role in discovery of new drugs. Plants contain a large number of spread of pharmacologically active ingredients and each herb has its own unique combination and properties. Most of the human populations are affected by inflammation related disorders worldwide. Review of herbal medicines used by different medicinal system and tribal/ ethnic people in pain and inflammation is essentially quite important in the face of treatment. Curcumin and its derivatives appear as attractive drug candidates against various neoplastic, inflammatory and skeletal disorders. However, due to problem of low aqueous solubility and limited bioavailability, a lot needs to be done in order to exploit this group of compounds to our advantage on a large, commercial scale. Hence it is need of time that all such herbal medicines should be considered for determination of their pharmacological activities by isolation of single entity responsible for anti-inflammatory activity and development of suitable formulation which would be beneficial against inflammatory disorders. So in this review article we discussed some herbal medicinal plants on behalf of their phytoconstituents which can be helpful in inflammation.

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Research Article

COMPARISON OF PESTICIDE RESIDUES IN POULTRY TISSUE IN VARIOUS DISTRICTS OF GARHWAL REGION OF UTTARAKHAND

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ABSTRACT

The present investigation was carried for comparison of drugs residues of Chlorpyrifos and Endosulfan in samples of poultry tissues (muscle, liver, fat and kidney) collected from different districts and respective sub-centers of Garhwal regionof Uttarakhand by using reverse phase HPLC. There were significant difference between district dehradun with the districts uttarkashi, chamoli and pauri Garhwal at $P<0.05$ in the residual concentrations of chlorpyrifos and endosulfan, irrespective of the sample type collected from Garhwal region of Uttarakhand. Contrast to that, residual concentrations of chlorpyrifos and endosulfan vary non-significantly with districts haridwar and tehri Garhwal.

Key words: Chlorpyrifos, endosulfan, HPLC, residue, poultry sample

INTRODUCTION

It is of great interest to investigate the toxic effects of insecticides in agriculture produce after oral administration of either insecticides treated crops or of low dose insecticides exposure for longer period**.** In India, the research on pesticide residue analysis especially in foods of animal origin is in infancy and need sincere efforts to meet out the global standards of food safety. Out of various pesticide residues likely to be encountered in meat industry, organochlorines, organophosphates and their metabolites are more prevalent. Endosulfan and chlorpyriphos are widely used for the control of pests, mites, flies and lice affecting the livestock and poultry (Loomi *et al.,* 1972) and detected in poultry egg, meat and cow milk and milk products (Rawat *et al.,* 2003).FAO/WHO has recommended the minimal risk level of different pesticides in human diet/ animal diet**.** According to the guidelines of Codex Alimentarious (2000) issued on various safety limits, the maximum residue limits for chlorpyrifos and endosulfan in poultry meat have been approved as 0.1 µg/g and 0.2 µg/g, respectively. In view of this fact, this study was undertaken for detection and comparison of pesticide residual concentration among various district of Garhwal regions of Uttarakhand.

MATERIAL AND METHODS

For sample collection, Garhwal region were divided into six centers (districts) and different samples were collected by multistage cluster sampling technique as described by Das (2000). The centers were selected on the basis of use of pesticides in various agricultural operations, public health programmes and animal husbandry practices in the Uttarakhand state. identified six centers were Dehradun,Pauri Garhwal,Tehri Garhwal,Uttarkashi,Chamoli andHaridwar. Twenty locally produced poultry samples were selected randomly from each districts sub-center. Each sample of poultry meat, egg, feed and water were collected for each specimen from respective sub centers and analyzed within 3 days of collection by HPLC. In the present study, reverse phase HPLC was used to detect and quantify the mentioned pesticides. Acetonitrile was used and a mobile phase (ACN : Water, 65 : 35). A wavelength of 220nm was used for detection of endosulfan and chlorpyrifos

Extraction from poultry tissue and water

The extraction of pesticide residues from different tissues (liver, kidney, muscle and fat) of poultry birds was done as per the method described by Loerger and Smith (1993) with slight modifications.Ten ml of HPLC grade acetonitrile was mixed with 5 gm of tissue and homogenized in a mortar. The homogenate was sonicated at 10 amplitude microns for 30secs, with a pause of 5 seconds (a total of 15 cycles) by using ultrasonic tissue disintegrator. The sonicated tissue was centrifuged at 12000rpm for 15 min and supernatant collected in a petridish and dried overnight at 37^0C. The residue was reconstituted in 2ml acetonitrile and subjected to clean-up procedure. Clean up process was done as per the technique described by Telling and Sissions (1977) with slight modifications using solid- phase extraction C_{18} cartridges.

Extraction from yolk

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

Table 1:
Comparison of mean difference residualconcentrationsof chlorpyrifos irrespective of the sample types within the districts of Garhwal region of Uttarakhand state.

Pesticide	District (I)	Districts (J)	Mean difference conc. I-J (µg/g)	Std. Error
Chlorpyriphos	Dehradun	Haridwar	-0.00356	0.005942
		Tehri Garhwal	0.014652	0.005953
		Uttarkashi	.0218365*	0.005964
		Chamoli	.0341113*	0.005953
		Pauri Garhwal	.0393916*	0.005964
	Haridwar	Dehradun	0.003556	0.005942
		Tehri Garhwal	.0182073*	0.005953
		Uttarkashi	.0253922*	0.005964
		Chamoli	.0376670*	0.005953
		Pauri Garhwal	.0429473*	0.005964
	Tehri Garhwal	Dehradun	-0.01465	0.005953
		Haridwar	-.0182073*	0.005953
		Uttarkashi	0.007185	0.005974
		Chamoli	.0194597*	0.005964
		Pauri Garhwal	.0247400*	0.005974
	Uttarkashi	Dehradun	-.0218365*	0.005964
		Haridwar	-.0253922*	0.005964
		Tehri Garhwal	-0.00718	0.005974
		Chamoli	0.012275	0.005974
		Pauri Garhwal	.0175551*	0.005985
	Chamoli	Dehradun	-.0341113*	0.005953
		Haridwar	-.0376670*	0.005953
		Tehri Garhwal	-.0194597*	0.005964
		Uttarkashi	-0.01227	0.005974
		Pauri Garhwal	0.00528	0.005974
	Pauri Garhwal	Dehradun	-.0393916*	0.005964
		Haridwar	-.0429473*	0.005964
		Tehri Garhwal	-.0247400*	0.005974
		Uttarkashi	-.0175551*	0.005985
		Chamoli	-0.00528	0.005974

*Mean difference concentrations differ significantly.

used for HPLC analysis.

Extraction from albumin

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

Extraction for poultry feed sample

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

RESULTS AND DISCUSSION

Mean residual concentrations difference of district Dehradun with the districts Uttarkashi, Chamoli and Pauri Garhwal vary significantly at $P<0.05$. Contrast to that; mean difference residual concentrations of chlorpyrifos vary non-significantly with districts Haridwar and Tehri Garhwal shown in the tables (1 and 2).

Mean residual concentrations difference of district pauri Garhwal with the districts Uttarkashi, Haridwar, Tehri Garhwal and pauri Garhwal vary significantly at $P<0.05$. Contrast to that; mean difference residual concentrations of chlorpyrifos vary non-significantly with district Chamoli.

Mean residual concentrations difference of district haridwar with the districts, chamoli and pauri Garhwal vary significantly at $P<0.05$. Contrast to that; mean difference residual concentrations of endosulfan vary non-significantly with districts Tehri Garhwal, Uttarkashi and Dehradun.

Mean residual concentrations difference of district Pauri Garhwal with the districts Haridwar, Tehri Garhwal and Pauri Garhwal vary significantly at $P<0.05$. Contrast to that; mean difference residual concentrations of

Table 2:
Comparison of mean difference residualconcentrationsof Endosulfan irrespective of the sample types within the districts of Garhwal region of Uttarakhand state.

Pesticide	District (I)	Districts (J)	Mean difference conc. I-J (µg/g)	Std. Error
Endosulfan	Dehradun	Haridwar	0.002562	0.008081
		Tehri Garhwal	-0.0032	0.008081
		Uttarkashi	0.019278	0.008081
		Chamoli	.0244907*	0.008081
		Pauri Garhwal	.0365929*	0.008081
	Haridwar	Dehradun	-0.00256	0.008081
		Tehri Garhwal	-0.00576	0.008081
		Uttarkashi	0.016716	0.008081
		Chamoli	0.021929	0.008081
		Pauri Garhwal	.0340308*	0.008081
	Tehri Garhwal	Dehradun	0.003196	0.008081
		Haridwar	0.005758	0.008081
		Uttarkashi	0.022474	0.008081
		Chamoli	.0276864*	0.008081
		PauriGarhwal	.0397886*	0.008081
	Uttarkashi	Dehradun	-0.01928	0.008081
		Haridwar	-0.01672	0.008081
		Tehri Garhwal	-0.02247	0.008081
		Chamoli	0.005213	0.008081
		Pauri Garhwal	0.017315	0.008081
	Chamoli	Dehradun	-.0244907*	0.008081
		Haridwar	-0.02193	0.008081
		Tehri Garhwal	-.0276864*	0.008081
		Uttarkashi	-0.00521	0.008081
		Pauri Garhwal	0.012102	0.008081
	Pauri Garhwal	Dehradun	-.0365929*	0.008081
		Haridwar	-.0340308*	0.008081
		Tehri Garhwal	-.0397886*	0.008081
		Uttarkashi	-0.01732	0.008081
		Chamoli	-0.0121	0.008081

* Mean difference concentrations differ significantly.

endosulfan vary non-significantly with districts uttarkashi and chamoli.

In Garhwal region mean residual concentration of chlorpyrifos and endosulfan irrespective of the sample type were detected maximum in district Haridwar and minimum in district Pauri Garhwal.Levels of chlorpyrifos and endosulfan residues were present in poultry meat eggs, feed and water samples. These findings are in conformity with the presence of pesticides in samples of feed grain of this region. The contamination of poultry meat, eggs and water with pesticide residues could be due to their accumulation in food grain used in preparation of poultry feed (Nobel,1990). Pesticide residue were also detected in samples of poultry meat collected from Trai, Kumaon region and adjoining plains (Taneja, 2000; Mishra, 2001). Various other several reports from India (Tripathi *et al.,* 1973; Singh and Chawla, 1988) and USA and Japan (Corneliussen,1969; Chen *et al.*, 2001) also revealed accumulation of pesticide residues in tissues of poultry and other animals. Pesticide residues were also detected in ground and surface water in India (Rao *et al.,* 1987, Flavio *et al.*,1999). Lino and Silveira (1994) analyzed chicken muscle and skin samples for residues of OP insecticides, chlorpyrifos, malathion collected in Coimbra; however, they could detect only ethion and methidathion in 4 and 2 samples, respectively. Mishra (2001) while screening poultry meat for pesticide residues reported that the mean residual concentration of chlorpyrifos was 0.35 µg/g in meat samples collected from different centers of Kumaon.

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

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Research Article

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

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ABSTRACT

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

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

INTRODUCTION

In recent years it has become common practice to incorporate antibiotics in rations fed to poultry and other domestic animals. Antibiotics are used in the poultry to enhance growth and feed efficiency and reduce the incidence of disease. Antibiotic usage has facilitated the efficient production of poultry, allowing the consumer to purchase high quality meat and eggs at a reasonable cost. In a recent consumer survey, Resurreccion and Galvez (1999) reported that 77% of consumers responding considered animal drug residues in meats to be an extreme health concern. Although antibiotics are approved for use in poultry. Another danger of receiving antibiotics residue is microbial resistance of body microflora to common antibiotics which may cause serious problems at microbial infections. There is some problems for soil microflora which receives antibiotics residue in birds manure (Lee *et al.,* 2000). Non detected effects of this problem in human communities are a wide spectrum resistance to antibiotics as a chronic effect.

Many of antibiotics used to treat bacterial infections in humans have veterinary application; prophylactics and growth promoters. In these two cases as growth promoters the antibiotics are used at concentration lower than therapeutic concentration for a longer period of time. This is potentially dangerous practice since it provide strong selective pressures for emergence of antibiotic resistant strain of bacteria (Simonsen *et al.,* 1998), induction of allergic reactions in human and technological problem of fermented meat products (Pavlov *et al.,* 2005).

Chlortetracycline (CTC), a member of tetracycline family, is a broad-spectrum antibiotic which is commonly used in poultry. Chlortetracycline can be used against *Escherichia coli*, *Salmonella* and *Staphylococcus* spp. Chlortetracycline is given orally in drinking water and by intramuscular injection. Chlortetracycline (CTC) supplementation in quantities of 50 to 20,000 ppm showed the presence of the antibiotic in chicken serum, tissues, and eggs (Durbin *et al*.,1953).

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

MATERIALS AND METHODS

Sample collection

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

Chemicals and apparatus

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

HPLC system comprising mobile phase reservoir, degasser, HPLC pump, sample injector, guard column, main column, detector, data collection unit, waste or fraction collector with NEXERA software was used for this study.

Chromatography conditions

The (UHPLC) unit of Liquid Chromatography Mass Spectrometer (LCMS- 8030, Shimadzu, Japan) with HPLC system equipped with photodiode array UV- Vis detector was used for this study. Chromatography conditions were maintained as described by Adewuyi *et al*. (2011) with

slight modification. Particle separation was done using with C18 column (Supelco, USA, column dimensions: 150× 2.1 mm, particle size: 1.9 µm) and the temperature of column was set at 30°C. The mobile phase comprised of 0.01M oxalic acid, acetonitrile and methanol respectively in 70:20:10 ratio (HPLC grade) with flow rate adjusted at 1ml/min. Peak separation was accomplished after 3.10 minutes and 20 µl treated samples were required for injection.

Standard preparation

Stock standard solutions of chlortetracycline were prepared by dissolving 10 mg of compound in 10 ml of methanol to obtain a ûnal concentration of 1 mg/ml. Stock standard solutions were stored at -20^{0}C and were stable for at least 4 weeks. These solutions were diluted to give a series of working standard solutions that were prepared daily as per the Cinquina *et al*. (2003).

Sample preparation

The extraction of chlortetracycline was done by using method describe by Cinquina *et al*. (2003) with slight modification. Briefly, five gram of muscle/liver/kidney of chicken meat sample were homogenised, placed in a glass centrifuge tube and 2 ml of 20 per cent trichloroacetic acid (TCA) added. Sample was extracted with 20 ml McIlvaine buffer and centrifuged at 4000 rpm for 20 min. The supernatant was then applied to a SPE cartridge, previously activated with 3 ml of methanol and 2 ml of water. After sample loading, the cartridge was washed with 2 ml of methanol 5 per cent in water. Finally chlortetracycline was eluted with 3 ml of methanol. The solvent was removed under a nitrogen stream and the residue was dissolved in 1 ml of methanol and ûltered with a 0.2 mm ûlter. An aliquot (20 ml) was injected into the UHPLC system.

RESULTS AND DISCUSSION

Mean residue concentration of chlortetracycline in chicken meat samples (muscle, liver and kidney) from different target areas of Jabalpur district was analyzed using UHPLC. Total 180 chicken meat samples were analyzed including 18 samples each from Jabalpur-1 to Jabalpur-10 target areas. Among the 180 samples, 48 samples (26.66 per cent) were detected positive for Chlortetracycline residues. Out of these 48 positive samples, 8 samples (16.66 per cent) were quantified with Chlortetracycline residue at violative levels while 40 samples (83.88 per cent) were detected with residue below the recommended MRLs. Chlortetracycline residue concentration was detected in meat samples (muscle, liver and kidney) of target area 1, 3, 7, 8 and 10 (Table 01 and Fig. 01).

Out of 48 positive samples, mean residue concentration of chlortetracycline was 65.91±13.87 µg/kg in muscle, 151.16±36.01 µg/kg in liver and 280.91±85.48 µg/kg in kidney samples. After boiling the same samples, the mean residual concentration significantly reduced to 45.94±9.74 µg/kg in muscle, 102.23±24.36 µg/kg in liver and 183.49±55.85 µg/kg in kidney samples (Table 02 and Fig.02). Mean residue concentration of Chlortetracycline significantly reduced to 30.12 per cent, 32.37 per cent and 34.67 per cent in muscle, liver and kidney samples respectively.

Mean residue concentration of Chlortetracycline in muscle, liver and kidney samples were analyzed by HPLC in different target areas of Jabalpur district. Total 180 chicken meat samples were analyzed including 18 samples each from 10 target areas. As revealed under the study, 48 samples (26.66 per cent) showed detectable levels of chlortetracycline residues. The mean residue concentration of chlortetracycline in muscles, liver and kidney samples were 77±30.51 µg/kg, 191±80.81 µg/kg

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

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

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

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

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

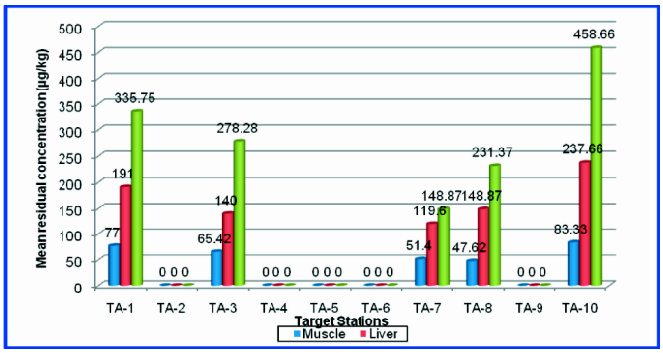

Fig 1:
Mean residue concentration of Chlortetracycline (µg/kg) in muscle, liver and kidney of broiler chicken from different target areas

and 335.75±183.85 µg/kg respectively in target area 1, 65.42±18.82 µg/kg, 140±48.41 µg/kg and 278.28±121.04 µg/kg respectively in target area 3, 51.4±17.26 µg/kg, 119.6±54.35 µg/kg and 206±113.39 µg/kg respectively in target area 7, 47.62±15.67 µg/kg, 148.87±38.34 µg/kg and 231.37±89.32 µg/kg respectively in target area 8, 83.33±38.36 µg/kg, 237.66±90.43 µg/kg and 458.66±201.32 µg/kg respectively in target area 10. Liver and kidney samples showed more positive results with higher residual concentration as compared to the muscle samples. Out of 180 samples, 8 samples (4.44 per cent) were detected with residue level above maximum residue limit. The findings are in accordance with Cinquina *et al.,* (2003) who also quantified the residual concentration of chlortetracycline in bovine meat.

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

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Research Article

SCREENING OF DISTILLER'S DRIED GRAINS WITH SOLUBLES (DDGS) FOR AFLATOXINS USING THIN LAYER CHROMATOGRAPHY (TLC)

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ABSTRACT

Distillers dried grain with solubles (DDGS), the by-product of alcohol industries, is an important source of domestic animal feed due to high nutritive value. A total number of 115 DDGS feeds samples were collected and analysed for total Aflatoxin (i.e. Aflatoxin B_1, B_2, G_1 & G_2) by thin layer chromatography (TLC) as per AOAC method. The average moisture percentage of DDGS ranges from 8-12%. The results showed that the number of contaminated samples in DDGS were highest with aflatoxin B_1 (27.82 %, range of 10-40 ppb) followed by Aflatoxin B_2 (20.86 %, 5-20 ppb) and both Aflatoxin G_1 & G_2 (5%, range of 5 ppb each). Thus, the presence of mycotoxins in DDGS may limit its use as feed in livestock and poultry. It is recommended from the findings of this study that every batch of DDGS should be examined in reference laboratories before reaching farmsteads for the presence of mycotoxins.

Key words: Aflatoxin, DDGS, Thin Layer Chromatography

INTRODUCTION

Distillers dried grain with solubles (DDGS) is the by-products of the alcohol industries, produced by the fermentation process of cereal grains such as maize, barley, wheat, sorghum and rye in dry mill ethanol plants (Abd El-Hack, 2015). DDGS contain high levels of protein, fiber, minerals and vitamins which are important source of animal feed and may be used to enrich human foods (Khatibi *et al.,* 2014). The level of mycotoxin in DDGS was reported to be approximately 3 times as high as the level in the grain that could limit its use in the animal feed industry (Zhang and Caupert, 2012). Among 300 mycotoxins known, Aflatoxin B1 is the most potent natural carcinogen classified as class I by IARC and is associated with both toxicity and carcinogenicity in human and animal populations. Thin layer chromatography (TLC) is a method still broadly used for quantitative and semi-quantitative measurements of mycotoxins with detection by fluorodensitometry or visual procedures. TLC based on silica gel, F_{254} fluorescent silica gel or silica gel impregnated with organic acid has been reported to be applied for detection of common mycotoxins (Lin *et al.,* 1998). Thus, the present study was designed to rapidly screen the presence of aflatoxin B_1, B_2, G_1 and G_2 in DDGS using TLC.

MATERIALS AND METHODS

All the chemical and solvents used for analysis were of analytical grade (Emerck). A total number of 115 DDGS feeds samples were collected randomly from different parts of Chennai, Tamil Nadu. Extraction of Aflatoxin B_1, B_2, G_1 & G_2 was done as per AOAC method with screening and quantification by TLC.

Determination of moisture content

About 500 grams of samples were collected and were finally ground using explosion proof laboratory blender. Then, the moisture content of each of the sample was determined as per the method described by Ubwa *et al*. (2012).

Extraction of aflatoxins

Sample analysis was carried out by taking a known quantity (25g) of the powdered sample in a 250 ml flask and treating with 19 ml distilled water and 106 ml acetone. This mixture was shaken for 30 minutes at 200 rpm on a shaker. It was then filtered through Whatman paper (No.1). To the 75 ml of filtrate, 1.5g of cupric carbonate was added. Another solution of 85 ml of 0.2N NaOH and 15 ml of 0.4M $FeCl_3$ was prepared. This solution was mixed with filtrate containing cupric carbonate thoroughly and then filtered through Whatman No. 1 filter paper. Transfer the 100 ml filtrate into a 500 ml separating funnel and add 100 ml of 0.03% H_2SO_4 and 25 ml of chloroform. Shake the mixture vigourously releasing the fumes or gases and allow for separation. Then the lower layer was transferred to a 100 ml separating funnel and treated with 40 ml 1% KCl in 0.02M KOH solution by gentle shaking and allowing for separation. The lower layer was collected in a vial, by passing through anhydrous sodium sulphate bed. The extract was evaporated in a hot plate under fume hood. Finally the dried extract was re-dissolved in 0.2 ml of chloroform and used for TLC (Ramesh *et al.,* 2013).

Sample assay

The dissolved residue was then spotted on to a

silica gel F_{254} 10 × 20 cm TLC plates of about 0.5 mm thickness as 5 µl drops using micro-syringes. The standard solution of aflatoxins B_1, B_2, G_1 & G_2 (Sigma Aldrich) was also spotted on to the same plate as drops of 1, 3, 5 ìl. These standards were calibrated and checked for its purity by UV Spectrophotometer (AOAC, 2000). Samples of chloroform extracts and standard solution of aflatoxins were spotted in 1 cm bands. The spotted plate was placed vertically in the development tank containing chloroform: acetone (9:1, v/v) mobile phase upto 80 mm from lower edge of plate and covered properly. It took 20 to 30 min for the solvent to reach the stop line (9 cm) from the base line. After each development, the plate was then removed and dried with a hair drier. This process was repeated for all the samples and the developed plates were viewed under a long wavelength UV lamp (366 nm). The aflatoxin B and G when present fluoresced blue and greenish blue, respectively. The fluorescence intensities of aflatoxin spots of sample were compared with those standard spots. The sample spot, which matches one of the standard spots, was selected. Standard was also used to compare the colour and R_f/ R_t value of unknown sample streak on the plate. The amount of aflatoxin B_1, B_2, G_1 & G_2 was estimated. For further confirmation TLC plates were sprayed with 20% H_2SO_4 and heated 10 min at 110° C, the fluorescence intensity increased. Method validation was performed in blank samples spiked with aflatoxin standards at 5µg kg^{-1} (Sarathchandra and Muralimanohar, 2013 and 2014).

RESULTS AND DISCUSSION

Our study showed that the average moisture percentage of DDGS is 8-12 %. Moisture is also important because it influences microbial growth and thus affects shelf life during storage. The moisture content data in this study are very similar to other reports (Bhadra *et al.,* 2009; Zhang and Rosentrater, 2013).

The results showed that maximum number of DDGS samples is contaminated with aflatoxin B_1, with the percentage of 27.82 % and within 10-40 ppb concentration range, followed by aflatoxin B_2 (20.86 %; 5-20 ppb), while both aflatoxin G_1 and G_2 have 5% of contamination with concentration range of 5 ppb each. Out of 115 DDGS samples, aflatoxin B_1 were detected in 32 samples, aflatoxin B_2 in 24 samples, aflatoxin G_1 and G_2 in 5 samples each. Most of the DDGS contaminated samples contain 10 ppb concentration of aflatoxin B_1, on an average of 15.93 ppb. While aflatoxin B_2 contaminated DDGS samples have an average of 9.16 ppb. The range of the above mentioned aflatoxins in DDGS feed samples are presented in Table 1. The aflatoxin contamination level of DDGS samples in our studies were below the guidelines provided by US FDA or European Union in animal feed. Garcia *et al.* (2008) reported that all the mycotoxins except DON examined in both coproducts, DDGS and WDG, were well below the FDA recommendations for each mycotoxin in animal feed. The report by Rodrigues (2008) showed that 99% of the 103 DDGS samples analysed contained at least one detectable mycotoxin, with 8% containing detectable aflatoxins. Among the 103 DDGS samples, 67% were from the United States and 33% were from Asia. Zhang *et al.* (2009) conducted an extensive literature review of published studies and evaluated samples from three large data sets of DDGS samples to determine the extent and level of mycotoxin contamination among U.S. DDGS sources. Concentrations of all mycotoxins in DDGS were generally below the FDA action levels for all mycotoxins. Similar finding was also reported by Caupert *et al*. (2011). Kathirvelan *et al.* (2014) reported that out of a total number of 20 DDGS samples analysed, 16 samples contained traces of aflatoxin B_1 and four samples contained 50-100 ppb using TLC. The higher percentage of positivity in their finding when compares with our present studies might be due to differences in sources of DDGS or the seasonal variation in sample collection. The source of the DDGS collected in our present study is from rice and wheat. It has been reported that sample with high aflatoxin, had high level of moisture (13-15%) at the time of collection of raw material sample. The environmental condition which includes the warm and humid storage conditions favor the aflatoxin development in feed ingredients. Like many grain-based feed ingredients, DDGS may contain amounts of mycotoxins that can negatively affect animal performance. Mycotoxins can be present in DDGS if the grain delivered to an ethanol plant is contaminated with them as they are not destroyed during the ethanol production.

Mycotoxin regulations have been established in more than 100 countries (Van Egmond *et al.,* 2007), and the maximum acceptable limits vary greatly from country to country. In the case of lots intended for industrial

Table 1:
Aflatoxin B_1, B_2, G_1 & G_2 contamination in DDGS feed samples by TLC

Type of mycotoxins	No. of sample tested	No. of contaminated samples	% of contaminated samples	Concentration range (ppb)		Average Conc.(ppb)in positive samples
				Low	High	
Aflatoxin B_1	115	32	27.82	10	40	15.93
Aflatoxin B_2	115	24	20.86	5	20	9.16
Aflatoxin G_1	115	5	4.7	0	5	5
Aflatoxin G_2	115	5	4.7	0	5	5

purposes (e.g., bioethanol or biopolymer production), neither maximum limits nor guidance levels have been established. The European Union has set a maximum level of aflatoxin in agriculture commodities with aflatoxine B_1 of 4µg kg^{-1} (Binder, 2007). The U.S. FDA has established maximum tolerable levels ranging 20-300ppb for aflatoxins in feed ingredients for various types of animal feeds (Zhang *et al.,* 2009).

Feeding of DDGS to livestock and poultry will be a promising strategy to reduce the feed cost on production with simultaneous maintenance of nutritive value of feed. One of the great advantages of DDGS is the possibility of its storage for a year but WDGS can be stored for a week only. The presence of mycotoxins is one of the major disadvantages in feeding DDGS to livestock and poultry. It is recommended from this study that every batch of DDGS reaching farmsteads be examined in reference laboratories for the presence of mycotoxins.

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Research Article

SUBCHRONIC TOXICITY OF THIAMETHOXAM AND THE AMELIORATIVE POTENTIALS OF QUERCETIN IN MALE WISTAR RATS

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ABSTRACT

This study was designed to evaluate subchronic toxicity of thiamethoxam and amelioration potential of quercetin on biochemical parameters of blood. Thiamethoxam was administered orally daily at two dose levels i.e. 2.5% and 5% of maximum tolerated dose (MTD = 4200 mg/kg b.wt.) and quercetin was administered orally daily at dose of 50 mg/kg for 60 and 90 days. A gap of 12 hours was maintained between thiamethoxam and quercetin administration. Blood samples were collected in heparinized vials and centrifuged and kept at -20°C till its use. Various enzymatic and non-enzymatic biochemical parameters were determined using standard methods. Thiamethoxam at two dose levels of 2.5% and 5% maximum tolerated dose (MTD = 4200 mg/kg) significantly increased aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in 90 days schedule, whereas alkaline phosphatase (ALP) increased at both dose rates in 60 days and decreased in 90 days schedule as compared to control. Thiamethoxam administered at lower dose rate increased serum glucose level in 60 and 90 days schedules while total protein, albumin and creatinine increased at both dose levels in 90days schedule as compared to control. Amelioration effect of quercetin on AST at higher dose level was observed in 90 days schedule and on ALT at both doses levels in 90 days, whereas on ALP at both dose levels in 60 and 90 days treatment schedule compared to control. Amelioration effect of quercetin on total plasma protein and albumin at both dose levels in 90 days and in glucose at lower dose level in 90 days schedule as compared to control was observed. Thus quercetin ameliorated thiamethoxam induced biochemical alterations inin a 90 days study in rats.

Key words: Biochemical parameters, quercetin, subchronic toxicity, thiamethoxam, rats.

INTRODUCTION

Thiamethoxam, a second generation neonicotinoid belonging to thioncotynyl subclass and marketed under the trademarks ACTARA(R) for foliar treatment, causes low toxicity to warm blooded animals. The action of thiamethoxam is based on interference with the transmission impulses in the nervous system of insects. Similar to the naturally occurring neurotransmitter acetylcholine, thiamethoxam acts by exciting certain nerve cells by acting on a receptor protein. The lasting effect of the product results in a disorder of the nervous system of the insect killing the treated insects (Pasqualini *et al.,* 2001). Thiamethoxam induced an increase in the anxiety behavior and decreased acetylcholinesterase activity in different brain regions and thus is likely to act on the central nAChRs and would produce an alteration on the cholinergic transmission, modulating the anxiety behavior, acetylcholinesterase levels (Rodrigues *et al.,* 2010). Because of its greater water solubility it moves readily in plant tissues. Among the vertebrates, humans are thought to be protected from neonicotinoid toxicity because of the poor permeability of blood brain barrier and high water solubility and slow metabolism in mammals (Tomizawa and Casida, 2005). The anti-oxidant activity of quercetin molecule is higher than other well-known anti-oxidant molecules such as ascorbyl, trolox and rutin because of the number and position of the free hydroxyl groups in the quercetin (Nuengchamnong *et al.,* 2004). The objective of this research is to ascertain the subchronic toxicity of thiamethoxam and the ameliorative potentials of quercetin on enzymatic and non-enzymatic biochemical parameters in male Wistar rats.

MATERIALS AND METHODS

Experimental animals

Ninety six male Wistar rats weighing 120-140 g were procured from Disease Free Small Animal House (DFSAH), Lala Lajpat Rai University of Veterinary and Animal Sciences (LUVAS), Hisar. The Wistar rats were acclimatized to laboratory conditions for 2-3 days before the experiment ands were provided with feed and water *ad libitum* and maintained at room temperature with a natural light-dark cycle with temperature ranging between 22 to 27º C throughout the study. The prior approval of institutional animal ethical committee (IAEC) was obtained for use of the animals in this study.

Drugs and chemicals

The formulation product of thiamethoxam (ACTARA®)25G (Syngenta India Ltd) and quercetin dihydrate was used in this experiment, gum acacia was used as a vehicle for oral administration of the compounds.

Experimental design

Thiamethoxam at two dose levels (2.5% and

5% of MTD = 4200mg/b.wt) was administered orally daily for 60 and 90 days in adult male rats for subchronic toxicity study. An ameliorative effect of quercetin at dose rate of 50 mg/kg orally was also studied in rats 60 and 90 days. Treatment schedule of different groups was followed as mentioned below:

Group 1: Vehicle control (16 rats): 2% gum acacia was given twice daily orally for 60 and 90 days.

Group 2: Thiamethoxam (2.5% of MTD) (16 rats): Thiamethoxam suspension in 2% gum acacia was administered once daily orally for 60 and 90 days.

Group 3: Thiamethoxam (5% of MTD) (16 rats): Thiamethoxam suspension in 2% gum acacia was administered once daily orally for 60 and 90 days.

Group 4: Quercetin (50 mg/kg) (16 rats): Quercetin suspension in 2% gum acacia was administered once daily orally for 60 and 90 days.

Group 5: Thiamethoxam (2.5% of MTD) and Quercetin (50 mg/kg) (16 rats): Quercetin and thiamethoxam suspension in 2% gum acacia were administered once daily orally for 60 and 90 days. The gap of 12 hours was maintained between thiamethoxam and quercetin administration.

Group 6: Thiamethoxam (5% of MTD) and Quercetin (50 mg/kg) (16 rats): Quercetin and thiamethoxam suspension in 2% gum acacia were administered once daily orally for 60 and 90 days. The gap of 12 hours was maintained between thiamethoxam and quercetin administration. Eight rats from each group were killed on the next day of completion of sub chronic exposure to collect blood.

Blood samples were taken by heparinized hypodermic syringe (5mg/ml in NSS) directly from heart after anaesthetizing animals with ether in heparinized vials and refrigerated till processing. 5 -7 ml blood was used for separation of plasma. Plasma was separated in a refrigerated centrifuge at 3000 rpm for 15 min and stored at -20°C for analysis of enzymes and biochemical constituents.

Biochemical parameters

Alkaline phosphatase, aspartate aminotransferase (AST), alanine aminotransferase (ALT), total protein, albumin, cholesterol, total triglycerides, creatinine, urea nitrogen and glucose were estimated in plasma using an Erba XL-200 model fully Automated Random Access Clinical Chemistry Analyzer (Model: EM 200, Erba®, Mannheim, USA).

Statistical analysis

The results were expressed as mean ± standard error of means (S.E.M.) followed by one way ANOVA along with Bonferroni multiple comparison tests using Graph Pad Prism Version -4.0 software and Microsoft Excel ($p<0.05$) was the critical criterion for the statistically significant differences between the data (Mead and Curnow, 1982).

RESULTS

As shown in Table 1, in 60 days schedule, AST level did not increased significantly in TMX-treated groups at both doses of (2.5%TMX and 5%TMX) compared to control and the rest of the treated groups. In 90 days treatment schedule there was significant increase in AST levels at dose rates of 2.5%TMX, 5.0% and in quercetin-treated groups compared to control. There is no significant effect of 2.5% TMX + Qu and 5.0% TMX + Qu dose on AST levels compared to control. In 60 days schedule, ALT level did not increase significantly in TMX-treated groups at both dose rates (2.5%TMX and 5%TMX) compared to control and the rest of the treated groups. In 90 days treatment schedule there was significant ($p<0.05$) increase in ALT levels in 2.5%TMX and 5.0% TMX-treated groups compared to control. The serum level of ALT in the other groups remains the same to that of control group. In 60 days treatment schedule, ALP level increased significantly ($p < 0.05$) in TMX-treated groups at both dose rates of (2.5%TMX and 5%TMX) compared to control. In 90 days treatment schedule the level of ALP decreased significantly ($p < 0.05$) at both dose rates (2.5%TMX and 5%TMX) compared to control group. The rest of the treated groups remain same as compared to control.

As shown in Table 2, in 60 days schedule, the plasma protein concentration is not significantly ($p<0.05$) influenced in TMX-treated group at dose (2.5%TMX), as compared to control and quercetin group. In 90 days schedule, plasma protein concentration was observed to have decreased significantly ($p<0.05$) in TMX-treated groups at both doses (2.5%TMX and 5.0%TMX) as compared to control. The levels were quite high in 2.5%TMX+Qu-treated and 5.0%TMX+Qu-treated groups though not significant compared to control group. In 60 days schedule, the plasma albumin concentration did not vary significantly ($p<0.05$) in TMX-treated group at both doses (2.5%TMX and 5.0%TMX), as compared to control and quercetin-treated groups. There is significant ($p<0.05$) increase in plasma albumin concentration in 2.5%TMX and 5.0%TMX treated group compared to control group. There is significant ($p<0.05$) effect in plasma albumin concentration in Quecertin, 2.5% TMX + Qu and 5.0% TMX + Qu compared to control group.

There is no significant change in the plasma globulin concentration in all the different groups of various treatment schedules.

There was no significant change in albumin/ globulin ratio among different groups of various schedules. In 60 days schedule, there was no significant change in plasma cholesterol level in 2.5%TMX and 5.0%TMX treated group as compared to control animals. In 90 days schedule, plasma cholesterol levels were observed to have decreased significantly ($p<0.05$) in Quecertin and 2.5%

TMX + Qu as compared to control. In 60 and 90 days schedule, plasma triglyceride levels were observed not to have increased significantly ($p<0.05$) in TMX-treated groups at both doses (2.5%TMX and 5.0%TMX) and quercetin-treated groups as compared to control. In 60 and 90 days treatment schedule, plasma glucose levels were observed not to have increased significantly ($p<0.05$) in group treated with 2.5%TMX as compared to control and quercetin-treated groups. Significant ($p<0.05$) decrease was observed in quercetin treated in 90 days treatment schedule compared to control. Significant ($p<0.05$) increase was observed at the dose rate of 2.5% TMX + Qu as compared to control (Table 2).

In 60 days schedule, the plasma creatinine concentration was not increased in TMX-treated groups at both doses (2.5%TMX and 5.0 %TMX) as compared to control, quercetin and 2.5%TMX + Qu -treated groups. There was significant decrease in plasma creatinine concentration in 5.0 %TMX+Qu-treated group as compared to control. In 90 days treatment schedule, plasma creatinine levels were observed to have increased significantly ($p<0.05$) in TMX-treated groups at both doses (2.5%TMX and 5.0%TMX) as compared to control. There was no significant effect in plasma creatinine concentration in quercetin, 2.5%TMX+Qu and 5.0%TMX+Qu-treated group compared to control respectively. In 60 days schedule, there was no significant increase in urea nitrogen level in 2.5%TMX and 5.0%TMX-treated group as compared to control and quercetin treated group. In 90 days schedule, there was significant ($p<0.05$) increase in plasma urea nitrogen levels in 2.5%TMX, 5.0%TMX and 5.0%TMX-treated group as compared to control treated groups (Table 2).

DISCUSSION

Thiamethoxam at two dose levels of 2.5% and 5% maximum tolerated dose (MTD = 4200 mg/kg) significantly increased AST and ALT in 90 days schedule, whereas ALP increased at both dose rates in 60 days and decreased in 90 days schedule as compared to control.

Liver functional AST and ALT are intracellular enzyme present in both cytoplasm and mitochondria widely distributed throughout the body tissues with the greatest amount in cardiac muscle, skeletal muscle and the kidneys that is used to transfer amino groups from aspartate (forming oxaloacetate) to 2 oxoglutarate (forming glutamate) and is a key enzyme in gluconeogenesis. These enzymes activity in the plasma are most frequently determined for the diagnosis of liver pathology especially in liver carcinoma, infective hepatitis, alcoholic cirrhosis, biliary obstruction and toxic hepatitis (Kaneko, 1997; Abdel-Wahab *et al.,* 2007)

ALT is an intracellular cytoplasmic enzyme widely distributed throughout the body's tissues with the greatest amounts in liver and the kidneys (Murray *et al.,* 2006). Serum ALT and AST indicate the concentration of hepatic intracellular enzymes that have leaked into the circulation indicating hepatocellular injury (Han *et al.,* 2012). Alkaline Phosphatases are a group of enzymes found primarily the liver (isoenzyme ALP-1) and bone (isoenzyme ALP-2). There are also small amounts produced by cells lining the intestines (isoenzyme ALP-3), the placenta, and the kidney in the proximal convoluted tubules (Kaplan *et al.,* 1998).

The administration of 2.5% and 5.0% TMX MTD in this study indicated that liver and kidneys were affected on prolonged administration. An elevation in the serum activity of AST is known to occur in association with liver diseases or pathology in a variety of animal species and it is of diagnostic importance in the assessment of the level of liver and kidney cellular damage especially if no disease exists in other tissues in which it is found in high concentration (Teitz, 1994). This finding is further substantiated by the lesions observed in the liver and kidney following histopathological examination of these organs. Liver toxicity was observed at higher dose administration of thiamethoxam manifested as hepatocellular hypertrophy, increased liver weights and associated changes in clinico-biochemical parameters including increased cholesterol levels though not observed in this study and increased activity of certain liver enzymes. It was postulated that the observed hyaline change in proximal convoluted tubules of the male rat kidney was due to accumulation of protein that is unique to male rats leading to kidney pathology. Similar elevations in ALT in rodentstreated with imidacloprid (neonicotinoid) were reported by several authors (Mohany *et al.*, 2012).

Plasma proteins are a significant indicator of health condition, metabolic and production features of animals due to physiological role exhibited by this macromolecule in the body (Kaneko *et al.*, 1997). Plasma proteins play important function in the maintenance of homeostasis because they play significant role in the maintenance of colloid osmotic pressure, blood volume and normal water composition in the body compartment. Liver is the primary site for the synthesis of this macromolecule. The most common causes of surge in serum total proteins level is hepatotoxicity which leads to the production of globulins called acute phase proteins and immunoglobulin's (Burton, 1994). Decrease in total protein and albumin levels is suggestive of development of disorder in protein synthesis and metabolism which is an indication of hepatotoxicity (Eraslan *et al.,* 2009). Albumin is the most abundant serum protein and is largely responsible for maintaining intravascular osmotic pressure. Globulins are a heterogenous population of proteins that include specific transport proteins, inflammatory mediators, acute phase

proteins, clotting factors, enzymes and immunogoblins. Thiamethoxam in this study administered at lower dose rate increased serum total protein and albumin increased at both dose levels in 90days schedule as compared to control.

Thiamethoxam in this study administered at lower dose rate increased serum glucose level in 60 and 90 days schedules as compared to control. Creatinine is produced by degradation of creatine and creatine-phosphate, an energy storing molecule mainly present in skeletal muscles, and its catabolism to creatinine occurs at a steady state. It mainly circulates in a free form in the plasma and is distributed into the whole body water compartment (Watson *et al.,* 2002). Increased levels of creatinine indicate degeneration of kidneys, heart muscle, and other muscles and its estimation is very useful in

Table 1:
Effect of subchronic oral exposure of thiamethoxam, quercetin and their combination on plasma enzyme biochemical parameters.

Parameter(IU/L)	Days	Control	2.5% TMX	5.0% TMX	Quecertin(Qu)	2.5% TMX + Qu	5.0% TMX + Qu
AST	60	193.7 ± 24.09	152.7 ± 15.42	161.5 ± 15.04	163.2 ± 23.00	179.2 ± 8.80	160.6 ± 6.78
	90	152.32 ± 22.89	266.58[a]± 12.35	330.68[a]± 21.95	445.90[a]± 94.63	252.49 ± 51.72	156.58[cd]± 16.73
ALT	60	42.8 ± 4.33	42.79 ± 1.93	36.5 ± 1.79	38.1 ± 2.88	44.4 ± 6.72	38.1 ± 2.45
	90	36.36 ± 3.74	64.60[a]± 6.75	63.90[a] ± 7.21	51.90 ± 4.93	43.58[b] ± 1.69	48.35[c] ± 3.47
ALP	60	128.1 ± 17.90	180.80[a]± 20.50	174.9[a]± 11.29	137.6 ± 5.71	150.4[b]± 12.7	144.1[c]± 19.0
	90	120.0 ± 20.14	93.25[a]± 24.08	78.00[a]± 4.79	105.25± 8.09	119.63[b] ± 23.39	118.5[c]± 7.29

Values are expressed as Mean±SEM of eight animals in each group.
a, b, c, d, e (p d" 0.05) vs. control, 2.5% TMX, 5.0% TMX, Qu and 2.5% TMX + Qu, respectively.
2.5%TMX – mean 2.5% MTD of thiamethoxam /kg b.wt. (105mg/kg b.wt orally), respectively. AST – Aspartate aminotransferase; ALT – Alanine aminotransferase; ALP – Alkaline phosphatase.

Table 2:
Effect of subchronic oral exposure of thiamethoxam, quercetin and their combination on plasma non enzyme biochemical parameters.

Parameters	Days	Control	2.5% TMX	5.0% TMX	Quecertin (Qu)	2.5% TMX + Qu	5.0% TMX + Qu
Total protein (g/dl)	60	7.36	8.12	8.07	10.1	15.7[abcd]	16.4[abd]
		0.39	0.36	0.8	1.68	0.59	0.54
	90	16.3	20.1[a]	20.7[a]	15.8[b]	16.7[bc]	16.3[bc]
		0.89	0.52	1	0.68	1.16	0.59
Albumin (g/dl)	60	3.93	4.06	3.62[b]	3.85	3.97[c]	3.94
		0.06	0.09	0.06	0.06	0.11	0.07
	90	3.69	4.53[a]	4.62[a]	3.56 [bc]	3.71 [bc]	3.61[bc]
		0.16	0.09	0.07	0.08	0.08	0.17
Globulin (g/dl)	60	2.25	1.81	1.89	2	2.43	2.45
		0.18	0.15	0.18	0.03	0.03	0.04
	90	2.34	2.1	2.08	2.39	2.08	2.38
		0.21	0.25	0.32	0.42	0.25	0.29
A:G Ratio	60	1.81	1.88	1.86	1.94	1.53	1.59
		0.12	0.2	0.27	0.19	0.13	0.15
	90	1.9	2.08	2.01	1.88	1.99	1.88
		0.24	0.26	0.25	0.21	0.2	0.22
Cholesterol (mg/dl)	60	90.6	86.5	81.9	86.3	86.3	86.3
		1.93	2.97	2.29	2.57	2.57	2.56
	90	90.6	86.5	92.3	77.6[ac]	76.0[ac]	81.5
		1.94	2.97	3.51	3.42	2.32	2.31
Triglycerides (mg/dl)	60	47.5	49.4	54.8	53.4	55.5	59.5
		3.38	4.19	2.84	3.22	3.87	1.79
	90	45.1	54.1	57.3	52.38	48.38	55.25
		5.55	4.67	4.16	1.94	4.06	5.04
Glucose	60	88.7	142.9 [a]	99.1	110.2	154.9[acd]	123.4
(mg/dl)		7.48	7.33	11	6.46	17.2	6.49
	90	111.8	112.7[a]	105.0[b]	61.1[abc]	97.0 [bd]	123.1[bd]
		11.2	3.07	5.99	4.69	7.23	8.85
Creatinine	60	1.12	1.02	0.99	0.97	0.99	0.86 [a]
(mg/dl)		0.06	0.02	0.04	0.03	0.04	0.02
	90	0.92	2.38[a]	1.85[ab]	0.95 [bc]	1.04[bc]	1.13 [bc]
		0.05	0.13	0.07	0.03	0.09	0.16
Blood urea nitrogen (mg/dl)	60	33.9	36.9	36.6	40.7	35.2	34.9
		1.58	1.56	1.5	1.75	1.19	1.79
	90	36.4	58.1	58.6	40.9	44.6	52.5
		3.05	3.59	2.09	5.39	10.29	14.19

Values are expressed as Mean SEM of eight animals in each group. a, b, c, d, e ($p<0.05$) vs. control, 2.5% TMX, 5.0% TMX, Qu and 2.5% TMX + Qu, respectively. 2.5%TMX – mean 2.5% MTD of thiamethoxam /kg b.wt. (105mg/kg b.wt orally), respectively.

early deduction of nephrotoxicity induced by exogenous compounds (kaneko, 1997). Urea is synthesized by the liver from ammonia that is absorbed from the intestine or produced by endogenous protein catabolism. Serum urea concentration is, therefore, affected by rate of urea production, glomerular filtration rate (GFR) and flow rate of urine through the renal tubule. The elevation of plasma levels of urea and creatinine are considered as significant markers of renal dysfunction (Almdal and Vilstrup, 1988). Thiamethoxam in this study administered at lower dose rate increased serum creatinine and blood urea nitrogen increased at both dose levels in 90days schedule as compared to control. Conclusively, thiamethoxam has been observed to produce toxic effects on the liver and kidney which is reflected by increase in enzymatic biochemical parameters and some non enzymatic biochemical parameters and quercetin was found to ameliorate subchronic toxic effects of thiamethoxam.

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Research Article

HEPATOGENIC EFFECT OF *ECLIPTA ALBA* ON EXPERIMENTALLY INDUCED LIVER TOXICITY IN ALBINO RATS

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ABSTRACT

The present study was planned to explore hepatogenic activity of ethanolic and aqueous extract of *Eclipta alba* leaves on the basis of haemato-biochemical and histopathological changes in paracetamol induced liver damage by single dose of 500mg/kg b.wt. in albino rats. The ethanolic extract of the *E. alba* treated rats revealed marked hepatogenicity in the form of moderate revival of haemato-biochemical and histopathological changes as evident from significant ($p<0.05$) reduction in AST, ALT, ALP, total bilirubin and direct bilirubin; and a significant ($p<0.05$) increase in total protein and albumin as compared to paracetamol treated group. On the other hand the *E. alba* aqueous extract proved less hepatogenic as compared to ethanolic extract treated albino rats. It was concluded that ethanolic and aqueous leaf extract of *Eclipta alba* had hepatogenic effect on paracetamol induced hepatotoxicity in albino rats as evidenced by moderate reversal of biochemical, haematological and histopathological alteration.

Key words: Albino rats, *Eclipta alba*, hepatogenic, paracetamol.

INTRODUCTION

Liver has the enormous job of maintaining the body's metabolic homeostasis. Liver damage results in a variety of histopathological changes such as fatty change (steatosis), necrosis, cholestasis, cirrhosis, and acute hepatitis but has immense functional reserve and regenerative capacity. Hepatocytes have high metabolic rate, which renders them extremely susceptible to degenerative and necrotic changes leading to hepatic damage (Thapa and Walia, 2007). This has necessitated the search for hepatogenic agents. The exploration of indigenous medicinal plants may yield such agents.

Eclipta alba commonly known as "Bhringaraja" is known since long for its action on wide variety of ailments. Its leaves contain beta-amyrin, wedelolacetone, triterpenoids, flavonoids, luteolin-7-O-glucoside, L-terthienyl methanol and stigmasterol (Prakash *et al.,* 2011). It has been used traditionally in treatment of hepatomegaly, spleenomegaly and various other chronic diseases (Uddin *et al.,* 2010). Meager information is available on the hepatogenic effect of ethanolic extract of *E. alba.* Thus, the present study was undertaken to study the hepatogenic activities of ethanolic and aqueous extracts of *E. alba* leaves on paracetamol-induced liver damage in albino rats.

MATERIALS AND METHODS

Collection of plant and preparation of extract

The leaves of plant *Eclipta alba* were taxonomically identified, authenticated and procured from Department of Aromatic and Medicinal Plants, Agriculture College, Jawaharlal Nehru Krishi Vishwa Vidyalaya, Jabalpur. Fresh leaves were shade-dried at room temperature and grounded to obtain fine power in mechanical grinder and ethanolic and aqueous extract were prepared by soxhlet extraction as per the method described by (Handa *et al.).,* 2008. The resultant extracts were stored in desiccators kept under refrigerator for further investigation. Fresh solution of each extracts was prepared by reconstituting with normal saline.

Experimental animal

Adult inbred rats of albino-strain (*Rattus norvegicus*) of either sex weighing 100-150gm were obtained from stock animal house. Animals were housed in polypropylene cages and maintained week before start of experiment. The experimental protocol was approved by Institutional Animal Ethics Committee (IAEC) and was conducted according to the Indian National Science Academy guidelines for the use and care of experimental animals.

Experimental design

After acclimatization for one week, the animals were divided into 4 groups having 6 rats in each group. Animals were fasted overnight before start of the experiments. Group-I (normal control) animals received feed and tap water *ad libitum* while remaining 3 groups of animals were subjected to a single dose of paracetamol administered intraperitoneally at the dose rate of 500mg/kg b.wt. on the first day of experimentation. To experimentally produced liver damaged animals of Group-II were maintained as toxicity control. The treatment was started one day after paracetamol administration. The

ethanolic and aqueous extracts of *E. alba* were administered separately to rats of Group-III and Group-IV, respectively, at a dose rate of 400 mg/kg b.wt. intraperitoneally for seven consecutive days (3rd to 9th day of experimentation) and followed for studying the hepatogenic effect of *E. alba* extracts.

Haemato-biochemical examination

The blood samples were collected (intra-orbital sinus puncture) from rats of group-I & II on 3rd day and on 10th day of experimentation from rats of all the groups except group-I. The blood samples were kept in sterile and anticoagulant added vials for estimation of various haematological parameters *viz,* total erythrocyte count (TEC), total leucocyte count (TLC), differential leucocyte count (DLC), packed cell volume (PCV), and hemoglobin (Hb) as per the methods of Feldman *et al.,* (2000). Serum was separated after clotting of blood for determination of aspartate transaminases (AST), alanine transaminases (ALT), total bilirubin and direct bilirubin was done by using diagnostic reagent kits (Transasia Biomedicals Ltd., Daman). Similarly, for the determination of serum alkaline phosphatase, total protein and albumin the diagnostic kits from Reckon Diagnostics Pvt. Ltd., Baroda were used. Their estimation was done by using fully Automated Auto-analyser (ERBA. CHEM.).

Hepatogenic activity (H) against hepatotoxin (paracetamol) of the test sample was calculated by considering the difference in biochemical values between the hepatotoxin treated and the control group as 100% level of reduction and was expressed as by the method given by (Mehta *et al.,* 2003).

$$H = 100 - \frac{\text{Test extract} - \text{Normal control}}{\text{Paracetamol control} - \text{Normal control}} \times 100$$

Where H is percentage hepatogenic activity.

Histopathological examination

The rats from each group were euthanized on 10th day of the experimentation. Liver was carefully dissected out and examined for gross and histopathological changes. Tissues processing was done and stained with haematoxyline and eosin by procedure described by (Kiernan, 1999). The criteria for histopathological assessment of liver damage were: hepatic cell necrosis, hydropic degeneration, fatty degeneration, inflammatory cell infiltration, vascular and other changes. To assess these changes arbitrary lesion score was made as minimal (+), moderate (++) and marked (+++).

Statistical analysis

The data collected for various parameters were statistically analyzed by Dunnett's test after one-way ANOVA. All the values in the text were expressed as Mean ± SE.

RESULTS

The percentage extractability of ethanolic and aqueous extract of *E. alba* was found to be 17.17% w/w and 14.87%w/w respectively. The effect on serum biochemical parameters following 7 days intraperitoneal administration of ethanolic and aqueous extract of *E. alba* are presented iin Table-1. The serum levels of AST, ALT, ALP, total bilirubin and direct bilirubin were found to be increased significantly($p<0.05$) in rats of toxicity control group as compared to the group I (normal control) whereas values were significantly ($p<0.05$) lower in the *E. alba* extract treated groups (Group III and IV) than group II. The level of total protein and albumin were decreased significantly ($p<0.05$) while the mean values recorded in ethanolic and aqueous extract treated group were significantly higher as compared to group II. However these values remained statistically lower than the group I. The percent hepatogenic activity of ethanolic and aqueous extract of *E. alba* against paracetamol toxicity with reference to different biochemical parameters had been shown in Table-2. It has been found that hepatogenic potential of ethanolic extract is higher than the aqueous extract of *E. alba*.

The effect on haematological parameters following 7 days intraperitoneal administration of ethanolic and aqueous extract of *E. alba* are presented in Table-3. The TEC, percentage hemoglobin and PCV were significantly ($p<0.05$) less in group II whereas a higher values of aforesaid parameters were observed in *E. alba* ethanolic extract treated rat ie group III and or in aqueous extract treated group IV rats as compared to Group I.

Arbitrary lesion score showing hepatogenic effect

Table 1.

Biochemical findings showing hepatogenic effect of *Eclipta alba* in paracetamol induced liver damage in rats.(n=6, Mean)

GROUP	AST(IU/L)	ALT (IU/L)	ALP (IU/L)	TotalBilirubin (mg/dl)	Direct Bilirubin (mg/dl)	Total Protein (g/dl)	Albumin (g/dl)
I	103.87^{e}	59.75^{e}	129.24^{e}	1.22^{d}	0.32^{d}	7.77^{a}	3.38^{a}
II (3rd Day)	189.36^{b}	211.84^{a}	257.41^{a}	3.35^{a}	1.11^{a}	6.75^{c}	3.27^{c}
(10ThDay)	196.29^{a}	184.87^{b}	224.39^{b}	3.31^{ab}	1.12^{a}	6.48^{d}	3.25^{c}
III	118.83^{d}	105.97^{d}	151.89^{d}	1.38^{c}	0.45^{c}	7.59^{b}	3.32^{b}
IV	150.65^{c}	138.63^{c}	176.17^{c}	2.27^{b}	0.71^{b}	6.79^{c}	3.25^{c}
SEM	1.69	1.52	1.53	0.02	0.01	0.02	0.02
CD atpdd 0.05	4.66	4.21	4.22	0.048	0.027	0.048	0.048

- The mean values with common alphabet as superscript don't differ significantly from each other.
- SEM : Standard Error Mean; CD : Critical Difference

Table 2.
Percent hepatogenic activity of *Eclipta alba*.

Group	TREATMENT	AST	ALT	ALP	Total Bilirubin	Direct Bilirubin	Total Protien	Albumin
III	Ethanolic Extract	83.81	63.05	76.19	92.34	83.75	86.05	53.85
IV	Aqueous Extract	49.38	36.95	50.67	49.76	51.25	24.04	00.00

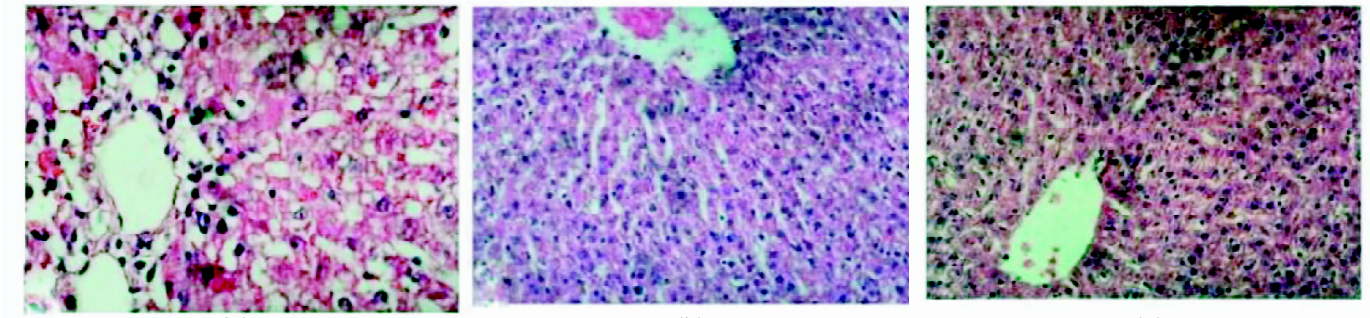
Table 3.
Haematological findings showing hepatogenic effect of *Eclipta alba* in paracetamol induced liver damage in rats.

Group	TEC	Hb	PCV	TLC	Differential Leukocyte Count				
	($x10^6$ /mm^3)	(gm %)	(%)	($x10^3$ /mm3)	N(%)	M(%)	L(%)	E(%)	B(%)
I	8.06^a	15.32^a	44.53^a	9.70^c	31.50^c	2.33^{bc}	60.50^a	1.83	1.31
II (3^{rd}Day)	7.27^d	14.56^{bc}	40.87^d	13.48^a	48.83^a	1.83^b	52.50^b	1.6	1.25
(10^{th} Day)	7.25^d	14.44^c	40.80^d	14.34^a	43.16^a	2.83^a	52.16^b	1.66	0.56
III	7.74^b	14.70^b	43.56^b	12.26^b	35.83^b	2.66^{ab}	56.66^a	1.33	0.78
IV	7.68^c	13.95^d	42.09^c	13.01^{ab}	34.16^b	3.33^a	60.33^b	1.16	0.68
SEM	0.01	0.05	0.01	0.56	1.03	0.79	1.03	0.92	0.43
CD at pd≤0.05	0.02	0.0143	0.023	1.556	2.855	2.196	2.844	NS	NS

• The mean values with common alphabet as superscript don't differ significantly from each other.
• Values are mean of six observations. • SEM : Standard Error Mean; CD : Critical Difference; NS: non significant

Table 4.
Lesion score for hepatogenic assessment of *E. alba*.

GROUP	TREATMENT	LESION SCORE				
		Hypereamia / Hemorrhage	Hydropic Degeneration	Fatty Change	Necrosis	Inflammatory Reaction
II	Paracetamol	+++	+++	+++	+++	+++
III	Ethanolic Extract	+	+	Nil	Nil	Nil
IV	Aqueous Extract	+	++	+	+	Nil



(a) (b) (c)

Rat liver showing Fig. (a): extensive hemorrhage, degenerative and necrotic changes in paracetamol treated group, (H&E X 400)
Fig. (b): telengiectesis and mild degenerative changes in ethanolic extract of *E. alba* treated group. (H&E X 400)
Fig. (c): mild regenerative changes and rearrangement of hepatocytes in aqueous extract of *E. alba* treated group. (H&E X 400)

of *E. alba* extracts on the basis of different microscopic lesion are displayed in Table 4. It was found that lesion score is highest for Group II and lowest for group III. The normal control rats did not reveal any pathological change in the liver parenchyma while paracetamol administration revealed varying degree of degenerative changes, extensive hemorrhage, and necrotic changes. At certain places mild to moderate inflammatory changes were noticed with marked infiltration of neutrophils and macrophages in the affected area (Fig.1). Histopathology of ethanolic extract treated group showed almost normal lobular structure of liver while foci of mild congestion and occasional foci of cellular swelling were also seen (Fig.2). Liver sections of aqueous extract treated group revealed apparently normal hepatocytes throughout the lobule. However cellular swelling was more extensive with foci of necrosis, sinusoidal obliteration and infiltration of inflammatory cells in the portal areas were evident (Fig.3).

DISCUSSION

In recent years, researchers have examined the effects of many herbs used traditionally by herbalists to support liver function and to cure hepatic diseases. The present investigation was designed to test the efficacy of *E. alba* as an hepatogenic agent in experimentally induced hepatotoxicity by paracetamol in albino rats and to extrapolate the findings of the present study for field application to utilize the beneficial effects of *E. alba* against different hepatopathies.

Liver function tests conducted through serum assays give information about the state of the liver, describing its functionality (bilirubin and albumin), cellular

integrity (transaminases) and its link with the biliiary tract (alkaline phosphatase) (Agbaje *et al., 2009*). The AST, ALT, ALP, total bilirubin and direct bilirubin serum level were markedly elevated in paracetamol treated animals compared to control rats indicating liver toxicity. Comparatively, the mean values of these biochemical parameters in *E. alba* aqueous extract treated group were less than Group II (toxicity control) but higher than those recorded in ethanolic extract treated rats. Earlier workers have also reported the reversal of these liver markers after indigenous medicinal treatment in paracetamol induced hepatotoxicity in rats. Total protein and albumin levels in paracetamol treated group were decreased significantly as compared to the control group. On comparison, quantities of total proteins and albumin in aqueous extract treated group were lower than that of ethanolic extract treated rats which corresponds with observation of (Bhaumik and Sharma.1996) in rabbits.

The result of hematology had shown the recoupment after treatment with *E. alba* extracts indicating the ethanolic extract treatment being better than the aqueous extract. (Gupta *et al*., 2003) also recorded significant reduction in haemoglobin, PCV, total erythrocyte and lymphocyte counts with increase in total leukocyte and neutrophils following paracetamol administration. The decrease in the haematological values in this study may be attributed to the failure of damaged liver parenchyma to produce erythropoietin and partly due to inappetance, leading to decreased availability of nutrients. Rats of paracetamol toxicity group exhibited marked histopathological lesions, where as liver of rats treated with ethanolic extract of *E. alba* revealed mild congestion and occasional foci of degenerative changes. Similarly a curative effect of ethanolic extract of *E. alba* was noticed by (Singh *et al.,* 2001) in rats and mice subjected to paracetamol toxicity.

Very few studies have been conducted regarding hepatogenic activity of the *E.* alba in albino rats . The findings of the present study indicate that ethanolic extract provided more regeneration of hepatocytes than that of the aqueous extract. The earlier investigations by (Kumar *et al.,* 2013) also reported the hepatoprotective activities of ethanolic extract of *E. alba* in rats. It can be concluded that *E. alba* extract in ethanolic base provided better hepatogenic effect to the paracetamol induced hepatotoxicity in rats.

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Research Article

PHARMACOKINETICS AND DOSAGE REGIMEN OF MOXIFLOXACIN FOLLOWING INTRAVENOUS ADMINISTRATION IN BUFFALO CALVES

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ABSTRACT

The present study was conducted to investigate intravenous pharmacokinetics of moxifloxacin in six healthy buffalo calves. High Performance Liquid Chromatography (HPLC) with fluorescence detector assay was employed to derive the moxifloxacin plasma concentration versus time data set after single dose intravenous administration of moxifloxacin. The mean initial plasma moxifloxacin level of 4.240 µg.ml^{-1} was observed at 5 min. following single dose intravenous administration at dose rate of 5 mg.kg^{-1} body weight. The mean ± S.E. values of $t_{½\beta}$, Vd_{area}, Vd_{ss}, Cl_B and AUC were 4.33 ± 0.02 h, 3.79 ± 0.06 L.kg^{-1}, 0.27 ± 0.03 L.kg^{-1}, 0.61 ± 0.01 L.h^{-1}.kg^{-1} and 8.25 ± 0.10 µg.h.ml^{-1}, respectively. Optimized IV dose of moxifloxacin in buffalo calves calculated would be 6.0 mg.kg^{-1} body weight, to be repeated every 24 hours to treat infections caused by susceptible bacteria having MIC values equal to or less than 0.50 µg.ml^{-1}.

Key Words: Moxifloxacin, Intravenous pharmacokinetics, Buffalo calves, Dosage regimen

INTRODUCTION

Fluoroquinolones are considered to have characteristics like large volume of distribution, low plasma protein binding, wide spectrum of bactericidal activity with low minimum inhibitory concentrations (MIC) against susceptible target micro-organism and concentration-dependent effect against majority of bacteria (Brown, 1996; Dalhoff *et al*.,1996). Moxifloxacin, a fourth generation newer fluoroquinolone, is an extended-spectrum fluoroquinolone which has improved coverage against Gram-positive cocci and atypical pathogens compared with older fluoroquinolone agents, while retaining good activity against Gram-negative bacteria (Balfour and Lamb, 2000). Thus, moxifloxacin is gaining an imperative use in human medicine as well as in veterinary medicine. However, there is limited literature available on pharmacokinetics of moxifloxacin in livestock species, including horse (Gardner *et al.,* 2004), goat (Fernandez-Varon *et al.,* 2006; Carceles *et al.,* 2007; Patel *et al.,* 2011), sheep (Goudah, 2008; Carceles *et al*; 2009, Modi *et al*.; 2012), cattle (Goudah and Hasabelnaby 2010) and camel (Abd el-atya *et al.,* 2007). Looking at differences found in pharmacokinetic characteristics of moxifloxacin due to variations in species, age, assay method and pharmacokinetic model, the present study was planned to investigate the plasma pharmacokinetics and dosage regimen of moxifloxacin following its single dose intravenous administration in buffalo calves.

MATERIALS AND METHODS

Animals

The study was conducted in six healthy female Mehsana buffalo calves, weighing between 80 to 100 Kg, kept at Livestock Research Station (LRS), Sardarkrushinagar Dantiwada Agricultural University, Gujarat, India. The buffalo calves were maintained on adequate antibiotic-free standard ration, and *ad libitum* water was provided throughout the experiment period. The study was prior approved by the Institutional Animal Ethics Committee (IAEC), College of Veterinary Science and A.H., Sardarkrushinagar, and all essential managemental measures were taken for the welfare of experimental animals.

Drug and reagents

Moxifloxacin pure powder and injectable formulation (10% w/v) were procured from Intas pharmaceutical Pvt. Ltd., Gujarat, India. All the chemicals and reagents used in the study were procured from S. D. Fine Chem. Ltd., Mumbai, India, and were of HPLC/ analytical grade.

Sample collection

A single dose of moxifloxacin injection (10% w/v) was injected at dose rate of 5.0 mg.kg^{-1} body weight through intravenous (IV) route via jugular vein in each of buffalo calves. Blood samples (approximately 5.0 ml) were collected from contra-lateral jugular vein in heparinized centrifuge tubes with the help of intravenous catheter (Venflon®) fixed into jugular vein at 0 time (before drug administration) and at 0.083 (5 min.), 0.25 (15 min.), 0.5 (30 min.), 1, 2, 4, 8, 12, 24 and 36 h after IV administration of moxifloxacin in order to know its persistence level and duration in plasma of the calves. After separation of plasma from blood, samples were transferred to cryo-vials (3 ml capacity) and stored at -20°C until analyzed.

Sample preparation and HPLC assay

Plasma moxifloxacin concentration was analyzed by HPLC method described by Siefert *et al*. (1999) with minor modifications. The optimized method was sensitive and reproducible, and assay linearity was observed from 0.001 to 10.0 µg.ml^{-1} with R^2 (mean correlation coefficient) value of 0.9992.

For sample preparation, plasma proteins were precipitated by addition of 200 µl each of plasma and acetonitrile in a centrifuge tube and shaken on a vortex mixture for 10 seconds. This was followed by centrifugation for 10 min. and finally supernatant was diluted 4-folds with diluent (prepared by 0.067-M disodium hydrogen phosphate in water, adjusted to pH 7.5.) This was transferred into inserts (automatic sampler vial) from which 50 µl prepared sample was injected into HPLC machine.

The HPLC system (Agilent 1100 series) consisted of gradient solvent delivery pump (LC-9A), fluorescence detector (RF-551), automatic sampler (SIL-6B) and column heater (CTO6A). Chromatographic separation was done using reverse phase C18 column (250 mm X 4.6 mm; 5 µ) at 20°C temperature. The mobile phase consisted of a mixture of buffer (prepared by dissolving 10 gm of tetrabutyl ammonium hydrogen sulphate in 1 L of deionised water) and acetonitrile (80:20). Mobile phase was pumped into HPLC column at a flow rate of 1.0 ml.min^{-1} at 20°C and effluents were monitored at the excitation wavelength of 296 nm and emission wavelength of 504 nm.

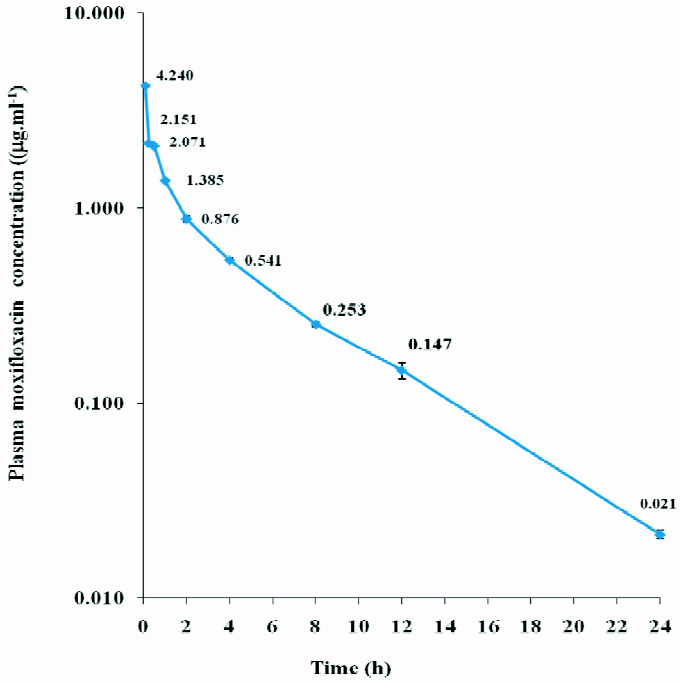

Fig 1:
The Semi logarithmic plot of plasma moxifloxacin concentration versus time following IV administration at the dose rate of 5.0 mg.kg^{-1} body weight in Mehsana buffalo calves (Each point represents mean ± S.E. of six calves).

Pharmacokinetic analysis

Various pharmacokinetic parameters like elimination rate constant (β), elimination half-life ($t_{1/2\beta}$), apparent volume of distribution (Vd_{area}), volume of distribution at steady state (Vd_{ss}), area under curve (AUC), mean resident time (MRT), total body clearance (Cl_B) etc. were calculated by PK Solutions Ver. 2.0 software, USA. This software program uses non-compartmental model approach for pharmacokinetic analysis.

For deriving dosage regimen of moxifloxacin in buffalo by IV route, the equations D (priming dose) = Cp. Vd. $e^{\beta\delta}$ and D' (maintenance dose) = Cp. Vd. ($e^{\beta\delta}$ -1) were used (Baggot, 2001), where, Cp is the required plasma concentration in terms of minimum inhibitory concentration (MIC), Vd is the volume of distribution at steady state, 'e' represents the base of natural logarithm, β is overall elimination rate constant and ô (tau) is the dosage interval.

RESULTS AND DISCUSSION

The semilogarithmic plot of plasma moxifloxacin concentrations versus time after single dose intravenous administration at the dose rate of 5.0 mg.kg^{-1} body weight in buffalo calves is given in Figure 1. The initial plasma level of moxifloxacin after IV dosing at 0.083 (5 min.) was recorded as 4.24 ± 0.07 µg.ml^{-1} which nearly halved to 2.15 ± 0.01 µg.ml^{-1} at 0.25 h (15 min). The plasma concentration of moxifloxacin at 24 h was 0.021 µg.ml^{-1} after IV administration, but could not be detected further in sample of 36 h.

The pharmacokinetic parameters of moxifloxacin calculated for buffalo calves are presented in the Table 1. The elimination rate constant (β) varied from 0.157 to 0.162 h^{-1} with a mean of 0.160 h^{-1}. The elimination half-life ($t_{1/2\beta}$) ranged between 4.266 to 4.395 h with a mean of 4.33 h. The mean value of AUC, AUMC, MRT, Vd_{area}, Vd_{ss}, and Cl_B were 8.25 µg h ml^{-1}, 39.89 µg h^2 ml^{-1}, 4.83 h, 3.79 L kg^{-1},

Table 1:
Pharmacokinetic parameters of moxifloxacin after single dose intravenous administration (5.0 mg.kg^{-1} body weight) in Mehsana buffalo calves (n=6).

PharmacokineticParameters	Unit	Mean ± SE
Cp^o	µg.ml^{-1}	4.24 ± 0.70
β	h^{-1}	0.16 ± 0.001
$t_{1/2\beta}$	h	4.33 ± 0.02
AUC	µg.h.ml^{-1}	8.25 ± 0.10
AUMC	µg.h^2.ml^{-1}	39.89 ± 1.34
MRT	h	4.83 ± 0.12
Vd_{area}	L.kg^{-1}	3.79 ± 0.06
Vd_{ss}	L.kg^{-1}	0.27 ± 0.03
Cl_B	L.h^{-1}.kg^{-1}	0.61 ± 0.01

Cp^o =Concentration of drug in plasma at zero time, β=Elimination rate constant, $t_{1/2\beta}$=elimination half-life, AUC= Area under curve, AUMC=Area under first moment of curve, MRT=Mean residence time, Vd_{area}=Apparent volume of distribution, Vd_{ss}=Volume of distribution at steady state, and Cl_B=Total body clearance.

0.27 L kg^{-1}, and 0.61 L h^{-1} kg^{-1}, respectively.

In the present study, mean elimination half life ($t_{½\beta}$) of moxifloxacin following its single dose IV administration (5.0 mg.kg^{-1} body weight) in buffalo calves was 4.33 h. However, lower values of $t_{½\beta}$ of moxifloxacin were reported in other species like 1.31 h in lactating goats (Carceles *et al.,* 2007), and 1.87 h in camel (Abd el-atya *et al.,* 2007), 1.77 h in lactating ewes (Goudah, 2008), 3.29 h in calves (Goudah and Hasabelnaby, 2010), whereas, similar value of $t_{½\beta}$ (4.12 ± 0.30 h) was determined in goats (Patel *et al.,* 2011). In buffalo calves, mean Vd_{ss} of moxifloxacin was observed to be 0.27 L.kg^{-1}, which was lower than those reported in lactating goats and ewes (0.79 and 0.84 L.kg^{-1}, respectively) (Fernandez-Varon *et al.,* 2006; Goudah, 2008). The good tissue distribution of moxifloxacin in various species may be related to its low molecular weight and high lipophilic nature. Moreover, low plasma protein binding is reported for moxifloxacin as 27 % in calves (Goudah and Hasabelnaby, 2010).

The mean AUC value of moxifloxacin in buffalo calves (8.25 µg.h.ml^{-1}) was markedly lower than the AUC value reported in ewes (14.74 µg.h.ml^{-1}) (Goudah, 2008) and male camels (14.72 µg.h.ml^{-1}) (Abd el-atya *et al.,* 2007). The AUC observed in present study was also lower than those reported in goats (11.71 µg.h.ml^{-1}) by Fernandez-Varon *et al*. (2006) and sheep (11.25 µg.h.ml^{-1}) by Modi *et al.* (2012). The difference in AUC values might be due to species variation or difference in drug formulations. In present study, mean value of Cl_B (0.61 L.h^{-1}.kg^{-1}) was higher than the Cl_B values reported in goats as 0.43 L.h^{-1}.kg^{-1} (Fernandez-Varon *et al.,* 2006), in sheep as 0.39 L.h^{-1}.kg^{-1} (Carceles *et al.,* 2009) and in lactating ewes as 0.34 L.h^{-1}.kg^{-1} (Goudah, 2008).

The eventual objective of present study was to compute dosage regimen of moxifloxacin in buffalo calves for i.v. route for the treatment of infectious diseases caused by susceptible bacteria. As the MIC range of moxifloxacin against most of susceptible bacteria was 0.1- 0.5 µg.ml^{-1} (Woodcock *et al.,* 1997), for maintaining MIC of 0.50 µg.ml^{-1} in plasma, the optimum dose of moxifloxacin was calculated to be given IV at the dose of 6.0 mg.kg^{-1} body weight (both priming and maintenance dose) to be repeated at time interval of 24 h.

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Research Article

AMELIORATIVE POTENTIAL OF AQUEOUS EXTRACT OF *PANAX GINSENG* AGAINST ACETAMIPRID INDUCED SUB ACUTE TOXICITY IN RATS

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ABSTRACT

The study was carried out to investigate the ameliorating potential of *Panax ginseng* in combating acetamiprid induced immunotoxicity, oxidative stress and genotoxicity in rats. Wistar rats were divided in four groups having six rats in each group. Group I was kept control (ground nut oil administered as vehicle). In group II, acetamiprid @ 52.5 mg/kg, in group III, acetamiprid and aqueous extract of root of *Panax ginseng* @ 50 mg/kg and in group IV, aqueous extract of *Panax ginseng* alone were administered daily to the rats orally by gavage for 28 days. The rate of gain in body weight was significantly decreased in group II, however, it was restored in Group IV. Acetamiprid exposure caused significant increase in serum level of ALT, AST, LDH and creatinine kinase activities as compared to all other groups. Treatment with acetamiprid significantly decreased the RBC, Hb, PCV values as compared to other groups. Acetamiprid produced toxicity in the form of enhanced lipid peroxidation and reduced GSH, SOD and catalase levels. *Panax ginseng* was significantly effective in restoration of these parameters towards normal.

Key words: Acetamiprid, Panax, sub-acute toxicity, rats

INTRODUCTION

Acetamiprid (ACE), a member of neonicotinoid synthetic chlorinated insecticide family was initially widely accepted as safe pesticides in the vicinity of animals and human beings. However, recent reports stated headache, dizziness, nausea, vomition and other symptoms in human beings by inhalation (Todani *et al.,* 2008) and genotoxicity in human peripheral blood lymphocytes (Kocaman and Topaktas, 2007, 2010). *Panax ginseng* is a traditional Chinese medicinal herb reported for its immunomodulatory, antioxidant and antistress properties (Abdel-Fattah *et. al.,* (2010); Fatma, (2002 and 2003)). The present study has been carried out to study the ameliorating potential of aqueous extract of *Panax ginseng* against acetamiprid induced toxicity.

MATERIALS AND METHODS

Panax ginseng plant was procured from commercial firm. The dried root of *Panax ginseng* was powdered and soaked in distilled water for 24 hours with continuous stirring at 40ºC. The mixture was filtered and concentrated in a rotatory vacuum evaporator at 40-50ºC. The final extract was produced after drying the filtrate in incubator with fan (40ºC) and lyophilized. The percent yield (w/w) of aqueous extract of *Panax ginseng* was 11.5%.

Wistar rats of 2 to 2.5 month old age, weighing between 150 to 250 gm were used in this study. The animals were kept in plastic cages and acclimatized for two weeks in the experimental lab of animal shed under standard managemental conditions. Standard rat feed and water was provided *ad libitum* throughout the experimental period. All the experimental animals were kept under constant observation during entire period of study. The present study was performed in accordance with the guidelines on regulation of scientific experiments as approved by the Institutional Animal Ethics Committee.

Technical grade Acetamiprid (96.8% pure) was used and the desired concentration of Acetamiprid was made in groundnut oil (dose selected on the basis of LD_{50} in rats) while *Panax ginseng* extract was dissolved in water.

Sub-acute toxicity was conducted in adult male Wistar rats. Rats were divided in four groups comprising six animals each. Group I was kept control (ground nut oil administered as vehicle). In group II, acetamiprid @ 52.5 mg/kg, in group III, acetamiprid and aqueous extract of root of *Panax ginseng* @ 50 mg/kg and in group IV, aqueous extract of *Panax ginseng* alone was given daily as gavage for 28 days. The parameters evaluated were body weights, organ weights, serum biochemical (ALT, AST, LDH and creatinine kinase) and haematological (Hb, PCV, TEC, TLC, DLC, MCV, MCH, MCHC).

RESULTS AND DISCUSSION

Administration of acetamiprid, Panax and their combination did not produce any mortality in Wistar male rats. Dullness, reduction in feed intake and rough body coat was observed in acetamiprid treated groups as compared to control. Some rats in acetamiprid treated groups exhibited mild oedema in sub mandibular region. Rats in other groups exhibited normal activities and behavior during the study period.

The body weight (gm) was recorded at weekly intervals (Fig 1a) and the percent body weight gain was calculated there from. Body weight gain in animals serves as an index of growth rate (Palani *et al.,* 1999). In the present study, general toxic effect of acetamiprid was manifested by significant loss in weight at subsequent intervals of observation and lower percent weight gain at the end in acetamiprid alone and in Panax- acetaprimid combination as compared to control. Zhang *et al.* (2011) has also reported a decrease in body weight gain of male mice administered with acetamiprid. The increase in body weight gain because of *Panax ginseng* in rats has also been reported by Aphale *et al.* (1998). Absolute organ weights and relative organ weights of control and Panax alone treated group were comparable to each other. Absolute weights of brain, heart, spleen, thymus and adrenal were found to be significantly decreased ($P<0.05$) in acetamiprid group as compared to control and Panax treated group, whereas, recorded absolute weight for liver and kidney were significantly higher ($P<0.05$) as shown in Fig 1b.

Absolute organs weights are also classical indices for development of toxicity. Lower body weight for most of the organs in acetamiprid treated group in the present study was proportionate with total body weight which was significantly lower in Acetamiprid treated group than control in the present study. As per US Environmental Protection Agency, acetamiprid causes generalized, nonspecific toxicity in mammals, and does not appear to have specific target organ toxicity. In the present study, simultaneous administration of *Panax ginseng* along with acetamiprid, reduced or partially antagonized the effects induced by Acetamiprid towards the normal values of control.

The relative organ weights were comparable in control and Panax treated group. The relative weight of spleen and thymus in acetamiprid treated group was significantly lower ($P<0.05$) than control and Panax treated group. In co-treatment group of Panax with acetamiprid, significantly ($P<0.05$) lower relative weight for spleen, comparable relative weight for brain, kidney, heart and significantly higher ($P<0.05$) relative weight for liver, lung, thymus and adrenal as compared to control and Panax treated alone group was recorded.

Dullness, rough body coat, reduced feed intake and retarded growth was observed in acetamiprid treated group which simulated the symptoms of general toxicity. Submandibular edema in the group might be due to decrease in serum total protein in this group. *Panax ginseng* restored the general conditions of rats in acetamiprid- *Panax ginseng* group, to almost equal to control because of its probable physiological and pharmacological actions.

It was observed that treatment with acetamiprid significantly decreased the RBC, Hb, PCV values in comparison to other groups. The MCV, MCH and MCHC values for acetamiprid treated groups were significantly higher than control and panax alone group. (Table 1)In acetamiprid and panax combination group, the values for RBC, Hb, PCV and MCHC were comparable to control and panax treated group, but MCV and MCH values were significantly lower than both. A significant decrease in RBC, Hb and PCV is indicative of adverse effects of Acetamiprid on hemopoiesis. Reduction in hemoglobin content could be due to impaired biosynthesis of heme in bone marrows (Shakoori *et al.,* 1992). Alterations in MCV, MCH and MCHC indicate that Acetamiprid produce adverse effects on hematopoietic system of rats after subacute exposure. Administration of Panax along with Acetamiprid revived the RBC, Hb and PCV values towards control. *Panax ginseng* supplementation in drinking water for 30 days induced significant positive effects on erythropoiesis in adult rats evidenced by increase of RBC counts and of haemoglobin concentrations (Simsek *et al.,* 2007).

Acetamiprid exposure caused significant increase

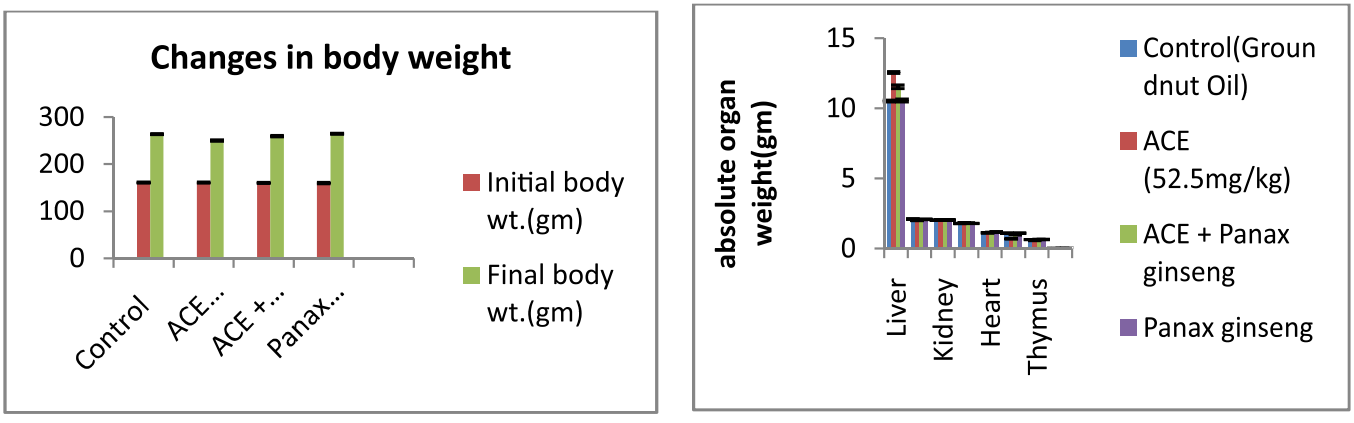

Fig 1:
Effect of *Panax ginseng* on a.body wt.(a) gain and b. absolute organ weights(b) in rats following 28 days exposure to acetamiprid (ACE).

Table 1:
Effect of *Panax ginseng* on hematological parameters in rats following 28 days exposure to acetamiprid (ACE).

Groups	RBC (×10^{12}/L)	Hb (g/dl)	PCV (%)	MCV (fl)	MCH (pg)	MCHC (g/dl)
Control (Groundnut oil)	7.25±0.03^{b}	14.33±0.06^{b}	44.86±0.20^{b}	61.85±0.01^{b}	19.75±0.00^{b}	31.94±0.00^{b}
ACE (52.5mg/kg)	6.28±0.03^{a}	13.59±0.07^{a}	43.60±0.23^{a}	69.44±0.05^{c}	21.64±0.01^{c}	31.17±0.00^{a}
ACE + *Panax ginseng*	7.26±0.04^{b}	14.28±0.09^{b}	44.71±0.30^{b}	61.51±0.01^{a}	19.64±0.00^{a}	31.93±0.00^{b}
Panax ginseng	7.27±0.03^{b}	14.37±0.05^{b}	45.01±0.19^{b}	61.85±0.00^{b}	19.75±0.00^{b}	31.93±0.00^{b}

Values (Mean+ SEM, n=6, bearing different superscripts in the same rows differ significantly (P<0.05) in Duncun Multiple comparison post hock test)

Table 2:
Effect of *Panax ginseng* on serum bio-chemical parameters in rats following 28 days exposure to acetamiprid (ACE).

Group	ALT(U/L)	AST(U/L)	LDHU/L)	Creatinine Kinase(U/L)
Control (Groundnut oil)	47.49±0.35^{b}	116.89±0.86^{b}	233.54±1.73^{b}	327.20±2.42^{ab}
ACE (52.5mg/kg)	53.16±0.38^{c}	130.83±0.93^{c}	281.38±1.86^{c}	379.37±2.61^{c}
ACE + *Panax ginseng*	48.33±0.44^{b}	118.96±1.08^{b}	237.68±2.15^{b}	333.00±3.02^{b}
Panax ginseng	44.80±0.36^{a}	110.26±0.90^{a}	227.16±1.79^{a}	324.65±2.51^{a}

Values (Mean+ SEM, n=6, bearing different superscripts in the same rows differ significantly (P<0.05) inDuncun Multiple comparison post hock test)

in serum level of ALT, AST, LDH and creatinine kinase activities as compared to other groups (Table 2). The serum levels of ALT, AST, LDH and creatinine kinase enymes in Panax -acetamiprid combination group were comparable to levels in control group. In Panax alone group, the level of enzymes were found significantly lowest (P<0.05) among the treatments and levels of ALT, AST and LDH were significantly lower (P<0.05) than control, however comparable value of creatinine kinase was observed. In the present study, the increase in AST, ALT and LDH after exposure to Acetamiprid could be due to liver dysfunction and disturbance in the biosynthesis of these enzymes with alteration in the permeability of liver membrane. Wang *et a*l. (2012) and Gokcimen *et a*l. (2007) have reported increment in ALT, AST, ALP and LDH enzyme activities in rats, when administered with graded doses of Diazinon, an organophosphate. The protective actions of *Panax ginseng* with recuperating in biochemical parameters in rats treated with carbon tetrachloride (CCl_4) and gentamicin sulphate has been reported by Kalkan et al. (2012), respectively. Improvement in biochemical parameters in cotreatment of acetamiprid with panax displays the ameliorative action of *Panax ginseng*.

Thus, it can be concluded from the study that aqueous extract of *Panax ginseng* has the ability to ameliorate the changes produced by acetamiprid induced toxicity in rats.

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Research Article

SCREENING STUDY FOR RISK ASSESSMENT OF HEAVY METALS IN FISH SAMPLES OF KONKAN REGION

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ABSTRACT

The coastal region of Maharashtra coast, stretching between Dahanu in the North and Redi/Terekhol in the South is about 720 km long and 30-50 km wide, is a place of hectic human activity, intense urbanization in pockets and enhanced industrialization, resulting in degradation of marine ecosystem through indiscriminate releases of domestic and industrial effluents. This study aimed to assess the concentrations of Lead (Pb), Arsenic (As), Cadmium (Cd), Copper (Cu) in the muscle sample of shell fish collected from the Konkan region of Maharashtra. The analysis was carried out by Inductively Coupled Plasma Atomic Emission Spectrometer (ICP-AES). The concentrations of lead and cadmium was found to be elevated (above MRL) in the certain pockets of coast whereas rest of the heavy metals analysed were not traceable or within MRL. This study provides useful information regarding heavy metal accumulation in fish around costal area of Konkan to support the safe marine production in Maharashtra. The findings from this study also provide a scientific basis for risk assessments regarding ecological protection and food safety.

Key words: Inductively Coupled Plasma Atomic Emission Spectrometer, heavy metals, Konkan region of Maharashtra, fish.

INTRODUCTION

Konkan region in Maharashtra shows fast industrial development due to major water resources in this region. The M.I.D.C. in this region is having number of manufacturing units of pesticides, chemicals, paints, dyes, pharmaceuticals, detergents etc. Most of the industries do not have effluent treatment plants and therefore, discharging their gaseous, liquid and solid effluents in sea or nearby water reservoirs, rivers, etc and thus polluting the environment. Use of pesticides and phosphate fertilizers also contribute in heavy metal pollution (Omarand Al-Khashman *et al.,* 2004; Boularbah *et al.,* 2006). Major categories of soil pollutants include nutrients (fertilizers, sewage sludge), acids, heavy metals, radioactive elements and organic chemicals (herbicides, insecticides, fungicides and other pesticides).

Fish being rich source of proteins, vitamins, minerals and trace elements, plays important part of the diet in the Konkan population. Fish are relatively situated at the top of the aquatic food chain; therefore, they normally can accumulate heavy metals from food, water and sediments (Zhao *et al.,* 2012). The content of toxic heavy metals in fish can counteract their beneficial effects; several adverse effects of heavy metals to human health have been known for long time (Castro-Gonzalez and Mendez-Armenta, 2008). This may include serious threats like renal failure, liver damage, cardiovascular diseases and even death (Al-Busaidi *et al.,* 2007, Rahman *et al.,* 2012). Therefore, many international monitoring programs have been established in order to assess the quality of fish for human consumption and to monitor the health of the aquatic ecosystem (Meche *et al.,* 2010).

The numbers of studies and surveys have been performed regarding the sources of heavy metal pollution, the bioavailability of metals, and other issues in aquatic system, agriculture, etc. However, there is a lack of information related to the heavy metals in fish population of Konkan area of Maharashtra. The need of study originated because of fish being important component of Konkan inhabitants diet. Therefore, a regional-scale survey was performed to study Arsenic, Lead, Mercury, Cadmium and Copper contamination in shell fish population of entire costal region of Konkan. The sample collection and analysis was carried out in year between 2015 to 2017.

MATERIALS AND METHODS

The entire coastal areas of Konkan were included for collection of samples which includes Mumbai, Ratnagiri, Raigad and Sindhudurg districts. In 2016-17 some centres from Goa were also included while sampling the fish. Place wise samples of shellfish collected from different areas are given in the Figure 1. Muscle samples of shell fish were considered for analysis by Inductively Coupled Plasma Atomic Emission Spectrometer (ICP AES)-(Model: ARCOS from M/s. Spectrometer, Germany at IIT, Powai, Mumbai) at the Indian institute of technology, Powai, Mumbai (Unanimous, 1989; Thompson and Walsh, 1989).

Processing of samples for metal analysis

Muscle sample of shell fish

The homogenized samples were kept for drying at 1000^{0}C and further subjected to ash in muffle furnace at 4500^{0}C by increasing temperature 50^{0}C /hr for 8 hrs or overnight. The samples were made wet with 1-3 ml of deionized water and evaporated on water bath. Re-ashing

at 450^{0}C for 1-2 hrs or longer in muffle furnace was repeated till white/grey color developed. Final aliquot in 50 ml of 0.1 M nitric acid (HNO_3) was prepared by addition of 5 ml of 6M HCl to crucible for collection of ash followed by evaporation of acid on water bath.

Analysis of heavy metals using ICP-AES

The wavelength of the spectral line of ICP-AES is generally unique for each element. The wavelength (nm) for Pb, As Cd, and Cu were 220.35, 189.04, 214.43 and 324.75 respectively., whereas the detection limit for these metal was 0.01 ppm. Spectrometer-wavelength range: 130 to 770 nm, Detector: Charge Coupled Device (CCD)

Standard, stock and working solutions

Arsenic standard solution @ 1000 mg/l in deionized water, and cadmium and lead standard solutions were prepared separately containing at 1000 mg/L of each in 0.5M HNO_3, respectively. Stock standard solutions were prepared as for arsenic: 1mg/l (10 µl standard solution was taken in 10 ml volumetric flask and diluted with 8M HCl to make the volume of 10 ml), cadmium (10 mg/l i.e., 100 µl standard solution was taken in 10 ml volumetric flask and diluted with 0.1M HNO3 to make the volume) and Lead and Cu (1mg/l (i.e.,10 µl standard solution was taken in 10 ml volumetric flask and diluted with 0.1M HNO3 to make the volume).

During analysis of arsenic, cadmium and lead, the stock standard solutions were diluted to make a range of working standards that covered the concentration of the element to be determined on the basis of tolerance levels prescribed by various regulatory agencies. Every time freshly prepared standard solutions were used for analysis.

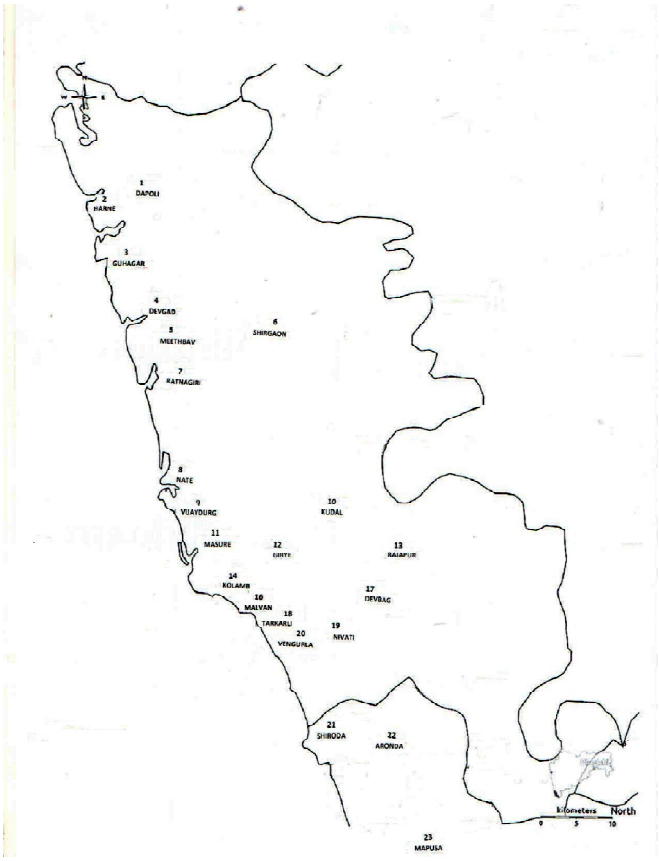

Fig 1. Map of Konkan region of Maharashtra

Arsenic: From the stock solution, 100, 500, 750, 1000 and 1500 µl solutions were taken in 10 ml volumetric flasks and diluted to the volume with 8M HCl to prepare the concentrations of 10, 50, 75, 100 and 150 µg/l, respectively.

Cadmium: From the stock solution, 0.1, 0.25, 0.5, 1, 1.5 and 2 ml solutions were taken in 10 ml volumetric flasks and diluted to the volume with 0.1M HNO_3 to prepare the concentrations of 0.1, 0.25, 0.5, 1, 1.5 and 2 mg/l, respectively.

Lead: From the stock solution, 100, 200 and 500 µl solutions were taken in 10 ml volumetric flasks and diluted to the volume with 0.1*M* HNO_3 to prepare the concentrations of 10, 20 and 50 µg/l, respectively.

RESULTS AND DISCUSSION

The concentrations of heavy metals in fish samples of coastal region of Konkan are presented in Table 2. Compared with the WHO standards. Out of 497 samples (383 prawns and 114 crabs)collected in 2015-16, 15 of prawns and 4 samples of crab revealed levels of lead above MRL. These prawn samples were 1 from Vengurla, 7 from Dapoli, 1 from Ratnagiri, 1 from Guhagar and 5 from Haarne Bandar, also showed levels of lead above MRL. 115 samples of prawn and 132 samples of crabs were collected in 2016-17 and processed of which results of all crab samples and of 48 prawn samples are available. None showed levels above MRL though traces of cadmium copper and lead are present in the samples which indicated that some heavy metals had accumulated in the surveyed samples.

This study indicates that non essential metal Pb and Cd might be present in the fish and continues input could risk the catchment ecosystem. The risk further will also depend upon the form in which metal is accumulated and the extent of its absorption from the gastrointestinal tract, frequency of consumption of fish, etc.

In order to assess the data generated during monitoring, it is crucial to have the baseline data against which the results of monitoring could be compared. Fixing the baseline in itself is not easy in the absence of long-term database because a natural marine environment is prone to spatial and temporal changes associated with tidal movements and seasonal fluctuations. Unfortunately, for several areas such data was not available for the Maharashtra coast. The database generated by present survey over the years through studies conducted from time to time in these areas will be considered as the best

available approach to assess the impacts.

The findings from present study have important implications for the development of pollution prevention and reduction strategies to reduce bioaccumulation of heavy metals in marine ecosystem of Konkan area. This study can serve as a reference for the regional management and environment quality assessment of the underlying study area of Konkan region.

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Research Article

EFFECT OF ENROFLOXACIN AND CIPROFLOXACIN ON HEMATOBIOCHEMICAL PROFILE IN RATS

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ABSTRACT

In this study, the effect of two fluoroquinolones employed in small and companion animal medicine were evaluated for their hematobochemical profile. 36 rats were divided into six different groups each containing 6 rats. the negative control group I was given one ml of distilled water orally, treatment groups were fed with enrofloxacin III and IV (@75mg/kg b wt and 150mg/kg b wt) and ciprofloxacin V and VI (@50 mg/kg b wt and 100 mg/kg b wt) orally for 28 days. Positive control group II was administered with Cyclophosphamide @ 20mg/kg b wt intraperitoneally 24 hrs prior to sacrifice. There was significant reduction ($P<0.05$) in Hb,PCV, TEC, MCV, MCH, MCHC and TLC levels in treatment groups as compared to control group I after 28 days. There was significant reduction ($P<0.05$) in total serum proteins and nochnage in lipid profile was observed. BUN, creatinine, ALT and AST levels were increased significantly ($P<0.05$) after 28 days in treatment groups. Thus study revealed at high doses enrofloxacin and ciprofloxacin pose a risk to hematopeoitc system and heapatobiliary system

Key words: Enrofloxacin, ciprofloxacin, hematological parameters, rats.

INTRODUCTION

Antibacterial agents are among the most widely used drugs in veterinary medicine. Antibacterials, like other drugs, may cause various adverse reactions or side-effects. Adverse drug reactions can be classified according to their etiology, such as pharmacological, biochemical, pathological, genotoxic and allergic reactions. Biochemical side-effects are generally considered to be indicators of pathological side-effects (Kayaalp, 1994). The fluoroquinolones have been widely used because of their long elimination half-lives, excellent antibacterial activity and wide antibacterial spectrum. While enrofloxacin, danofloxacin and ciprofloxacin are used in veterinary medicine, six fluoroquinolones (norfloxacin, ciprofloxacin, ofloxacin, pefloxacin, fleroxacin and enoxacin) are used in human medicine.

Although there is knowledge about side-effects of fluoroquinolones related to blood and biochemical parameters in humans, the knowledge related to their side-effects in animals is rather limited. Biochemical and haematological side-effects of fluoroquinolones in humans are anaemia, thrombocytopenia, leukopenia, neutropenia, reversible decreases in haemoglobin, haematocrit levels and blood glucose concentration, reversible increases in serum concentrations of alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), β-glutamyltransferase and bilirubin concentration and metabolic acidosis (Jones and Smith, 1997). The purpose of this study was to determine the influences of enrofloxacin and ciprofloxacin on haematological and biochemical parameters in wistar rats.

MATERIALS AND METHODS

Experimental animals

Wistar rats of 2 to 2.5 months age, weighing between 200-250 gm, were selected for the study. They were kept in plastic cages and acclimatized for two weeks in the experimental animal house of the department under standard managemental conditions. Standard feed and water were provided *ad libitum* throughout the experimental period to the animals and they were kept under constant observation during entire period of study.

Experimental design

Rats were randomly divided into six groups (four test groups and two control groups, positive and negative) with 6 rats in each group. Experiment was designed as per OECD guidelines. Group I which is negative control was fed with 1 ml of distilled water daily per oral for 28 days, group II which is positive control was given Cyclophosphamide @20mg/kg b wt 24hrs prior to sacrifice. Groups III and IV are administered with enrofloxacin (Enrox®10%) @ 75 and 150 mg/kg b wt per oral respectively. Groups V and VI are ciprofloxacin(Ciprox®10%) treated groups @50 and 100mg/kg b wt respectively for 28 days. On 28th organs were examined for gross lesions and the blood samples were collected to evaluate hematological and biochemical parameters. Haemeto blood samples were evaluated for Hemoglobin, packed cell volume, RBC and WBC counts, Differential leucocyte counts. Serum separated was subjected to ALT, AST, ALP, cholesterol, urea, creatinine, glucose, total protein, albumin, globulin and albumin: globulin ratio. Haematological parameters were estimated in blood as per the method given by Jain (1986). Data obtained in the present study were statistically compared by analysis of variance (Snedecor and Cochran, 1994).

RESULTS

Haematobiochemical profile of rats treated with

enrofloxacin and ciprofloxacin is shown in Table 1. There was a significant (P<0.05) reduction in Hb, PCV,TEC, MCH,MCHC and TLC observed in treatment groups (III,IV, V & VI) as compared to control group I. Lymphocytes counts shown significant (P<0.05) increase in treatment group and neutrophils, monocytes, esniophils, basophil counts were decreased significantly (P<0.05) as compared to control group I after 28 days.

Total serum proteins, albumin, globulin levels was significantly (P<0.05) increased in treatment groups after 28 days. There was no change was observed in triglycerides and cholesterol levels. BUN and creatinine levels were increased significantly (P<0.05) in treatment groups as compared to control group I. the enzymes AST and ALT levels increased significantly (P<0.05) in treatment groups after 28 days.

DISCUSSION

The significant difference in Hb,PCV and TLC counts may be due to their toxic effect on hematopoietic system including bone marrow which resulted in decreased biosynthesis of Hb (Palma-Carlos *et al.,* 1971) and reduction in TEC might have occurred either due to direct hemolytic action of the drug or the drug induced auto immune disorder leading to anemia (Packman, 2001). Eryhthrocyte indecises like MCV,MCH and MCHC values were in agreement with the findings of Hb concentration in this study.

Total serum proteins, albumin and globulin levels were increased significantly (P<0.05) in treatment groups, and A:G ratio increased in reatment groups, as albumin and globulin are globular proteins, which are mainly synthesized by liver and other cells particularly by immune cells (Dhinaa and Palanisamy, 2010).

Ghaly *et al*. (2015) reported that significant decrease in total serum proteins, albumin and globulin levels after administration of fluoroquinolones may be due to partial hydrolysis of proteins in blood In this study rats showed significant decrease in total serum proteins, albumin and globulin which might have occurred either due to hepatotoxic effects of fluoroquinolones at high doses or direct hydrolytic action on their plasma proteins and this may be attributed to the hepatotoxic effect of enrofloxacin and ciprofloxacin.

There was also significant (P<0.05) decrease of glucose level was observed in treatment groups, this may be due to hepatotoxic action of enrofloxacin and ciprofloxacin. Shailer *et al*. (1997) also reported reduced blood glucose levels in human following administration of clinofloxacin @ 400mg/kg through intravenous route.

Alanine aminotransferase (ALT; formerly serum glutamic pyruvic transaminase, SGPT) and aspartate amino transferase (AST; formerly serum glutamic oxaloacetic transaminase) are the important biomarkers for the hepatic function in the body (Amacher, 2002). In this study a significant increase in the levels of AST and ALT were attributed to the toxic effect of enrofloxacin and ciprofloxacin on liver function at higher doses. These findings are in agreement with the findings of Greene *et al*. (2002) who reported an increase in levels of these enzymes in dogs following treatment with enrofloxacin.

BUN and creatinine values also increased in treatments groups indicating possible nephrotoxicity induced by enrofloxacin and ciprofloxacin.

Thus, enrofloxacin and ciprofloxacin at high doses pose a serious risk of hepatotoxicity, mild nephrotoxicity

Table 1:
Effect on hematobiochemical parameters following oral administration of enrofloxacin and ciprofloxacin for 28 days in rats (Mean+_SE; n=6).

Parametres	Group I	Group II	Group III	Group IV	Group V	Group VI
Hb (g/dl)	16.5±0.50	14.92±1.24	13.08±0.57[a]	13.17±0.60[a]	13.28±0.44	11.50±0.72[a]
PCV (%)	50.67±1.55	45.33±1.47	39.00±1.57[a]	37.67±0.88[a]	38.30±1.37[a]	33.15±1.07[a]
TEC (×106/µl)	7.16±0.20	6.11±0.38	6.30±0.26	5.95±0.17[a]	6.04±0.27[a]	5.22±0.17[a]
MCV (femto litre)	67.62±0.30	82.36±0.51[a]	66.46±0.26[a]	65.18±0.28[a]	71.71±0.35[a]	69.27±0.31[a]
MCH (picogrem)	24.70±0.16	21.49±0.69[a]	20.69±0.29[a]	20.32±0.05[a]	22.70±0.41[a]	21.91±0.37[a]
MCHC (%)	36.12±0.24	34.44±0.26[a]	31.15±0.32[a]	30.60±0.35[a]	30.82±0.24[a]	30.19±0.13[a]
TLC (×103/µl)	8.37±0.29	7.51±0.28[a]	7.56±0.25[a]	7.33±0.14[a]	7.36±0.13[a]	7.21±0.15[a]
TSP (g/dl)	7.41±0.76	6.30±0.11[a]	6.80±0.46	6.75±0.25[a]	6.66±0.12[a]	6.42±0.20[a]
Albumin (g/dl)	5.06±0.58	4.11±0.31[a]	4.40±0.17	4.06±0.21[a]	4.25 ±0.88[a]	4.16±0.35[a]
Globulin (g/dl)	4.13±0.08	2.43±0.23[a]	2.65±0.28[a]	2.53±0.06[a]	2.35 ±0.03[a]	2.30±0.02[a]
A:G	1.19±0.12	1.73±0.25[a]	1.69±0.09[a]	1.62±0.19[a]	1.82 ±0.26[a]	1.64±0.32[a]
Glucose	93.37±2.71	86.67±2.85	73.63±2.57[a]	73.33±3.45[a]	76.67 ±3.14[a]	72.00±2.97[a]
BUN	8.04±0.40	8.62±0.21	8.33±0.32	8.65±0.45	9.14±0.26[a]	9.66±0.08[a]
Creatinine	0.51±0.05	0.60±0.03	0.61±0.08	0.76±0.03[a]	0.73±0.03	0.79±0.10[a]
Tryglycerides	53.67±4.87	53.21±3.97	59.17±6.67	58.83±4.57	69.50 ±5.34	64.07±6.98
Cholesterol	51.67±1.59	54.67±3.27	53.67±6.58	53.17±3.58	56.33 ±4.33	55.80±7.73
AST	59.17±2.16	69.13±2.62[a]	58.67±2.02	61.87±1.08	62.33±5.54	68.69±4.47[a]
ALT	57.02±4.03	69.77±2.21[a]	58.01±3.25	61±5.03	61.25±1.15	66.33±2.02[a]
ALP	114.7±9.81	119.00±6.12	117.70±9.41	119.56±4.38	108.30±11.54	121.30±5.78

Values in table are Mean ± S.E. (n = 6); a= Significant (P<0.05) as compared to group I with same column.

and suppression of hematopoietic system.

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Research Article

ARSENIC INDUCED REPRODUCTIVE TOXICITY AND ITS POTENTIATION BY CYPERMETHRIN IN MALE RATS

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ABSTRACT

The reproductive toxicity produced by arsenic and its potentiation by cypermethrin in male Wistar rats was investigated. The animals were divided into five groups (Group I to V) having seven animals in each group. All groups were given different treatments for 28 days. Group I and II were naïve and vehicle controls and were given plain drinking water and groundnut oil (1ml/kg, once daily orally), respectively. In Group III, cypermethrin (25 mg/kg) was administered once daily orally in groundnut oil (as vehicle). Group IV rats were administered 20 ppm arsenic in drinking water. Group V rats were given combined exposure of the treatments given to Group IV and V. All animals were sacrificed on twenty ninth day under ether anaesthesia. After the trial, significant decrease was observed in percent weight gain in all the treatment groups. All three treatments significantly decreased absolute organ weights of testis, epididymus and seminal vesicles. Total epididymal sperm count, live sperm count and sperm motility were significantly decreased, whereas dead sperm count, head and tail abnormalities in sperm were significantly increased in all treatment groups. In addition, testicular levels of specific enzymes (3β-HSD and 17β-HSD) and plasma testosterone levels were significantly reduced in all the treatment groups. The results demonstrated that there is potentiation in reproductive toxicity on combined exposure of arsenic and cypermethrin.

Key Words: Arsenic, Cypermethrin, Reproductive Toxicity

INTRODUCTION

Today, Cypermethrin, a synthetic pyrethroid has become one of the most important insecticide in wide scale use. Cypermethrin is classified by the World Health Organisation (WHO) as moderately hazardous chemical (Class II).Cypermethrin has been shown to produce various deleterious effects viz. genotoxicity (Suman *et al.,* 2005) and oxidative stress(Muthuviveganandavel *et al.,* 2007).

Arsenic concentration in ground water ranges from 50-1200 microgram/l, far above the current maximum permissible limit of 10 µg/l as per WHO (WHO, 1996). Arsenic (40 ppm) causes increased lipid peroxidation, decreased total epididymal sperm count, plasma testosterone level and testis specific enzymes in male rats (Khan*et al.*, 2011). At present, arsenic is considered to be one of the most relevant environmental global single substance toxicants (ATSDR, 2000).

MATERIALS AND METHODS

Experimental animals

The study was conducted on adult male Wistar rats weighing 100-120 g procured from Laboratory Resource Section, Indian Veterinary Research Institute, Izatnagar. The animals were maintained under standard managemental conditions and provided nutritionally balanced pelleted feed and water *ad libitum*. Prior to the start of experiment, animals were kept in laboratory conditions for a period of 7 days for acclimatization. All the experimental animals were handled as per the Institutional Animal Ethics Committee Guidelines.

Chemicals and reagents

Sodium arsenite was purchased from M/s Sigma Aldrich, USA. Cypermethrin (Technical grade) was purchased from Gharda Chemicals, Mumbai. All other chemicals used in the study were of analytical grade.

Experimental design

Animals were divided into five groups comprising seven animals each. Animals in the first group were given plain drinking water and served as naïve control group. Second group served as vehicle control group, which received groundnut oil (vehicle for cypermethrin) once daily orally at the dose rate of 1ml/kg body weight. In third group, cypermethrin was given to animals once daily orally at the dose rate of 25mg/kg body weight in groundnut oil (vehicle). In fourth group, animals were given drinking water containing 20ppm arsenic. In fifth group, animals were given combined exposure to cypermethrin at the dose rate of 25 mg/kg once daily orally in groundnut oil and arsenic in drinking water (20ppm arsenic). Length of experimental trial was 28 days.

Toxicity parameters

Clinical signs

Animals of all the treatment groups were observed daily for the presence of any abnormal clinical signs/ symptoms during the entire period of the experiment.

Body weights and organ weights

The body weight of the rats was measured at weekly intervals. Per cent body weight gain was calculated at the end of the experiment. The testes, epididymis, seminal vesicles and prostate were removed at the end of

the treatment period; blotted with tissue paper and their weights were properly recorded. Lateron relative organ weights (per 100 g body weight) were calculated.

Semen Evaluation

Epididymal total sperm count

During sacrifice the scrotal region was dissected to expose the cauda epididymis. Epididymes were minced aseptically into sterile petri dishes containing 1 ml of pre-warmed (35°C) Dulbecco's PBS and filtered through 80 μm pore-size nylon mesh. The filtrate was used for the evaluation of sperm parameters. The filtrate samples were diluted with 5 ml of pre-warmed (35°C) Dulbecco's PBS and the spermatozoa were counted by haemocytometer using the Improved Neubaur (Deep 1/10 mm. LABART, Germany) chamber as described by Pant and Srivastava (2003).

Live and dead sperm count

Per cent live spermatozoa were estimated by differential staining technique using Eosin-Nigrosin stain (NE). These slides were also used for estimating the per cent morphologically abnormal sperm on the basis of observable abnormalities of head, neck, mid-piece and tail region of the spermatozoa.

Sperm abnormalities analysis

Two hundred spermatozoa (heads only or intact sperm) per animal were evaluated for head and/or flagellar defects by microscopy by using 100X (oil immersion). The head and tail abnormalities were counted per 100 sperm count. Sperm motility was recorded as percentage of progressively motile spermatozoa after the dilution of semen.

Hormonal assay

The testicular 3β-HSD activity was measured according to the method of Talalay*et al*. (1962).The testicular 17β-HSD activity was measured according to the method of Jarabak*et al*.(1962). Testosterone concentration in plasma samples was determined by using standard RIA kits supplied by Immunotech, France.

RESULTS

There were no abnormal signs in the behavioural pattern of the animals in any of the groups over the entire period of study. The body weight and percent weight gain of the rats treated with arsenic plus cypermethrin were significantly decreased. (Table 1)

Cypermethrin treatment alone and arsenic plus cypermethrin treatment significantly decreased the absolute organ weights of testis, epididymis and seminal vesicles. Arsenic treatment alone followed the same trend except absolute organ weight of testis. Arsenic plus cypermethrin treatment significantly decreased the relative organ weights of testis, epididymis and seminal vesicles. (Table 2 &3)

Total epididymal sperm count, live sperm count, sperm motility and head & tail abnormality were significantly decreased, while dead sperm count were increased in all treatment groups as compared to naïve and vehicle control groups (Table 4& 5).

Activities of 3β-HSD, 17β-HSD enzymes and plasma testosterone levels were significantly decreased in cypermethrin alone, arsenic alone, and arsenic plus cypermethrin treated groups as compared to naïve and vehicle control groups.(Table 6)

DISCUSSION

In this study, arsenic plus cypermethrin treatment significantly decreased the body weight,absolute and relative organ weights of testis, epididymis, seminal vesicles and testis. In a previous study, moderate (10 mg/kg) and high (20 mg/kg) doses of β-cypermethrin not only decreased body weight but also weight of the testes, epididymes, seminal vesicles and prostate of mice (Wang *et al.,* 2009).

In the present study, total epididymal sperm count, live sperm count and sperm motility decreased, while dead sperm count, head abnormality and tail abnormality were

Table 1:
Effects on body weights (g) and % weight gain of male rats given exposure to arsenic, cypermethrin and their combination for 28 days.

Treatment	Dose	0 day	7 day	14 day	21 day	28 day	% weight gain
Naïve control	-	100.71±1.70^{a}	120.00±1.54^{ab}	137.14±2.14^{ab}	155.00±3.61^{ab}	180.00±3.93^{ab}	79.10±5.45^{ab}
Vehicle control	1 ml/kg	100.71±1.30^{a}	122.85±1.84^{a}	140.71±2.54^{a}	160.00±2.67^{a}	190.00±3.08^{a}	80.01±5.17^{a}
Cypermethrin	25 mg/kg	102.14±1.48^{a}	115.71±1.30^{ab}	132.14±1.48^{ab}	150.00±1.88^{ab}	169.28±1.7^{bc}	65.96±3.17^{b}
Arsenic	20 ppm	102.85±1.84^{a}	115.71±1.70^{ab}	137.85±1.84^{ab}	152.14±1.01^{ab}	172.14±3.4^{bc}	68.49±4.66^{b}
Arsenic+Cypermethrin	20 ppm+ 25 mg/kg	102.85±1.84^{a}	113.57±2.82^{b}	130.71±2.29^{b}	148.57±2.10^{b}	166.42±2.1^{c}	62.14±3.73^{b}

Table 2:
Effects on absolute weights (g) of different organs of male rats given exposure to arsenic, cypermethrin and their combination for 28 days.

Treatment	Dose (g)	Testis (g)	Epididymis (g)	SeminalVesicles (g)	Prostate (g)
Naïve control	-	2.17±0.04^{a}	0.43±0.02^{a}	0.43±0.01^{ab}	0.35±0.01^{a}
Vehicle control	1 ml/kg	2.17±0.14^{a}	0.42±0.03^{a}	0.46±0.01^{a}	0.34±0.01^{ab}
Cypermethrin	25 mg/kg	1.81±0.12^{cd}	0.32±0.02^{cd}	0.35±0.01^{cd}	0.32±0.01^{ab}
Arsenic	20 ppm	1.97±0.05^{abcd}	0.34±0.03^{bc}	0.39±0.01^{bc}	0.33±0.01^{ab}
Arsenic +Cypermethrin	20 ppm+25 mg/kg	1.75±0.08^{cd}	0.28±0.03^{d}	0.31±0.01^{d}	0.33±0.02^{b}

Table 3:
Effects of on relative weights (g/100g b.wt.) of different organs of male rats given exposure to arsenic, cypermethrin and their combination for 28 days.

Treatment	Dose	Testis	Epididymis	Sem vesicles	Prostate
Naïve control	-	1.20±0.02^{a}	0.24±0.01^{a}	0.24±0.01^{a}	0.19±0.01^{a}
Vehicle control	1 ml/kg	1.14±0.13^{ab}	0.22±0.01^{ab}	0.24±0.01^{a}	0.18±0.01^{a}
Cypermethrin	25 mg/kg	1.07±0.02^{ab}	0.19±0.01^{cd}	0.20±0.01^{bc}	0.19±0.01^{a}
Arsenic	20 ppm	1.14±0.01^{ab}	0.19±0.01^{bcd}	0.23±0.01^{ab}	0.19±0.01^{a}
Arsenic +Cypermethrin	20 ppm+25 mg/kg	1.05±0.01^{b}	0.17±0.01^{d}	0.18±0.01^{c}	0.18±0.01^{a}

Table 4:
Effects on different sperm related parameters in male rats given exposure to arsenic, cypermethrin and their combination for 28 days.

Treatment	Dose	TESC	Live SC	Dead SC	Sperm motility
Naïve Control	————-	60.28±5.94^{a}	93.42±7.83^{a}	6.57±1.36^{a}	93.14±7.89^{a}
Vehicle control	1ml/kg	60.57±5.30^{a}	93.00±8.65^{a}	7.00±1.09^{a}	92.71±9.45^{a}
Cypermethrin	25mg/kg	36.71±5.04^{b}	60.57±8.32^{b}	39.42±3.13^{b}	50.57±6.34^{b}
Arsenic	20ppm	36.42±4.80^{b}	61.71±7.86^{b}	38.28±3.71^{b}	60.85±7.52^{c}
Arsenic +Cypermethrin	20ppm + 25mg/kg	24.42±3.60^{c}	57.28±6.83^{b}	42.71±3.88^{b}	38.00±4.82^{d}

TESC= Total Epididymal Sperm Count (10^{6}), Live SC= Live Sperm Count (%), Dead SC= Dead Sperm Count (%)

Table 5:
Effects on different sperm abnormalities in male rats given exposure to arsenic, cypermethrin and their combination for 28 days.

Treatment	Dose	Head abnormalities	Tail abnormalities
Naïve Control	———	1.8±0.34^{a}	2.0±0.43^{a}
Vehicle control	1 ml/kg	3.28±0.56^{a}	3.71±0.68^{a}
Cypermethrin	25 mg/kg	9.00±1.25^{bc}	8.51±0.94^{b}
Arsenic	20 ppm	11.71±0.91^{bc}	11.00±0.92^{bc}
Arsenic +Cypermethrin	20 ppm+25 mg/kg	12.42±1.64^{c}	13.85±1.01^{c}

Table 6:
Effects on 3-beta-HSD, 17-beta-HSD and plasma Testosterone levels in male rats given exposure to arsenic, cypermethrin and their combination for 28 days.

Treatment	Dose	3-beta-HSD (Unit/mg protein/h)	17-beta-HSD (Unit/mg protein/h)	Plasma Testosterone (nanogram/ml)
Naïve control	———	27.68±1.85^{a}	20.96±0.55^{a}	10.07±0.29^{a}
Vehicle control	1 ml/kg	27.35±2.22^{a}	19.47±2.07^{ab}	9.41±0.48^{ab}
Cypermethrin	25 mg/kg	14.35±1.12^{b}	12.74±1.11^{cd}	5.49±0.57^{cd}
Arsenic	20 ppm	15.65±1.25^{b}	13.68±1.10^{bcd}	6.02±0.56^{cd}
Arsenic + Cypermethrin	20 ppm+25 mg/kg	11.01±1.03^{b}	9.76±1.23^{d}	4.58±0.56^{d}

n=7. Values (Mean ± SEM); values bearing different superscript in the same column differ significantly ($p<0.05$) in Tukey's multiple comparison post hoc test.

increased in all treatment groups. Earlier also, β-cypermethrin administration decreased sperm concentration, sperm motility and intact acrosome rate but also reduced serum testosterone concentration (Wang *et al.,* 2009). Arsenic (III) was shown to interfere with spermatogenesis (Sarkar*et al*., 2003).

3β-HSD and 17β-HSDare the prime enzymes in testicular androgenesis. In our study, activities of 3β-HSD and 17β-HSD enzymes in testicular tissue and concentration of testosterone hormone in plasma were decreased in all the treatment groups. In a previous work, spermatogenesis and plasma levels of testosterone were affected by arsenic(III), when it was given to Wistar rats via i.p. injections at 4, 5, or 6 mg/kg/day for 26 days (Sarkar *et al.,* 2003). A dose-dependent decrease in plasma and intra-testicular concentrations of testosterone in arsenic-treated rats may occur due to the inhibition of these (17β-HSD & 5β-HSD) testicular androgenic enzymes activities, because these enzymes are responsible for the regulation of testosterone biosynthesis (Ghosh*et al*., 1990).

The findings of our study, indicates that reproductive toxicity caused by arsenic is potentiated by cypermethrin, therefore the concurrent exposure of arsenic and cypermethrin may lead to significant male reproductive toxicity.

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Research Article

COMPARATIVE CORRELATION BETWEEN SERUM CYPERMETHRIN CONCENTRATION AND TOXICOLOGICAL MANIFESTATIONS IN DERMAL SUB-ACUTE AND ORAL SUB-CHRONIC CYPERMETHRIN EXPOSURE

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ABSTRACT

Cypermethrin, a type II synthetic pyrethroid insecticide, at dose rate of 0.5 mg/kg/day for 14 consecutive weeks produced mild signs of toxicity in buffalo calves. Peak serum cypermethrin concentration was observed on 4th week (1.53 ± 0.24 ppm) of exposure and then declined slowly till 14th week of treatment and no cypermethrin was detected after 2 weeks of post-treatment period. Dermal cypermethrin exposure produced more toxic signs as compared to the oral exposure. However, all cypermethrin treated animals recovered within 7 days of withdrawal of cypermethrin treatment. The peak serum cypermethrin concentration was observed on 14th day (0.588± ppm) of dermal exposure.

Key words : Buffalo calves, cypermethrin, subacute toxicity, pesticide residue.

INTRODUCTION

Pesticides have made valuable contributions to human and animal health by increasing food and fibre production and by reducing the occurrence of vector-borne diseases. An increase in global food demand has resulted in a significant increase in the use of pesticides in agriculture. In addition, an awareness of their utility in agriculture, animal husbandry, post-harvest technology and public health has lead to an exponential rise in their usage.

Cypermethrin is widely used against pests all over the world to increase the production of food grains and other agricultural products (Usmani and Knowles 2001) and it constitutes the most widely used Type II pyrethroid pesticide in India (Nagarjuna et al 2008). Although cypermethrin was considered safe and widely used on agricultural crops and forests as well as in public and animal husbandry practices (Igbedioh 1991), there is accumulating evidence that chronic exposure or high doses of cypermethrin have toxic effects on humans and animals. Cypermethrin, is a photostable, potent synthetic type II pyrethroid insecticide with low mammalian toxicity. There is an increased risk of food being contaminated with the insecticide, which may harm humans as well as domesticated animals. In animals, cypermethrin additionally is being used as a chemotherapeutic agent against ectoparasite infestations (Velisek *et al.,* 2006). It is a fast acting neurotoxin with good contact and stomach action.

In general, pyrethroids, are considered less toxic to mammals and are good substitutes to replace the toxicologically potent organophosphorus insecticides (OPs). Pyrethroids are easily converted to nontoxic derivatives through hydrolysis in mammalian species. However in insects, this hydrolytic activity is quite low (Cantalamessa 1993). Hydrolysis of pyrethroids is generally considered to be a detoxication process (Cantalamessa 1993). Yang et al (2009) mentioned that the magnitude of induction of carboxylesterases and cytochrome 3A4 by pyrethroids was correlated inversely with the rate of hydrolysis, but positively with the activation of the pregnane X receptor (PXR). It is known that after absorption, pyrethroids rapidly distribute throughout the body (Gray et al 1980) and readily enter the brain because of their high lipophilicity and lack of exclusion by the multi-drug transporter glycoprotein (Bain and LeBlanc 1996) at the blood–brain barrier.

Cypermethrin binds to bovine serum albumin (BSA) and bovine haemoglobin (BHb) (Yong *et al.,* 2006). Cypermethrin bonding with BSA is significantly stronger than its bonding with BHb. The binding with blood proteins affects the distribution, metabolism and excretion of insecticides. Binding with proteins will effectively decrease the concentration of free insecticides, benefit their metabolic modification and transport them to the disposal sites, alleviating the corresponding toxicity (Silva *et al.,* 2004, Sukowska et al 2004). In other words, binding with proteins will significantly affect the distribution, metabolism, and excretion of insecticide (Gao *et al.,* 2004).

MATERIALS AND METHODS

Cypermethrin (superfast, 25% EC) insecticide was commercially obtained from Insecticide (India) Ltd., SIDCO, Jammu. Fifteen healthy male buffalo calves (6-12 months) were randomly divided into three groups of five animals, each. Group I served as control where as the group II animals were administered cypermethrin orally @ 0.5 mg/kg/day for 14 consecutive weeks and Group III animals were sprayed with cypermethrin (0.25% solution) for 14 consecutive days. The permission of Institutional Animal Ethics Committee to conduct the experiment was

taken.

Estimation of serum cypermethrin concentration by gas chromatography

The method developed by Gill *et al.* (1996) was followed with some modifications for extraction of cypermethrin in serum. The serum samples were thawed and vortexed. An aliquot (1 ml) was placed into a centrifuge tube (15 ml). After equilibration at room temperature for 15 min, acetic acid (1.0 ml) was added and the tube vortexed for 1 min. The analytes were extracted with hexane/ dichloromethane (DCM) (9:1 v/v, 3.0 ml), vortexed for 1 min and centrifugation was done at 1800 rpm for 2 min. The top organic layer was drawn into a clean centrifuge tube (15ml). The extraction was repeated twice with 3ml of hexane/DCM solution. The combined organic phase was concentrated to 0.5 ml for clean up.

Clean up of pesticide residues in serum

One gram of activated florisil was packed in a glass column in between 2 layers of anhydrous sodium sulphate. The column was then washed with 8 ml of hexane and concentrated sample extract was added to the column. This was later extracted into a test tube by washing with 0.5ml of hexane. Elution was carried out with 8ml hexane and the resultant elute was concentrated to 2-5 ml and stored in labeled glass stoppered vials for analysis in GLC.

The insecticide in cleaned up extracts was quantified using the Electron Capture Detector (ECD of Gas Liquid Chromatography (GLC). The cleaned up extract measuring 2-5 µl was injected in GC and Winacid 6.2 software on PC was used for integration and computation of signals. Cypermethrin was identified and quantified by comparison of retention time and peak area of the sample chromatographs with those of standards run under the same operating conditions. The standards were run before and after each analysis.

The formula used for the quantification of the serum concentration was:-

$$\text{ppm (serum)} = \frac{\text{Pesticide standard injected (ng)}}{\text{Area of the standard}} \times \frac{\text{Area of sample}}{\text{Sample extract injected (µl)}} \times \frac{\text{Final volume of sample extract (ml)}}{\text{Initial volume (ml)}}$$

Table 1:

Concentration of cypermethrin in serum of buffalo calves after oral exposure of cypermethrin @ 0.5 mg/kg/day in buffalo calves.

Time (weeks)	Control	Treatment
Treatment		
0	ND	ND
2	ND	0.52±0.07
3	ND	1.25±0.18
4	ND	1.53±0.24
5	ND	1.32±0.14
6	ND	1.16±0.17
7	ND[a]	1.04±0.21
8	ND[a]	0.98±0.10
9	ND[a]	0.90±0.18
10	ND[a]	0.94±0.14
12	ND[a]	0.85±0.11
13	ND[a]	0.80±0.09
14	ND[a]	0.71±0.11
Post-treatment		
2	ND[a]	ND

Values given are expressed as ppm and represent the Mean ± S.E. of 5 animals unless otherwise stated.
a= Mean ± S.E. of 4 animals. ND stands for not detected.

RESULTS AND DISCUSSION

Toxic symptoms

The daily oral administration of cypermethrin at the rate of 0.5 mg/kg in buffalo calves for 14 consecutive weeks produced very mild signs of toxicity. There was a moderate degree of anorexia in all cypermethrin treated animals. Signs like lacrimation, slight froathing, listlessness, slight muscular tremors and weakness of limbs were observed in a milder form during later period of the cypermethrin treatment. All the 5 animals exposed to cypermethrin treatment recovered within 2 weeks of cypermethrin discontinuation. Peak serum cypermethrin concentration was observed on 4th week (1.53 ± 0.24 ppm) of exposure and then declined slowly till 14th week of treatment and no cypermethrin was detected after 2 weeks of post-treatment period.

The daily dermal exposure of 0.25% concentration of cypermethrin in buffalo calves for 14 days produced mild signs of toxicity. There was a mild to moderate degree of inappetance in all cypermethrin sprayed animals. The peak serum cypermethrin concentration was observed on 14th day (0.588± ppm) of dermal exposure. However the degree of signs like hyperaesthesia, lacrimation, salivation

Table 2:

Serum cypermethrin concentration after dermal exposure of 0.25% cypermethrin in buffalo calves.

Time (days)	0	3	7	10	14	7
Treatment						Post-treatment
Serum cypermethrin concentration (ppm)						
Control	ND	ND	ND	ND	ND	ND
Treatment	ND	0.126±0.05	0.259±0.07	0.549±0.10	0.588±0.08	ND

Values are the Mean ± S.E. of 5 animals unless otherwise stated.
ND stands for not detected.

and listlessness, were observed more in dermal exposure in comparison to oral cypermethrin treatment. There was however, no severe toxicity and lethality in any of the routes. Reduction of flaccidity in the hydrophobic region of the plasma membrane bilayer takes place where cypermethrin is preferentially localized (Nasuti et al 2003). This lowers the threshold of sensory nerve fibers for the activation of further action potentials, which may progress to hyper-excitation of the entire nervous system (Narahsahi et al 1995). Delayed sodium channel closure thus increases cell membrane excitability (Murphy 1993), which leads to multiple nerve impulses, causing the nerve to release neurotransmitter acetylcholine and stimulate other nerves (Eells *et al.,* 1992 J.T. Eells, P.A. Bandettini, P.A. Holman and J.M. Propp, Pyrethroid insecticide induced alteration in mammalian synaptic membrane potential, *J Pharmacol Exp Therap* 262 (1992), pp. 1173–1181. View Record in Scopus | Cited By in Scopus (31) Eells and Dubocovich 1988). Cypermethrin also inhibits the gamma aminobutyric acid receptor, causing excitability and convulsions in rats (Ramadan et al 1988). This may be the probable reason for symptoms observed in buffalo calves intoxicated with cypermethrin.

Concentration of cypermethrin in serum

The serum cypermethrin concentration of both treatment as well as control groups are presented in table 1. Peak cypermethrin concentration was observed on 4th week of the investigation and thereafter declined slowly.

Peak cypermethrin concentration was observed on 14th day of the exposure and cypermethrin was not detected in serum on 7th day of post treatment period as shown in table 2.

The initial incline could be due to continuous exposure of cypermethrin, thereafter the decline in concentration could be attributed to the induction of the metabolizing enzymes in present study. This indicates faster metabolism of cypermethrin in buffalo calves due to presence of carboxyl-esterase enzymes. Nishi et al (2006) stated that carboxylesterases hydrolyze a large array of endogenous and exogenous ester-containing compounds, including pyrethroid insecticides and observed that the *trans*-isomers of cypermethrin were hydrolyzed much faster than corresponding *cis*-counterparts by both enzymes. Crow et al (2007) described that hydrolytic metabolism of pyrethroid insecticides in humans is one of the major catabolic pathways that clear these compounds from the body. The primary metabolite of pyrethroids is 3-phenoxybenzoic acid, which is mostly used as biomarker for pyrethoid exposure. The more serum cypermethrin concentration in dermal exposure could be due to more absorption than oral exposure because of lipophilic nature of cypermethrin and metabolism of cypermethrin by gut microbes.

Oral cypermethrin produced varying degree of mild toxic signs in buffalo calves viz, lacrimation, slight froathing, listlessness, mild muscular tremors weakness of limbs and alopecia. Dermal cypermethrin exposure produced toxic signs of lachrymal discharge, dullness, depression, decrease body weight gain, muscle weakness, mild nervous signs and hyperaesthesia. However, all cypermethrin treated animals recovered within 14 and 7 days of withdrawal of cypermethrin treatment in oral and dermal exposure respectively. The high concentration achieved earlier in dermal exposure than in oral exposure could be due to the reasons of high dermal absorption due high lipophilicity and high degradation in rumen by rumen microbes.

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Research Article

PROTECTIVE EFFECT OF *ACORUS CALAMUS* RHIZOME IN PARACETAMOL INDUCED HEPATOTOXICITY IN RATS

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ABSTRACT

The present study was carried out to evaluate hepatoprotective activity of aqueous and alcoholic extract of *Acorus calamus* rhizomes against the paracetamol induced hepatotoxicity in rats. Hepatotoxicity was induced by oral administration of paracetamol and chemical parameters such as glutathione peroxidase, glutathione reductase, glutathione, catalase, lipid peroxidation and histopathological changes in liver were determined by comparing with a standard hepatoprotective drug silymarin. Treatment of rats with aqueous and alcoholic extracts of *Acorus calamus* rhizome after paracetamol administration normalized the altered levels of above parameters which may comparable with silymarin and Vit-E. The hepatoprotective activity was confirmed by histopathological examination of the liver tissue of control and treated animals. Based on the result it can be concluded that *Acorus calamus* rhizome possesses hepatoprotective effect against paracetamol induced hepatotoxicity in rats

Key words: *Acorus calamus* rhizome, Paracetamol, Silymarin, Vit-E,Hepatoprotection

INTRODUCTION

Hepatotoxicity is one of the major adverse effects produced by many of the drugs. Hepatic disorders are the most common and serious health problems in animals and human beings. *Acorus calamus* is a semiaquatic perennial aromatic herb with creeping rhizomes belonging to the family Araceae. It is commonly called as vacha, vasa, sweet flag etc and used to protect the children from kapha disorders and for the improvement of the intelligence and as memory enhancer. The rhizome powder of *A. calamus* is used in the training of talking birds. The medicated oil of calamus roots is used externally for massages to relieve vata and kapha disorders (Kulkarni, 1998).

The rhizomes of *A.calamus* has been reported to have various pharmacological and ectoparasiticidal activities (Ghosh *et al.,* 2011). However, perusal of available literature revealed no reports on antihepatotoxic potential of *Acorus calamus* extract of rhizome. Hence the present study was designed to evaluate the ethanolic and aqueous extracts of rhizome of *Acorus calamus* for its hepatoprotective effect in male rats.

MATERIALS AND METHODS

Paracetamol (M/S Granules India) and silymarin (Microlabs, Bangalore) and other chemicals of analytical grade were used in the study. Whole plant of *Acorus calamus* was collected from the local market and surrounding areas of Tirupati, Andhra Pradesh, India. The plant was identified and authenticated by the herbarium specialist, Department of Botany, S.V.University, Tirupati.

Preparation of alcoholic and aqueous extracts

Acorus calamus rhizomes were dried in shade, later they were powdered and extracted (1.5 kg) successively with 30 liters of 60% alcohol in a soxhalet extractor for 18-20 hours. The extract was distilled and concentrated to dryness under reduced pressure and controlled temperature (40-50°c) and finally freeze-dried. The ethanolic extract yielded a weight of 150 g (10% w/w). For ethanolic extract, the dried rhizomes of *Acorus calamus* were powdered and the powdered material was taken in a round bottom flask and was extracted with water for 48 h at room temperature. After 48 h, the solution was concentrated in a rotatory evaporator. Aqueous and alcoholic extract of *Acorus calamus* was suspended in 0.5% CMC.

Experimental animals

Male albino rats of *wistar* strain weighing 150-200g were obtained from Department of Laboratory Animal Medicine, TANUVAS, Madhavaram milk colony, Chennai. The animals were maintained under standard laboratory conditions with food and water *adlibitum.* Approval of the experimental protocol was obtained prior to the conduct of the experiment from the institutional animal ethics and biosafety committee.

Twenty four hours after the last day of treatment (Table 1), blood was collected from all the experimental animals by retrobulbar plexus puncture under ether anesthesia and whole livers were collected after sacrificing the animals by decapitation.Blood samples were used for the estimation of RBC enzyme profile viz glutathione peroxidase (Paglia and Valentine, 1967), glutathione reductase (Raghuramulu,1983), erythrocytic catalase (Britton and Machlly, 1956), lipid peroxidation hepatic tissue homogenate (Yagi, 1984). The liver specimens obtained from control and treated groups were fixed in 10% formalin ad stained with H&E stain.

Statistical analysis

The data was subjected to statistical analysis by applying one way ANOVA (Snedecor and Cochran, 1994).

Differences between means were tested using Duncan's multiple comparison test at 1% and 5% leaves as significance.

RESULTS AND DISCUSSION

Effects of aqueous and alcoholic extracts of *Acorus calamus* on various antioxidant parameters is given in Table 2. In the present study the levels of glutathione peroxidase were reduced in group II when compared to group I which is in accordance to the findings of Kumar *et al.* (2005). Silymarin prevented the reduction of glutathione peroxidase by protecting the glutathione depletion. *Acorus calamus* prevented the altered glutathione peroxidase levels due to its antioxidant activity as also reported by Manikandan and Sheeladevi (2005).

Glutathione reductase activity was reduced in group II which are in accordance with the findings of Kumar *et al* (2005). Vitamin E antagonized the glutathione reductase levels due to its antioxidant activity (Suhail and Ahmed, 1995). *Acorus calamus* attenuated the altered glutathione reductase level due to its antioxidant activity. Catalase, primarily antioxidant defence component is a tetramerichemoprotein present in peroxisomes and catalyzes dismutation of H_2O_2 reduces methyl and ethyl hydroperoxides. The liver, kidney and RBC possess relatively high levels of catalase (Yu, 1994). The catalase activity in the paracetamol treated group was reduced significantly ($P<0.01$) when compared to control group which is in correlation with the report of Kim *et al.* (2004). Silymarin normalized the altered catalase levels (Linn *et al.,* 2001).

Vitamin E increased catalase levels due to its antioxidant property (Suhail and Ahmad, 1995). *Acorus calamus* prevented the reduction in catalase activity which is in accordance with the findings of Manikandan *et al.* (2005). In the present study glutathione levels were reduced significantly ($P<0.01$) in paracetamol toxic group when compared to the control group. The results are in accordance with the previous findings. Silymarin antagonized the depletion of glutathione due to its free radical scavenging activity. Alcoholic extracts of *Acorus calamus* showed marked protective effect than aqueous extract in glutathione depletion. The protective action of Vitamin E may be due to its antioxidant activity and also due to its increased red cells level of reduced glutathione (Suhail and Ahmad, 1995).

Lipid peroxidation serves as a marker of oxidative stress. Lipid peroxidation is oxidative deterioration of poly unsaturated lipids and it involves ROS and transition metal ions. It is a molecular mechanism of cell injury leading to generation of peroxides and lipid peroxides, which can be decomposed to yield a wide range of cytotoxin products, most of which are aldehydes such as MDA. A substantial increase in hepatic lipid peroxidation was evident by elevated MDA level in liver homogenate of paracetamol administered group which was similar to the findings of Kumar *et al.* (2005). Silymarin reduced the MDA levels in tissue and Vit.E inhibits the lipid perodixation due to its antioxidant activity. *Acorus calamus* reduced the paracetamol induced lipid peroxidation. The results of the present study revealed that treatment with aqueous and alcoholic extracts of *Acorus calamus* normalized the activity of lipid peroxidation in liver tissue. The present study revealed the antilipid peroxidation activity of *Acorus calamus* against paracetamol induced lipid peroxidation in rats.

As shown in Fig 1, histopathological findings such as degeneration of hepatic cells sinusoidal haemorrhages and bile duct proliferation were noticed in group II (Kumar *et al.,* 2005). Treatment with silymarin (group V), Vit.E (group VI) and *Acorus calamus* (Group III and IV) preserved structural integrity of hepatic cellular architecture. The present study demonstrates antioxidative capacity of *Acorus calamus.* The antioxidative effect in turn could have protected the decrease of GSH and other enzymes restoring the free radical scavenger's enzymatic activity. Thus, the use of *Acorus calamus* may be recommended for life style related diseases including hepatic diseases

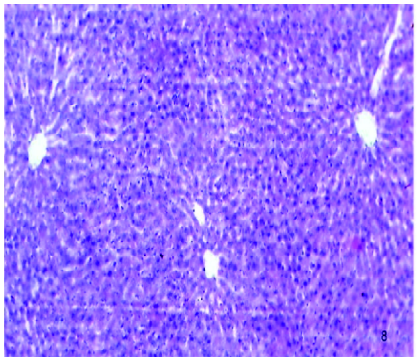
Table1:
Experimental design

Group I	0.5% Carboxy methyl cellulose *p.o* for ten days
Group II	Paracetamol @ 2g/kg *p.o* on Day one + 0.5% Carboxy methyl cellulose *p.o* for ten days
Group III	Paracetamol @ 2g/kg *p.o* on Day one + Ethanolic extract of *Acorus calamus* rhizome(600mg/kg) *p.o* for ten days
Group IV	Paracetamol @ 2g/kg *p.o* on Day one + Aqueous extract of *Acorus calamus* rhizome (600mg/kg) *p.o* for ten days
Group V	Paracetamol @ 2g/kg *p.o* on Day one + Silymarin @ 25mg/kg *p.o* for ten days
Group VI	Paracetamol@ 2g/kg *p.o* on Day one + Vitamin E (30mg/kg) *p.o* for ten days

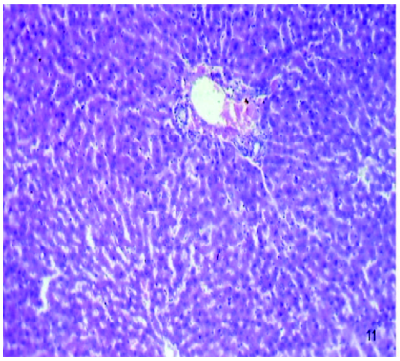
Table 2:
Effects of alcoholic and aqueous extracts of *A.calamus* rhizome on erythrocyte antioxidant parameters.

Groups	Glutathione peroxidase (U/ml)	Glutathione reductase(U/ml)	Catalase	Glutathione(mg dl^{-1})	Lipid peroxidation (nmol g^{-1})
Group I	72.45±1.75^{a}	40.81±0.77^{a}	2.98±0.02^{a}	6.40±0.27^{a}	32.80±0.35^{d}
Group II	47.58±3.65^{c}	27.35±1.12^{d}	1.50±0.11^{c}	1.45±0.07^{d}	42.95±0.86^{a}
Group III	60.38±1.19^{b}	34.13±1.12^{b}	2.58±0.07^{b}	5.58±0.15^{b}	36.78±0.41^{b}
Group IV	62.50±1.33^{b}	31.50±0.72^{c}	2.63±0.11^{b}	3.98±0.14^{c}	38.00±0.44^{b}
Group V	67.38±1.17^{ab}	39.65±0.75^{a}	3.02±0.01^{a}	5.78±0.09^{b}	34.78±0.59^{c}
Group VI	61.81±2.19^{b}	36.95±1.25^{b}	2.49±0.17^{b}	5.30±0.21^{b}	36.18±0.75^{bc}

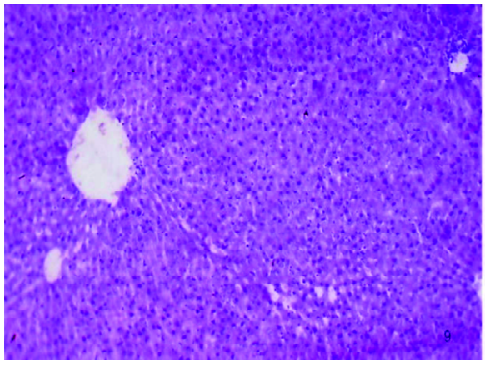
One way ANOVA, the values are mean±SE, n=6, Different superscripts a, b, c, d are statistically significant at $P < 0.001$ and $P<0.005$



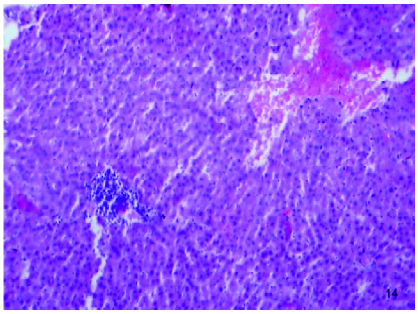
Group 1: Normal architecture of hepatic cells, H&E x 70



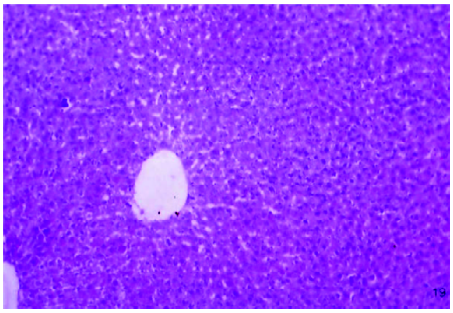
Group 2: Perivascular infiltration of mononuclear cells, H&E x 70



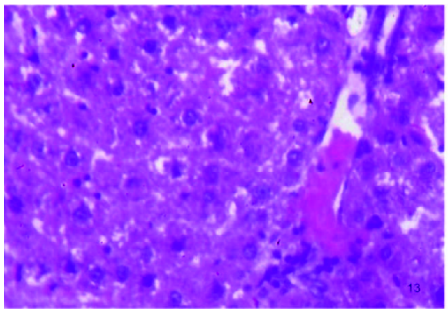
Group 5: Normal architecture of hepatic cells, H&E x 70



Group 2: Section showing sinusoidal haemorrhages, H&E x 70



Group 6: Mild degenerative changes in liver parenchyma, H&E x 70



Group 3 & 4: Mild proliferation of bile duct, H&E x 70,

Fig 1: Histoarchitecture of liver in different treatment groups

and also to improve the general health condition in animals

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Research Article

IN VITRO ASSESSMENT OF CYP-MEDIATED METABOLISM AND KINETICS OF LINCOMYCIN IN SHEEP USING S9 FRACTION

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ABSTRACT

The present study determines *in vitro* drug metabolism-kinetics of lincomycin and was conducted using S9 liver fraction of sheep. *in-vitro* metabolism-kinetics of lincomycin was performed by adding lincomycin (50 µg.ml^{-1}) in S9 fraction. The total protein and cytochrome P450 content was 20.41± 0.62 mg/g of wet tissue and 0.59±0.17 nmol/mg of protein, respectively. Exposure of licomycin with S9 fraction in sheep caused a decrease in lincomycin concentrations in time dependant manner i.e. 15, 30, 45 and, 60 min were 26.6±0.71, 24.17±1.09, 21.43±1.08 and, 18±1.64 µg.ml^{-1}, respectively. In sheep, kinetic parameter $t_{1/2}$ and CL_{int} of lincomycin in S9 fraction was found to be 46.92±4.24 min and 4.81±0.53 mL/min/g, respectively. Around 65% of lincomycin was metabolized within 1 hour by S9 fraction. Drug metabolism inhibition study indicates that the partial involvement of CYP 1A2, 3A4, 2C9 and 2D6 in lincomycin metabolism but did not show any clear-cut involvement. Further, studies are needed to check the involvement of other cytochrome P450 isoenzymes and cytosolic fractions in drug metabolism.

Key words: Cytochrome P450, sheep, S9 fraction, lincomycin.

INTRODUCTION

Lincomycin belongs to lincosamide antibiotic which comes from the *Streptomyces lincolnensis* (Birkenmeyer and Kagan, 1970). It has typically feature a 6,8-dideoxy-6-aminooctose lincosamine. In lincomycin A, this sugar moiety methylthiolincosamide is linked via an amide bond to an amino acid derivative propylhygric acid (Spizek and Rezanka, 2004). Lincomycin exerts its antibacterial action by inhibiting RNA-dependent protein synthesis by acting on the 50S subunit of the ribosome. and recommended for the treatment for gram-positive aerobic and anaerobic infections associated with bone, upper respiratory tract and skin diseases in livestock (Papich and Riviere 2009), especially against penicillin-resistant strains of *Staphylococcus spp*. and *Streptococcus spp*. It is also effective against other organisms including such as *actinomycetes* and some species of *Mycoplasma* and *Plasmodium*.

Drug metabolism by cytochrome P450 enzymes is one of the major determinants of drug clearance resulting in a potential loss of efficacy. These enzymes play critical roles in the biotransformation of drugs, carcinogens, steroid hormones and environmental toxicants (Coon, 2005).

In DMPK, "drug-like" properties commonly refer to respectable absorption, distribution, low metabolism, S9 and microsomal fractions, are used in drug metabolism studies to address various questions. The S9 fraction contains a group of soluble drug-metabolizing enzymes responsible for specific drug metabolism. The S9 fraction is the simplest system of the three containing both cytosol and microsomes, and represents a nearly complete collection of all drug metabolizing enzymes and provide complete metabolite profile to be achieved as they contain both phase I and phase II activity (Nakano *et al.,* 2004; Zhang *et al.,* 2008; Albaugh *et al.,* 2012).

Lincomycin is a promising drug candidate; however, despite its pharmacological importance no study has yet shown role of any cytochrome P450 (CYP) enzymes in lincomycin metabolism. This study was undertaken to determine the involvement of different CYP isoenzymes in lincomycin metabolism and lincomycin metabolism-kinetics using S9 fraction of liver in sheep under *in vitro.*

MATERIALS AND METHODS

Chemicals and reagents

Lincomycin standard (Lincomycin hydrochloride, CDH, Delhi, India) was procured. Tris HCl buffer (Merck, Mumbai, India), NADP, Glucose-6-phosphate, Glucose-6-phosphate dehydrogenase (SRL, Mumbai), Alpha-naphthoflavone, Ketoconazole (Sigma Aldrich, USA), Fluconazole, Hydroquinidine (Himedia, Mumbai, India) were procured commercially. All other chemicals/reagents used in this study were of analytical grade, obtained from reputed companies.

S9 Fraction preparation

S9 fraction was prepared by the method of Masengu *et al.* (2014). Briefly, livers from 5 sheep were collected at the time of slaughter from local slaughter house in chilled phosphate-buffered saline (PBS). Perfusion *in situ* was done using normal saline to remove blood from the organs. Livers of the animals were blotted

and collected in a labelled container. Then, liver was weighed, and cut into smaller pieces followed by homogenization in 50 mM Tris-HCL buffer (pH 7.4) containing 0.154 M potassium chloride (1 part of liver with 3 parts of buffer). Homogenates were centrifuged at 9000×g at 4°C for 15 minutes. Supernatant was stored at -32°C.

Functional characterization

Functional characterization of S9 fraction was done by estimating total protein by Lowry's Method (1951). Cytochrome P450 estimated by the method of Omura and Sato (1964). Briefly, S9 fraction was diluted with 0.1M potassium phosphate buffer 7.4 at 4°C. 950 µl of diluted S9 fraction were then added to reference cell and sample cell and were reduced with 50 µl 0.9M sodium dithionite to make it 1 ml. After 2 min baseline was scanned between 450- 490 nm. The sample cell was then bubbled with carbon monoxide for 30 sec at the rate of approximately 2 bubbles/sec and the cytochrome P450 spectrum was again recorded between 450-490 nm. The difference in absorption between 450 and 490 nm was used for the calculation of cytochrome P450 content using extinction coefficient of 91 Cm^{-1} $mM^{-.1}$ Carbon monoxide gas was generated freshly by the reaction of formic acid and concentrated sulphuric acid in the ratio of 1: 2 and purified by passing through potassium hydroxide pellets.

$$\text{CYP 450} = X \times \frac{1000}{91} \times \frac{\text{Volume of S9 fraction (ml)} + \text{Volume of phosphate buffer (ml)}}{\text{Volume of S9 fraction (ml)}}$$

Where,

$$X = \frac{(A_{450\text{-}490}) \text{ observed} - (A_{450\text{-}490}) \text{ baseline}}{(A_{450\text{-}490}) \text{ observed} - (A_{450\text{-}490}) \text{ baseline}}$$

***In-vitro* time-dependent kinetics**

To study *in vitro* metabolism of lincomycin, S9 fraction from 5 sheep were pooled together and used for the study. Similarly, total protein and cytochrome P450 content was measured again in pooled S9 fraction as discussed earlier and adjusted to protein content 1mg/mL. Lincomycin of concentration of 50 µg was used in the study. The assay procedure for the metabolism-kinetics of lincomycin consists of 100 µl S9 fraction, 630 µl of 0.02 M phosphate buffer (KH_2PO_4, pH 7.2), 20 µl of lincomycin (final concentration of 50 $\mu g.ml^{-1}$), and 250 µl of NRS (NADPH regenerating System). The NRS consisted of 1.7 mg/ml of NADP, 7.8 mg/ml of glucose-6-phosphate, and 6 units/ml of glucose-6-phosphate dehydrogenase. The resultant reaction mixture was incubated in shaking water bath at 37°C for the period of 60 min. The reactions were initiated by adding NRS and were terminated at different time points between 0-60 min (0, 15, 30, 45, 60 min) by adding 1 ml acetonitrile. After this, mixture was centrifuged at 14,000 g for 5 min at 4°C. The reaction performed in triplicate for each time point and for control without NRS in sample. Supernatant from each reaction was examined for disappearance of lincomycin using HPLC (high performance liquid chromatography).

HPLC assay for lincomycin

The concentrations of lincomycin in supernatant were determined using high performance liquid chromatography (HPLC) as per the method of Nielsen and Gyrd-Hansen (1998) with slight modifications.

Instrumentation and chromatographic conditions

The HPLC (Perkin Elmer) consisted of a single pump, a degasser and an autosampler injector with 200 µl loop (Perkin Elmer series 200). The detection was performed using an UV/VIS detector (Perkin Elmer 200 series) set at 210 nm. The reverse-phase chromatography was performed with an analytical C_{18} column (Sun fire®, Particle size 5µ, 4.6 × 250 mm, Waters, USA). The optimized method used binary-gradient mobile phase with acetonitrile (ACN) as mobile phase A and phosphate buffer, pH 7 as mobile phase B (Table 1). The flow rate was 1 $ml.min^{-1}$, and the injection volume was 50 µl. The Total Chrom software® (version 6.1) was used for instrument control and data analysis.

Stock solutions and standard preparation

The concentration of the standard stock solution (1 $mg.ml^{-1}$) was prepared. The primary stock solution of lincomycin was diluted quantitatively with water for the preparation of calibration standards and quality control (QC) samples. Lincomycin calibration standards were prepared fresh daily at concentrations of 0,0.05, 0.1, 0.25, 0.5, 1, 5, 10, 15, 40, 60, 80 and 100 $\mu g.ml^{-1}$ by spiking 900 µl blank S9 fraction with 100 µl of water (for the "zero" standard sample) or lincomycin working solutions. Prior to HPLC analysis of samples, the calibration standards and QC samples were processed accordingly. Aliquots of calibrators, QC samples and other S9 fraction samples (1000 µl) were added to centrifuge tubes. Subsequently, to all samples, 1 ml of acetonitrile was added and mixed for 10 seconds and the samples were centrifuged at 14,000 g for 5 min. After centrifugation, 1800 µl of clear supernatant was pipette out into a fresh test tube. The mixed clear supernatant (200 µl) was pipetted into an auto sampler vial.

Method for validation

(a) Selectivity

Selectivity of the method was evaluated by analyzing blank S9 fraction, S9 fraction spiked with lincomycin, and S9 fraction samples obtained during *in vitro* kinetic studies of lincomycin in sheep livers. For all S9 fraction samples, chromatograms were visually examined for chromatographic interference from endogenous compounds. The retention time of lincomycin was about 3.25 min, with a total run time of 10 min (Fig. 1).

(b) Calibration curve

The linearity of the method was evaluated by a

calibration curve in the range of 0.05 to 50 µg.ml^{-1} lincomycin. Calibration standards were run before and after the samples. The calibration curve was constructed by weighted (1/*y*) least-squares linear regression analysis. The calibration curves were described by the following linear equation: $y = ax \pm b$. *w*here, *y:* analyte area, *a:* slope of the calibration curve, *b: y* intercept and *x:* concentration (µg.ml^{-1}). The slope, intercept and correlation coefficient were calculated for each standard curve. The correlation coefficients (r), indicating the functional linear relationship between the concentration of analyte and the area under the peak, were above 0.998 across the concentration range used. Unknown lincomycin concentration in S9 fraction was computed from the equation of the calibration curve.

(c) Sensitivity

The limits of detection (LOD) and quantification (LOQ) were determined by signal-to-noise ratio evaluations of samples spiked from 0.01 to 1 µg.ml^{-1}. The LOQ was established by determining the concentrations of spiked calibration standards from 0.01 to 1 µg.ml^{-1}. The LOQ of the method was found to be 0.6 µg.ml^{-1}for lincomycin for S9 fraction of sheep liver, with acceptable accuracy and precision. The LOD was determined to be 0.4 µg.ml^{-1}, based on a signal-to-noise ratio of 3:1.

(d) Precision and accuracy

Lincomycin QC s amples at low, medium, and high concentrations were spiked for the determination of precision and accuracy and prepared as described above from six replicates at each level. The intra- and inter day precision and accuracy of the assay were determined by percent coefficient of variation (CV) and percent bias values, respectively. The coefficient of variation was calculated as: CV (%) = (standard deviation/mean) × 100. Inter- and intraday precision, in terms of the CV, was obtained by subjecting the data to one-way analysis of variance (ANOVA). The accuracy and precision of the method were evaluated with QC samples at concentrations of 0.5, 5 and 25 µg.ml^{-1}. At all levels, intra- and inter day assay precision levels were lower than 5 and 6%, respectively. The intra- and inter day assay accuracy levels ranged from 0.71 to 1.99 % and from 0.2 to 2.3%, respectively. These values indicated that the proposed method was precise and accurate.

(e) Recovery

The concentration of lincomycin was determined from the linear regression of the analytical standard curve. The absolute recovery was calculated by comparing the peak areas of the prepared QC samples with those of the standard solutions. The extraction recoveries of lincomycin from S9 fractions were 84.0 ± 4.56%, 90.7 ± 4.12% and 94.9 ± 3.29% for the low, medium and high QC samples, respectively. The recovery of lincomycin was consistent and efficient.

(f) Quantification

Regression formula obtained from the calibration curves was used to quantify the concentration of lincomycin in S9 fractions by substituting respective analyte area. Regression equation *y = 11065x+2705* was used for quantify the concentration of lincomycin. Where, *y:* analyte area and *x:* concentration (µg ml^{-1}). Lincomycin concentration at different time interval (0, 15, 30, 45 and 60 min) in incubated S9 fraction was calculated.

(g) Kinetic analysis

In vitro kinetic parameters were calculated by taking initial reaction velocity (v0) was calculated by multiplying the nominal substrate concentration at time 0 ($[S]_0$) by the slope of the log-linear regression from the concentration versus time relationship (k) and dividing by the protein concentration of the incubation, $v_0 = - k [S]_0 / [P]$. The velocity data were plotted in double reciprocal plot was rearranged to Linweaver-Burk equation. As the Lineweaver–Burk transformation as it is relies on the use of a double reciprocal plot, it is very sensitive to change at low concentrations. *In vitro* enzyme kinetic parameters were calculated by using Linweaver-Burk plot and kinetic parameters K_m and V_{max}. Where, K_m is the Michalis-Menten constant and V_{max} is the maximum rate of metabolism. K_m is the substrate concentration at half the maximum velocity (V_{max}).

Further, elimination half-lives ($t_{1/2}$) of the lincomycin was calculated from the slope of the line obtained by linear regression of the lincomycin remaining versus incubation time. The correlation coefficients in all cases were 0.99. The intrinsic clearance (Clint) of the lincomycin was calculated as described by Wen and Walle (2006), from total cytochrome P450 activity of S9 fraction per gram of liver and assuming that liver comprises 2.6% of total body weight in sheep and with ideal weight of 30 kg.

$$\textit{In vitro} \text{ half-life} = \frac{\ln 2}{\text{Rate constant(s)}} = \frac{0.693}{\text{Rate constant(s)}}$$

Where, β, rate constant for the disappearance of parent compound i.e. 2.303 x m and 'm' is the regression coefficient calculated by the method of "least square technique. Here, X is the concentration of the lincomycin and Y is the time of incubation.

$$m = \frac{\sum XY - \frac{\sum X \sum Y}{n}}{\sum X^2 - \frac{(\sum X)^2}{n}}$$

CYP Inhibitory assay

The reaction mixtures used in this study consisted of sheep liver S9 fraction (100 µL of S9 fraction with protein content 1mg/ml), 20µL lincomycin (final concentration 50 µg), 610 µL of 0.02 M phosphate buffer (KH_2PO_4, pH 7.2), 20 µL of inhibitors which includes alpha-naphthoflavone for CYP1A2 (10 µM), fluconazole for CYP2C9 (10 µM),

hydroquinidine for CYP2D6 (10μM), and ketoconazole for CYP3A (10 μM). Finally, reaction was initiated by addition of 250 μL of NRS (NADPH-Regenerating system: containing-NADP+ (10 mg/mL), glucose-6-phosphate (0.1 M) and glucose-6-phosphate dehydrogenase (1.0 U/mL). The reaction mixture without NRS was pre-incubated at 37°C for 5 min and then further incubated with NRS for 60 min in a water bath. After incubation, the reaction was terminated by adding equal volume of ice-cold acetonitrile. The assay was performed in pooled liver samples from five sheep in triplicates. The mixture was vortex and centrifuged at 14,000 g for 5 min at 4°C. Lincomycin concentration was measured in the supernatant by HPLC as described earlier.

RESULTS AND DISCUSSION

Effect on S9 protein and cytochrome P450 content

The protein and cytochrome P450 content in S9 fraction of sheep livers was estimated (Table 2). The protein content was found to be 20.41± 0.62 mg/g of wet tissue and the cytochrome P450 content was 0.59±0.17 nmol/ mg of protein. Total cytochrome P450 content per gram of liver was measured to be 12.04 nmol/g (range 4.16 - 20.81 nmol/g of liver) in sheep.

Effect on *in vitro* metabolism of lincomycin

Exposure of licomycin with S9 fraction along with NADPH regenerating system caused a decrease in lincomycin concentration with respect to the time. Lincomycin concentration was found to be decreased from 0-60 min (Table 3 and Fig. 2). In initial 15 min, the rate of metabolism was very high about 46.8% but subsequently 15-60 min the turnover/metabolism was very low.

Table 1.
HPLC mobile-phase gradient conditions for analysis of lincomycin.

Time (min)	Flow rate (ml.min^{-1})	% A	% B
0.5	1	40	60
10	1	40	60

A: acetonitrile; B: phosphate buffer

Table 2.
The Protein and cytochrome P450 content in S9 fractions from sheep liver.

Animal ID	Protein concentration (mg/g of wet tissue)	Cytochrome P450 (nmol/mg of protein)
1	20.92	0.79
2	21.64	0.26
3	21.64	0.6
4	18.92	1.1
5	18.92	0.22
	20.41± 0.62	0.59±0.17

Bold values are Mean ± SEM, n = 5.

Effect on *In-vitro* $t_{1/2}$ and CL_{int} in S9 fraction

In order to study the *in vitro* half life ($t_{1/2}$) of lincomycin in S9 fraction, the amount of parent compound remaining was plotted against time. The $t_{1/2}$ was calculated based on time verses concentration of lincomycin curve was 46.92±4.24 min, while, CL_{int} was calculated to be 4.81±0.53 ml/min/g of tissue in sheep liver (Table 4).

Effect on K_m and V_{max} in S9 fraction

In case of sheep, kinetic parameters such as K_m and V_{max} was found to be 30.3 μg and 0.22 nM/min/mg of protein, respectively. This indicates that, the drug concentration used in present study in sheep was two time higher than K_m value (Table 5). The metabolism of lincomycin study can be conducted even at lower concentration than 50.0 μg, which was used in our study.

Effect of CYP inhibitors on lincomycin metabolism in S9 fraction

The metabolic profile of lincomycin with CYP1A2, CYP2C9, CYP2D6 and CYP3A enzyme were evaluated in sheep liver. Involvement of these enzymes in lincomycin metabolism were investigated by using specific CYP enzymes inhibitors α-napthoflavone for CYP1A2, fluconazole for CYP2C9, hydroquinidine for CYP2D6, and ketoconazole for CYP3A in liver S9 fraction (Table 5). When the inhibitor of CYP1A2 (alpha-napthoflavone) was added, the concentration of lincomycin was reduced from 50 μg.ml^{-1} to 22.17±0.84 μg.ml^{-1}. In the presence of CYP2D6 inhibitor (hydroquinidine), lincomycin concentration was reduced to 14.03±0.69 μg.ml^{-1}, while, presence of CYP2C9 inhibitor (fluconazole) responsible for reduction in 50 μg.ml^{-1} to 24.9±0.76 μg.ml^{-1}. When the inhibitor of CYP3A4 (ketaconazole) was added the concentration of lincomycin reduced from 50 μg.ml^{-1} to 20.86±1.41 μg.ml^{-1}. The percent of drug metabolism in the presence of inhibitors in assays were found to be slightly higher as compared to the respective control (without inhibitor).

The cytochrome P450 activity in S9 fraction of

Table 3.
In-vitro kinetics of lincomycin, added at a concentration of 50 μg.ml^{-1} in S9 fractions of sheep liver.

Time	Lincomycin cocnentration					Lincomycin concnentration
	1	2	3	4	5	
15min	25.55	29.02	25.61	24.85	27.95	**26.60±0.71** (46.8)
30min	21.01	26.37	22.14	23.78	27.5	**24.17±1.09** (51.66)
45min	19.7	25.0	19.36	21.7	20.41	**21.43±1.08** (57.14)
60min	19.18	21.63	18.83	19.41	10.9	**18.00±1.64 (64.00)**
0 min (Control)	48.86	49.31	48.72	49.09	49.09	**49.00±0.09 (2.00)**

Bold values are Mean±SEM; n=5, Unit of all values are μg.ml^{-1}., Values in parentheses represents percent metabolism of the lincomycin.

our study was found to be lesser in sheep, as compared to earlier reports (Wisniewski *et al.,* 1987) in other species as they estimated in microsomal fractions. After ultracentrifugation post mitochondrial supernatant contains mostly microsomal enzymes, thus microsomal fraction might be reach in cytochrome enzymes content (Wisniewski *et al.,* 1987). The variation the protein and CYP content also indicates the species variation. The variation in cytochrome P450 activity in S9 fraction of sheep with slightly lower values in present study indicated the normal cytochrome P450 content, good metabolic activity and, functionally healthy animal status.

S9 fraction or hepatic microsomal liability represents one of the simplest and most widely used tools to predict *in vivo* clearance during the drug discovery process (Obach, 1999). Two methods are commonly employed, including the $t_{1/2}$ method, in which the first order rate of metabolism is determined at low substrate concentrations, while at higher concentration of substrate usually zero order rate of metabolism, and the enzyme kinetic method, in which V_{max} and K_m are determined (Obach *et al.,* 1997). In the present study the K_m<S was (two time more) indicating that the lincomycin concentration used in the present study was quite higher. As a result of this graph shows zero order kinetic pattern after 15 min of incubation. Further, rate of drug metabolism was very low, independent of concentration. The rate of the reaction becomes constant for a given enzyme concentration. Under these conditions, the enzyme is saturated with the drug, and the reaction is proceeding at maximal velocity. In other words, a further increase in drug concentration will not alter the velocity of reaction

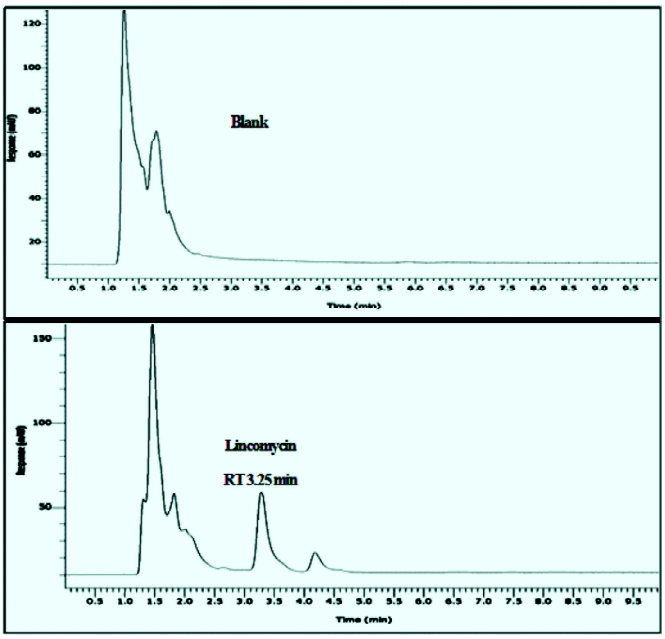

Fig 1.
Chromatograms of blank S9 fraction and S9 fraction containing lincomycin showing RT 3.25 min.

Table 4.
In vitro half life ($t_{1/2}$) and intrinsic clearance (CL_{int}) of lincomycin in S9 fraction of sheep livers.

Animal	Half life (min)	Intrinsic clearance (ml/min/g of tissue)
1	48.8	4.45
2	56.87	3.81
3	45.14	4.81
4	51.98	4.17
5	31.8	6.82
	46.92±4.24	**4.81±0.53**

Bold values are Mean±SEM; n=5

Table 5.
In vitro K_m and V_{max} derived from enzyme metabolism of lincomycin with respect to time in S9 fraction of sheep livers.

K_m (µg)	V_{max}(nM/min/mg of protein)
30.3	0.22

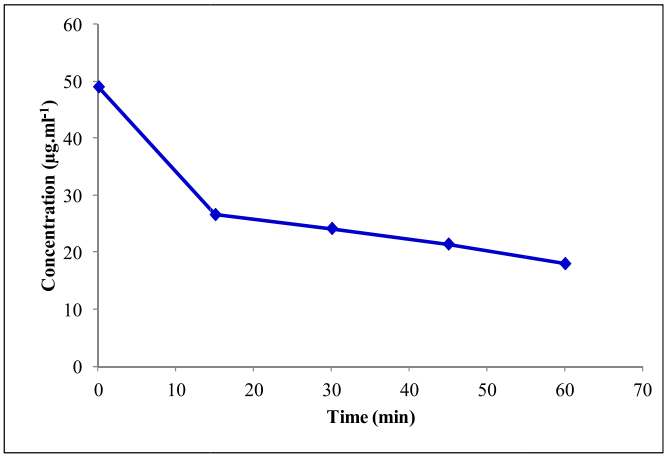

Fig 2.
In-vitro metabolic-kinetic plot of lincomycin, added at a concentration of 50 µg.ml^{-1}, in S9 fraction of sheep liver.

Table 6.
Effect of different cytochrome P450 inhibitors on the metabolism of lincomycin, added at a concentration of 50 µg.ml^{-1}, in S9 fraction of sheep livers.

CYP Inhibitors	Lincomycin (Presence of inhibitor)	Lincomycin(Absence of inhibitors)
α-napthoflavone(CYP1A2)	22.17±0.84(55.66)	19.1±0.05(61.80)
Hydroquinidine(CYP2D6)	14.03±0.69(71.94)	10.22±0.17(79.56)
Ketaconazole(CYP2C9)	20.86±1.41(58.28)	17.67±0.15(64.66)
Fluconazole(CYP3A4)	24.9±0.76(50.20)	21.31±0.26(57.38)

Values are Mean ± SE and unit of all are expressed in µg.ml^{-1}.
Values in parentheses represents percent metabolism of the lincomycin.

(zero-order reaction). When the drug concentration is smaller than Km the rate is proportional to the substrate concentration and first order reaction is obtained (Jia *et al.,* 2007). Here, plot clearly indicates the zero-order reaction; there is a linear relationship between the rate of product formation in time dependant manner and the substrate concentration.

Estimates of *in vivo* intrinsic clearance are typically made through the use of scaling factors applied to the observed *in vitro* data on the basis of protein concentration (Houston, 1994). If there is increasing concentrations of nonspecific binding components within the incubation may decrease the apparent intrinsic clearance of compounds that bind to liver microsomes (McLure *et al.,* 2000). CL_{int} is defined as the maximum activity of the liver (microsomal proteins or hepatocytes) towards a drug in the absence of other physiological determinants such as hepatic blood flow and drug binding within the blood matrix (Baranczewski *et al.,* 2006). In order to extrapolate *in vitro* clearance data on any new chemical entity metabolism to *in vivo* metabolic clearance, two critical factors have to be considered. The first one is the scaling factor (SF) to re-calculate *in vitro* CL_{int} to CL_{int} expressed per gram of liver, and it is known as the amount of microsomal protein per gram of liver when microsomal proteins were used to determine the CL_{int} *in vitro.* In the case of hepatocytes this value is known as the amount of hepatocytes per gram of liver. The scaling factor is very difficult to determine because of inter-individual variability in levels of microsomal protein as well as hepatocellularity per gram of liver. The second scaling factor is needed for expression of metabolic clearance per whole liver. Two crucial values are required for determination of this factor, namely liver size and its co-variation with body size. In the present study total CYP content based on these aspects had been considered and same was being used for calculation of $t_{1/2}$ and CL_{int} *in vitro* (Houston and Kenworthy, 1999).

No information available on the *in vitro* kinetics pattern of same compound in any other related species, however, *In vivo* pharmacokinetics of lincomycin in different species has been reported. In pigs, single i.m. lincomycin @11 mg/kg administrations showed peak plasma concentrations in 0.5 and 1 hour with concentration 5.33 and 10.92 $mg.kg^{-1}$ and gradually declined over 8 hours (Russel, 1979). After i.v. administration 10 mg/kg, the plasma clearance was 0.28 ± 0.09 L/h/kg and elimination half life ($T_{1/2}$) 3.56 ± 0.62 h (Albarellos *et al.,* 2011). Chickens received twice daily doses of 0.47-0.76 mg/kg lincomycin for 12 days showed half-life 8.3 hours (Goseline *et al.,* 1978). Whereas, female dog received a single dose of lincomycin 500 mg equivalents, after oral application peak plasma concentration of 4.5 mg/kg was reached at 4 hours and half-life was 4.1 hours, while, i.m. application, peak plasma concentration of 25.2 mg/kg was attained within 10 minutes, with half-life of 4 hours (Eberts *et al.,* 1963). In another report, adult male rats received single lincomycin dose of 100 mg/kg i.m. showed rapidly absorbed and peak plasma levels 20.9 $mg.kg^{-1}$ and declined biphasically to a concentration of 2.5 $mg.kg^{-1}$ at 8 hours (Davis and Balcom 1969). Fateeva and Poliak (1977) reported repeated dose administered via parenterally or orally revealed significant differences in distribution of the drugs in the animals and lower excretion rate of the lincomycin in rabbits was observed. Gouri *et al.*(2014) studied the pharmacokinetics of lincomycin after single i.v. administration @10 $mg.kg^{-1}$ in buffalo calves and found that distribution half-life of the drug to be 0.06 ± 0.01 h and elimination half-life 3.30 ± 0.08 h. The variation in elimination half life and peak concentration was noticed among the different animal species. In present study, *in vitro* half life 46.92 min was noticed with intrinsic clearance value 4.81 ml/min/g of tissue. These values are in the absence of many factor such as blood and endogenous metabolic factors that may have influence over kinetics behaviour of lincomycin.

Various drug metabolism studies of lincomycin indicated that the principal metabolites as parent lincomycin, *N*-desmethyl lincomycin, and lincomycin sulfoxide. Unchanged lincomycin known to eliminated in urine and faeces in dog and human after oral administration. There was no evidence of glucuronide or sulphate conjugation (Hornish *et al.,* 1987). This suggests that an equivalent form of these liver cytochrome P-450 isozymes, with similar catalytic activities, is present in the species tested. In cattle a form of cytochrome P-450 isozyme exhibiting a high catalytic activity for N-demethylation for other substrates having a N-methyl group structure. Present study does not show clear-cut involvement of selected CYPs: CYP1A2, CYP2C9, CYP2D6 and CYP3A in lincomycin metabolism. It means that other than these CYPs playing important role in the lincomycin metabolism in sheep.

CONCLUSION

The protein and cytochrome P450 concentration in S9 fraction of sheep was comparable to each other species. Drug metabolism inhibition study indicates the partial involvement of CYP 1A2, 3A4, 2C9 and 2D6 in lincomycin metabolism in sheep. The role these enzymes at this point can't clearly rule out. Further studies are needed to check the involvement of other cytochrome P450 isoenzymes and cytosolic fractions in drug metabolism.

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Research Article

EVALAUTION OF HEMATOBIOCHEMICAL CHANGES INDUCED FOLLOWING IMIDACLOPRID TOXICITY AND ITS AMEILIORATION BY COW URINE DISTILLATE IN WHITE LEGHORN BROILERS

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ABSTRACT

India is major producer of pesticide and the indiscriminate use of pesticides may lead to various hazardous troubles to human and animals. Imidacloprid, a neonicotinoids, launched in 1991, has now been used by a vast group of farmers engaged in agriculture practice. The toxic effect of pesticide is attributed to the lower productivity and feed conversion ratio. The poultry industry is one of the fastest growing livestock sector merchandise and the toxicity of modern day pesticides thus retarded the growth of birds and the egg and meat production. Cow urine, since ancient age had been recommended as daily used medicine as it has immune booster potential.The present study was aimed towards the evaluation of ameliorative effect of cow urine distillate following imidacloprid toxicity in white leghorn cockerels in continuous exposure for 60 days.Initially 48 birds were divided into six different groups randomly and equally. Group I served as control, whereas, group II and III were provided with 1/10th and 1/20th doses of LD50 and group V and VI were given 1/10th and 1/20th doses of LD50 with cow urine distillate @ 1ml/bird/day for 60 days. Group IV was provided with cow urine distillate only @ 1ml/bird/day for 60 days. The hematobiochemical indices were recorded to justify the ameliorative action after every fortnight.There was significant changes in the hematobiochemical values in the intoxicated groups as compared to control group. The study is the indicative of the beneficial role of cow urine supplement on daily basis as it protects the birds from being severely intoxicated. Cow urine has hematinic and immune boosting potential that may help the birds to counteract the undergoing stress following imidacloprid mediated toxicity.The study concluded that imidacloprid produced the dose dependent toxicity in white leghorn cockerels and the cow urine distillate was proved to be a handy immune booster as kept the values nearly towards normal side.

Key words: Imidacloprid, Cow urine distillate, Immune booster, White leghorn Cockerels

INTRODUCTION

India, the second largest populated country in the world, is by large infested with the uncontrolled and indiscriminate use of insecticides and other fertilizers to fulfil the desired need of food and other supplements to the society. Pesticides are the agents used to control or kill the pests causing degradation of crops and other fauna. India is one of the largest producers of pesticides in Asia and ranks twelfth in the world for the use of pesticides (Gunnell and Eddleston, 2003). In 1991, Bayer Crop Science introduced a new type of insecticide into the USA; imidacloprid, the first member of a group now known as the neonicotinoids. Imidacloprid (IMC) 1[(6-chloro-3-pyridinyl) methyl]-N-nitro-2-imidazolidinimine, a chloronicotyl was the first representative of neonicotinoids insecticides to be registered for use and presently the most important commercial product because of its high efficacy against insects and low soil persistence (Chao and Casida, 1997). Imidacloprid produces thinning of egg shell, and reduced egg hatchability which are indicators of the endocrine disruption potential of imidacloprid (Berny *et al*., 1999 and Matsuda *et al.,* 2001). Thus in this context imidacloprid induced endocrine disruption may change the normal physiology of the birds and to study the impact over the hematological and biochemical parameters will show some mechanistic pathway to elucidate the toxicity of imidacloprid in dose dependent manner. It had been proven that cow urine distillate had immunomodulatory action. In Sushrita Samhita the cow urine has been described as the most effective substance of animal origin with innumerable therapeutic values (Dhama *et al.*2005).The aim of the present study was to evaluate the ameliorative potential of cow urine distillate in imidacloprid mediated toxicity in white leghorn cockerels.

MATERIALS AND METHODS

The present study was conducted in 48 white leghorn cockerels, procured from IPF, GBPUAT, Pantnagar. These birds were acclimatized for two weeks in the experimental animal shed of Instructional poultry farm, Nagla, Pantnagar under standard managemental conditions. Poultry feed and water were provided *ad libitum* and kept under constant observation throughout study. All the chemicals required for this study were procured from Hi Media. ERBA diagnostics kits were used for biochemical analysis of glucose, serum total proteins, albumin, serum creatinine, urea, and cholesterol. Initially 48 birds were divided randomly and equally in six different

groups having eight (n=8) in each group. Group I was kept as control, group II and III were provided with imidacloprid @ 1/10th and 1/20th of LD50 respectively and Group IV was given only cow urine distillate @1 ml/bird/day, whereas group V and VI were given imidacloprid @1/10th and 1/ 20^{th} of LD50 dose with cow urine distillate treatment @1 ml/bird/day, respectively for a period of sixty days continuously by oral dosing (Table 1). After every fifteen days interval, blood samples were collected for hematobiochemical analysis. Experiment was conducted after the permission of the Institute animal ethics committee (IAEC) and adequate measures were taken to minimize pain or discomfort to animals.

Hematology

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

Biochemical parameters

The serum total protein**,** albumin**,** globulin, total cholesterol**,** glucose**,** creatinine and blood urea nitrogen was estimated by using ERBA diagnostic kits.

Statistical analysis

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

RESULTS

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

Hematological changes

The hematological parameters, including haemoglobin, PCV, TEC and TLC have shown significant decline in groups II, III, V and VI as compared to groups I and IV after 60 days exposure to imidacloprid (Table 2 and 3). Group I and IV revealed the significantly ($P<0.05$) higher value of the above mentioned parameters as compared to group II, III, V and VI. The ameliorative effects of cow urine distillate is justified by the less significant change in hematological values in treatment groups V and VI as compared to group II and III.

Biochemical changes

A significant ($P<0.05$) decrease in the total protein and albumin were observed in the groups II, III, V and VI as compared to the control group I and IV in dose dependent manner after 15th days onwards till end of study (Table 4). The treatment group IV, V and VI showed marked increase in comparison to without treatment group i.e. group II and III. A significant ($P<0.05$) decrease in blood glucose level was recorded in group II, III, V and VI as compared to the group I and group IV (Table 5). There was significant ($P<0.05$) increase in serum cholesterol and serum creatinine level levels in all intoxicated groups II, III, V and VI in comparison with control group I and IV after 60 days post exposure of imidacloprid in white leghorn cockerels (Table 5).

DISCUSSION

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

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

Table 1:
Experimental design of in vivo toxicity of imidacloprid following treatment with cow urine distillate for 60 days in WLH cockerels

Group	Treatment	Dose	Route	Period of exposure
I	Control untreated	-	-	-
II	Imidacloprid	$1/10^{th}$ LD_{50}	Oral	60 days
III	Imidacloprid	$1/20^{th}$ LD_{50}	Oral	60 days
IV	Control treated with cow urine	-	Oral	60 days
V	Imidacloprid + Cow urine (I)	$1/10^{th}$ LD_{50} Imidacloprid + cow urine @ 1ml/bird/day	oral	60 days
VI	Imidacloprid + Cow urine (II)	$1/20^{th}$ LD_{50}+ Cow urine @	Oral	60 days

beneficial effect on the health of the birds.The reduction in Hb, PCV, TEC and TLC might be due to transferring of fluid from extra vascular compartment to the intravascular compartment in order to maintain the normal cardiac output and stroke volume and also pooling of the blood cells in the blood reservoirs such as spleen etc. Group I and IV revealed the significantly (P<0.05) higher value of the above mentioned parameters as compared to group II, III, V and VI. The ameliorative effects of cow urine distillate is justified by the less significant change in hematological values in treatment groups V and VI as compared to group II and III. This is an indicator of immunomodulatory activity of cow urine distillate that stimulate the bone marrow and reticulo endothelial system to generate the more number of erythrocytes and other blood cells to fight against the intoxication with imidacloprid. Reduction in hematological values could be due to impaired biosynthesis of heme in bone marrow (Shakoori *et al.,* 1990). The clinical signs seen in IMC intoxicated birds were similar to those reported in rats (Sheets, 2001). The anaemia might have been occurred due to the hemolytic action of imidacloprid as evidenced in this study. Furthermore, damage to the GIT and liver could also have resulted in malabsorption and maldigestion of nutrients required for erythropoiesis. It has been suggested that compounds having benzene ring or other ring structure acts as a hapten that combines with protein components of white blood cells to develop an antigen that triggers the generation of antibodies which cause either the lysis or agglutination of leucocytes. Imidacloprid is also having a ring structure thus may have caused the leucopoenia as evident from the present study on white leghorn cockerels. Bapu (2001) proved that due to the presence of traces of iron in cow urine, it has been found to posses the haematinic properties. Continuous exposure to insecticide may then leads to lymphopenia which may have an immunosuppressive effect. The adverse effect on differential leucocyte count may be due to altered functioning of bone marrow and stress (Jain, 1986). Marked decrease in total protein, albumin and globulin was also observed in imidacloprid treated groups. According to Kori-Siakpere (1995) decrease in plasma protein, albumin and globulin could be attributed to renal excretion or impaired protein synthesis or due to liver disorders. The decrease in serum total protein also may be attributed to loss of protein either by decrease protein synthesis or increased proteolysis or degradation (Shakoori *et al.*, 1990). The persistent hypoglycaemia might have occurred due to hepatotoxic effect caused by the imidacloprid. Being the primary metabolising site, imidacloprid induced liver toxicity might hamper normal glycolysis, gluconeogenesis and glycogenolysis processes. The present findings were in agreement with the findings of Balani *et al.*, (2011). Significant increase in cholesterol, serum creatinine and blood urea nitrogen was noticed in intoxicated groups. However, group V and VI showed less significant alteration in the clinical values in comparison with group II and III cementing the beneficial and protective role of cow urine distillate against the toxicant. Garg (2004) also reported that supplementation of cow urine to white leghorn layer showed significant amelioration in serum total cholesterol. Liver is the major site of cholesterol synthesis and metabolism. Hepatic cholesterol homeostasis is maintained by equilibrium between the activities of the hydroxyl methyl glutryl Coenzyme A reductase and acyl co enzyme A cholesterol acyl transferase (Jain, 1986). The injury to renal epithelial cells has been associated with the increase in such markers as serum creatinine and blood urea nitrogen, of renal injury. In addition; increased hepatic urea production from amino acid metabolism could also be responsible for an increase in urea concentration in the serum .Treatment significantly (P<0.05) reduced the level of urea and creatinine in groups IV, V and VI as compared to group II and III. Cow urine distillate has the potent germicidal and antimicrobial activity in association with immune modulating ability (Chauhan *et al.,* 2009).

It is concluded from the study that imidacloprid causing toxicity in a dose dependent manner following sub chronic exposure to white leghorn cockerels and the cow urine distillate has produced significant ameliorative

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

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

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

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

	TOTAL PROTEIN					ALBUMIN				
Groups /Days	DAY O	DAY 15	DAY 30	DAY 45	DAY60	DAY O	DAY 15	DAY 30	D AY 45	DAY60
Group I	3.92 ± 0.013^{aA}	3.92 ± 0.018^{aC}	3.90 ± 0.016^{aC}	3.92 ± 0.023^{aC}	3.91 ± 0.027^{aC}	1.55 ± 0.031^{aA}	1.53 ± 0.016^{aC}	1.53 ± 0.026^{aD}	1.53 ± 0.013^{aC}	1.53 ± 0.015^{aD}
Group II	3.92 ± 0.010^{dA}	3.84 ± 0.033^{cAB}	3.32 ± 0.018^{bA}	3.24 ± 0.036^{bA}	3.06 ± 0.017^{aA}	1.54 ± 0.016^{eA}	1.39 ± 0.018^{dA}	1.34 ± 0.017^{cA}	1.26 ± 0.011^{bA}	1.16 ± 0.009^{aA}
Group III	3.92 ± 0.016^{eA}	3.83 ± 0.020^{dAB}	3.45 ± 0.033^{cB}	3.27 ± 0.034^{bA}	3.18 ± 0.018^{aB}	1.55 ± 0.016^{dA}	1.51 ± 0.015^{dC}	1.43 ± 0.015^{cBC}	1.29 ± 0.020^{bA}	1.22 ± 0.013^{aB}
Group IV	3.91 ± 0.018^{aA}	3.89 ± 0.004^{aABC}	3.91 ± 0.031^{aC}	3.92 ± 0.011^{aC}	3.93 ± 0.033^{aC}	1.56 ± 0.013^{aA}	1.59 ± 0.019^{aD}	1.58 ± 0.013^{aD}	1.60 ± 0.023^{aD}	1.61 ± 0.016^{aE}
Group V	3.92 ± 0.015^{dA}	3.90 ± 0.018^{dBC}	3.53 ± 0.039^{cB}	3.40 ± 0.018^{bB}	3.23 ± 0.021^{aB}	1.56 ± 0.007^{eA}	1.45 ± 0.020^{dB}	1.38 ± 0.015^{cAB}	1.30 ± 0.016^{bA}	1.21 ± 0.019^{aB}
Group VI	3.94 ± 0.018^{eA}	3.82 ± 0.037^{dA}	3.48 ± 0.014^{cB}	3.38 ± 0.021^{bB}	3.25 ± 0.022^{aB}	1.59 ± 0.013^{dA}	1.49 ± 0.006^{cBC}	1.46 ± 0.020^{cC}	1.36 ± 0.022^{bB}	1.27 ± 0.013^{aC}

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

Table 2:
Effect on Hb (g %) , PCV (%), TEC (10^6) following daily oral administration of *IMIDACLOPRID* with and without treatment for 60 days in white leghorn cockerels (Mean value± S.E., n=8).

	Hemoglobin					PCV					TEC				
Groups /Days	DAY 0	DAY 15	DAY 30	DAY 45	DAY60	DAY 0	DAY 15	DAY 30	DAY 45	DAY60	DAY 0	DAY 15	DAY 30	DAY 45	DAY60
Group I	8.51±0.151^{aA}	8.95±0.128^{abB}	9.10±0.193^{aC}	9.00±0.146^{aC}	8.88±0.101^{abC}	32.50±0.957aA	32.67±0.715aA	31.17±0.946aAB	32.83±1.447aB	31.83±0.601aCD	3.34±0.012aA	3.34±0.027aBC	3.33±0.022aD	3.36±0.011aB	3.36±0.009aD
Group II	8.46±0.142^{aA}	8.06±0.095^{cA}	7.61±0.127^{bA}	7.53±0.117^{bA}	6.80±0.085^{aA}	31.17±0.601^{bA}	31.33±0.494^{bA}	29.33±0.760^{aA}	29.00±0.577^{aA}	27.83±0.477^{aA}	3.33±0.012^{aA}	3.27±0.015^{bA}	3.09±0.015^{cA}	3.01±0.030^{dA}	2.83±0.020^{eA}
Group III	8.60±0.085^{aA}	8.26±0.122^{cA}	7.90±0.051^{bAB}	7.75±0.067^{bAB}	7.11±0.119^{aB}	31.00±0.577^{aA}	31.17±1.014^{aA}	30.50±0.764^{aAB}	30.00±0.577^{aA}	29.17±0.792^{aAB}	3.35±0.009^{aA}	3.28±0.010^{bA}	3.12±0.022^{cAB}	3.07±0.041^{dA}	2.96±0.032^{eB}
Group IV	8.68±0.098^{aA}	8.78±0.079^{abB}	8.98±0.060^{acC}	9.03±0.088^{cC}	9.00±0.057^{bcC}	31.50±0.428^{aA}	33.00±0.365^{aA}	32.17±0.477^{abB}	33.00±0.428^{aB}	32.67±0.715^{aB}	32.50±0.428^{aB}	3.32±0.042^{aA}	3.36±0.015^{abC}	3.38±0.008^{abE}	3.39±0.009^{aB}
Group V	8.50±0.057^{aA}	8.11±0.060^{cA}	8.00±0.077^{bcB}	7.83±0.055^{bB}	7.38±0.119^{aB}	31.33±0.422^{aA}	31.00±0.447^{abA}	30.00±0.516^{abAB}	29.67±0.715^{bA}	29.67±0.422^{aAB}	3.33±0.006^{aA}	3.30±0.021^{abB}	3.17±0.013^{cC}	3.08±0.017^{dA}	2.96±0.019^{eB}
Group VI	8.46±0.071^{aA}	8.10±0.073^{cA}	7.91±0.030^{bcAB}	7.78±0.040^{bAB}	7.41±0.110^{aB}	31.17±0.477^{aA}	31.33±0.494^{aA}	30.67±0.667^{aAB}	30.50±0.563^{aAB}	30.33±0.803^{aBC}	3.35±0.006^{aA}	3.28±0.012^{bA}	3.14±0.017^{cBC}	3.08±0.028^{dA}	3.04±0.026^{eC}

¹%Mean bearing common superscript with small letters differ significantly (P<0.05) when compared horizontally with in the same row & mean value bearing capital alphabets differ significantly (P<0.05)when compared vertically with in the same column.

Table 5:
Effect on glucose, cholesterol and serum creatinine following daily oral administration of *IMIDACLOPRID* with and without treatment for 60 days in white leghorn cockerels (Mean value± S.E., n=8).

Glucose	Cholesterol					Serum creatinine									
Groups /Days	DAY 0	DAY 15	DAY 30	DAY 45	DAY60	DAY 0	DAY 15	DAY 30	DAY 45	DAY60	DAY 0	DAY 15	DAY 30	DAY 45	DAY60
Group I	302.18±1.695^{aA}	299.11±1.906^{aC}	302.25±1.862^{aD}	299.50±1.258^{aC}	298.48±0.834^{aD}	132.76±0.424^{aA}	133.87±0.385^{aA}	133.39±0.497^{aA}	133.50±0.590^{aA}	133.95±0.341^{aA}	0.33±0.011^{aA}	0.33±0.007^{aA}	0.33±0.004^{aA}	0.32±0.008^{aA}	0.33±0.004^{aA}
Group II	299.68±2.895^{Ea}	282.33±3.303^{cA}	260.16±1.701^{cA}	227.33±2.538^{bA}	209.22±1.234^{aA}	132.99±0.513^{aA}	137.76±1.005^{bB}	145.63±0.776^{cC}	156.06±0.787^{dD}	162.41±0.900^{eD}	0.32±0.007^{aA}	0.41±0.011^{bB}	0.51±0.013^{cD}	0.61±0.015^{dC}	0.82±0.015^{eE}
Group III	300.20±2.210^{aA}	283.96±2.325^{bAB}	269.69±3.161^{cBC}	246.47±3.011^{dB}	228.62±2.081^{eBC}	133.19±0.508^{aA}	135.17±0.468^{aA}	141.50±0.680^{bB}	147.81±0.868^{cB}	156.74±1.171^{dC}	0.34±0.003^{aA}	0.40±0.008^{bB}	0.43±0.008^{cB}	0.54±0.012^{dB}	0.74±0.011^{eD}
Group IV	301.22±1.842^{aA}	300.46±0.742^{aC}	299.43±0.636^{aD}	298.27±1.982^{aC}	301.32±0.353^{aD}	132.61±0.430^{aA}	133.63±0.646^{aA}	133.76±0.589^{aA}	133.50±0.834^{aA}	133.18±0.333^{aA}	0.33±0.006^{aA}	0.33±0.003^{aA}	0.32±0.004^{aA}	0.32±0.004^{aA}	0.32±0.004^{aA}
Group V	301.22±1.842^{aA}	289.11±0.997^{bB}	266.39±2.125^{cB}	246.45±2.507^{dB}	224.80±3.698^{eB}	133.68±0.443^{aA}	135.31±0.874^{aA}	140.40±0.858^{bB}	151.63±0.809^{cC}	157.55±1.136^{dC}	0.33±0.003^{aA}	0.40±0.012^{bB}	0.48±0.011^{cC}	0.53±0.011^{dB}	0.69±0.008^{eC}
Group VI	300.47±0.739^{aA}	288.36±2.388^{abB}	272.86±2.043^{bC}	248.92±2.884^{cB}	234.12±1.564^{dC}	133.92±0.433^{aA}	134.46±0.302^{aA}	139.53±0.543^{bB}	146.27±0.810^{cB}	152.21±1.157^{dB}	0.32±0.009^{aA}	0.39±0.006^{bB}	0.42±0.009^{cB}	0.51±0.018^{dB}	0.64±0.014^{eB}

¹%Mean bearing common superscript with small letters differ significantly (P<0.05) when compared horizontally with in the same row & mean value bearing capital alphabets differ significantly (P<0.05)when compared vertically with in the same column.

action against the imidacloprid toxicity by restoring the values towards normaly by continuous administration @1 ml/bird/day for 60 days.

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Research Article

EFFECT OF *WITHANIA SOMNIFERA* ROOT POWDER ADMINISTRATION ON HEMATOLOGICAL PARAMETERS IN LEAD TREATED CHICKENS

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ABSTRACT

For this study, one hundred and fifty, one day old broiler chicks were divided into five groups of 30 birds each. Group I was kept as control. Groups II and III were administered 250 ppm and 400 ppm lead as lead acetate in triple glass distilled water, respectively. Groups IV and V were administered 250 ppm and 400 ppm lead in triple glass distilled water along with *Withania somnifera* root powder (WSRP) in standard recommended feed at the rate of 1% concentration. The treatment started from 2 weeks of age till 60 days post treatment (DPT). Blood was collected from each group at 0, 7th , 14th, 21st , 28th , 35th, 42nd, 49th and 60th DPT for hematological examination. There was a significant ($P<0.05$) decrease in values of Hb, PCV, TEC, MCH, MCHC and TLC. In groups II and III, significant increase in heterophil was recorded, whereas a significant decrease in lymphocyte was observed as compared to group I. In groups IV and V, significantly ($P<0.05$) lesser decrease in Hb, PCV, TEC, MCH, MCHC, lymphocyte and significant ($P<0.05$) increase in TLC and heterophil was observed as compared to groups II and III, respectively. It was concluded from the present study that 1 % WSRP administration significantly ameliorate the effect of lead administration @ 250 ppm and 400 ppm on hematological parameters in chickens.

Key words: Chickens, hematology, lead, *Withania somnifera*

INTRODUCTION

Lead is one of the most toxic among heavy metals and ubiquitous in nature. In most of the developing countries including India, the main source of lead pollution is automobile exhaust. Lead is discharged in waste and effluents of silver refineries, basic steel works and foundries, plating, mining, steel melting and metal processing, motor vehicle and air-craft industries in addition to printing industries (Nashikkar and Chakrabarti, 1994). Heavy metals including lead become toxic when they are not metabolized by the body and accumulated in the soft tissues. Health hazards due to increased lead exposure as a result of industrial and environmental pollution found to produce wide range of biochemical and physiological dysfunctions in animals and humans (Courtois *et al.*, 2003) including central and peripheral nervous systems (Bressler *et al.*, 1999), cardiovascular system (Khalil-Manesh *et al.*, 1993), hemopoietic system (Lanphear *et al.*, 2000), liver (Sharma, 1980) and kidneys (Damek-Poprawa and Sawicka-Kapusta, 2003). The absorbed lead is conjugated in the liver and passed to the kidney. From kidney, a small quantity of lead is excreted in urine and the rest accumulates in various body organs and interferes with their function, specially the kidney being a target site for lead toxicity (Jarrar, 2003).

Withania somnifera (Ashwaganda) is known since the ancient ages in our country for its medicinal values. *Withania* contains number of phyto constituents and withanolides as the major constituent. The main constituents of this plant are alkaloids and steroidal lactones but the withanine, the main alkaloid found in its roots and leaves is thought to be responsible for its biological activity. Hepatoprotective and nephroprotective potential of root extract of this plant due to reduction in lead-induced oxidative damage has already been reported (Chaurasia *et al.*, 2000). The present investigation was carried out to know the effect of *Withania somnifera* administration on hematological parameters in lead treated chickens.

MATERIALS AND METHODS

The research protocol for this investigation was approved by IAEC (IAEC/VPP/CVASc/134 dated 9.11.2012). One hundred and fifty broiler chicks of one day of age were divided randomly into five groups of 30 birds each viz. group I (control), group II (250 ppm lead), group III (400 ppm lead), group IV (250 ppm lead + WSRP) and group V (400 ppm lead + WSRP). After two weeks of adaptation period, groups II and III were administered 250 ppm and 400 ppm lead as lead acetate anhydrous ($C_4H_8O_6Pb_2$) in triple glass distilled water, respectively. Groups IV and V were administered 250 ppm and 400 ppm lead in triple glass distilled water along with *Withania somnifera* root powder orally in standard recommended feed at the rate of 1% concentration. The treatment in respective groups was given from 2 weeks of age of birds till 60 days post treatment (DPT). Blood was collected from 4 birds from each group at 0, 7th , 14th, 21st , 28th , 35th, 42nd, 49th and 60th DPT. Hematological parameters viz. Hemoglobin and packed cell volume (PCV) were estimated using the method of Jain (1986). Total erythrocyte count (TEC) and total leucocyte count (TLC)

were determined according to the method of Natt and Herrick (1952) using poultry blood diluting fluid. Differential leucocyte count (DLC) was done by preparing thin blood smear from a drop of fresh blood without anticoagulant. The leucocytes were counted as described by Lucas and Jamroz (1961). Data generated was statistically analyzed by using standard statistical procedures (Snedecor and Cochran, 1994) with help of SPSS software 2007.

RESULTS AND DISCUSSION

The results pertaining to different serum hematological parameters are given as under.

Hemoglobin concentration

Table 1 shows variation in values of various erythrocyte related parameters in different groups of experimental birds at various time intervals. In groups I and IV, no significant variation was recorded from 0 to 60th DPT. In groups II and III, the value of hemoglobin showed a significant decline from 0 to 60th DPT. In group V, the average hemoglobin value showed a significant decline from 0 to 60th DPT. The hemoglobin value when compared between different groups at particular time intervals, at 7th and 14th DPT, group I values showed a significant more as compared to group III. But, groups II and III values showed no significant difference with the values of groups IV and V. From 21st DPT to 35th DPT, group I had highest value of hemoglobin, followed by groups II and III but, did not show any significant variation with groups IV and V values. From 42nd to 60th DPT, the values of hemoglobin in group I did not differ significantly with group IV but in rest of the groups, the average hemoglobin values were significantly different where group III had lowest value as compared to group I, followed by groups II and V.

Average PCV values showed variation in values in different groups at various time intervals. The PCV values within groups I, IV and V did not show any significant variation from 0 DPT to 60th DPT (Table 1). The TEC values in group I and IV, values showed significant increase from 0 to 60th DPT. In the groups II and III, the TEC values significantly decrease from 0 to 60th DPT. In group V, the values significantly increase from 0 to 60th DPT. The TEC values when compared between different groups at particular time interval, at 7th DPT, the values in groups I, II, III and V did not show any significant variation but the values in group IV were significantly lower compared to these groups. At 14th DPT, all groups showed significant variation where values in group IV were significantly higher as compared to values in group V followed by groups I, II and III. At 21st and 28th DPT, the values in groups I and II did not show any significant variation but were significantly more than than the values in groups V, II and III. From 35th to 42nd DPT, the TEC values showed significant variation, where groups II and III had lowest values as compared to group IV followed by groups V and I. At 49th DPT, group IV had significantly higher values than group III followed by groups I, V and III values. At 60th DPT, the TEC values in groups I and IV were significantly more compared to group III values, followed by groups IV and II values (Table 1).

As shwn in Table 1, the values of MCV 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. The mean MCH values in groups I, II, III, IV and V, showed no significant difference from 0 to 60th DPT. When these values were compared between different groups at varying time intervals, no significant difference could be recorded between any group upto 14th DPT. But, at 21st DPT, the values in group I and IV were significantly higher in comparison to group III followed by groups V and III. At 28th DPT, the values in groups I, IV and V did not differ significantly but significantly higher from group II followed by group III values. From 35th to 42nd DPT, the values in group I showed significant increase with the value in group III but showed an insignificant difference with the values in groups II, IV and V. From 49th to 60th DPT, the values in group I did not vary significantly from the values in groups IV and V but showed significantly higher values than the values in group III.

The mean MCHC values in groups I, IV and V, the mean value of MCHC no significant variation from 0 to 60th DPT. The values in groups II and III decreased significantly from 0 to 60th DPT. When the MCHC values were compared between different groups at varying time intervals, no significant difference could be recorded between any groups from 0 to 21st DPT. From 28th to 35th DPT, group I values were significantly higher than group III only. At 42nd and 60th DPT, group I had significantly higher values when compared with group III, followed by group II but showed no significant difference than values of groups IV and V. At 49th DPT, values in groups I, IV and V did not show any significant variation but these were significantly higher than group III values only (Table 1).

The values of TLC and DLC related parameters of different groups at various time intervals are presented in Table 2. In group I, the average TLC values depicted a significant increase from 0 to 60th DPT. In groups II and III, the values showed a significant decrease from 0 to 60th DPT. In groups IV and V, the values had a significant increase from 0 to 60th DPT. The TLC values when compared between different groups at varying time intervals, no significant difference was observed between groups I, II, III, IV and V at 0 DPT. At 7th DPT, group I showed significantly higher values when compared with groups II and III values but did not show any significant variation with groups IV and V values. From 14th to 21st and 49th to 60th DPT, groups I and IV values did not show any significant variation but these groups showed significantly

Table 1:

Erythrocyte related parameters in different groups of experimental birds at different time intervals.

Group DPT	Group I	Group II	Group III	Group IV	Group V
Hemoglobin concentration (g/dl, (Mean±SE))					
0 DPT	9.48±0.076^{Aa}	9.50±0.160^{Aa}	9.46±0.059^{Aa}	9.46±0.079^{ABa}	9.48±0.110^{Aa}
7th DPT	9.50±0.147^{Aa}	9.28±0.105^{ABab}	9.10±0.091^{Bb}	9.38±0.067^{ABab}	9.23±0.062^{ABab}
14th DPT	9.50±0.093^{Aa}	9.10±0.099^{Ba}	8.50±0.127^{Cb}	9.30±0.134^{ABa}	9.20±0.197^{ABa}
21st DPT	9.45±0.068^{Aa}	8.50±0.126^{Cb}	8.13±0.072^{Dc}	9.29±0.115^{ABa}	9.20±0.138^{ABa}
28th DPT	9.44±0.134^{Aa}	8.20±0.122^{CDb}	7.50±0.072^{Ec}	9.21±0.118^{Ba}	9.10±0.073^{Ba}
35th DPT	9.40±0.122^{Aa}	8.20±0.179^{CDb}	7.40±0.156^{Ec}	9.37±0.012 ^{ABa}	9.13±0.101^{Ba}
42nd DPT	9.40±0.032^{Aa}	7.90±0.023^{Dc}	6.90±0.015 ^{Fd}	9.37±0.019 ^{ABa}	9.00±0.075^{Bb}
49th DPT	9.50±0.011^{Aa}	7.10±0.017^{Ec}	6.48±0.103^{Gd}	9.48±0.023^{Aa}	9.03±0.023^{Bb}
60th DPT	9.55±0.014^{Aa}	6.20±0.015^{Fc}	5.90±0.016^{Hd}	9.50±0.012^{Aa}	9.00±0.030^{Bb}
Packed cell volume (PCV, %, Mean±SE)					
0 DPT	33.25±0.750^{Aa}	33.25±0.479^{Aa}	33.50±0.500^{Aa}	32.50±0.500^{Aa}	33.50±0.289^{Aa}
7th DPT	33.50±0.500^{Aa}	31.75±0.479^{Bb}	31.00±0.408^{Bb}	31.50±0.289^{Ab}	31.25±0.479^{Cb}
14th DPT	33.75±0.629^{Aa}	30.00±0.408^{Cc}	29.50±0.500^{BCc}	32.00±0.707^{Ab}	31.75±0.479^{BCb}
21st DPT	33.50±0.289^{Aa}	29.00±0.707^{CDb}	29.25±0.479^{Cb}	32.25±0.479^{Aa}	32.00±0.707^{ABCa}
28th DPT	33.75±0.750^{Aa}	27.75±0.479^{DEb}	28.25±0.750^{CDb}	32.50±0.869^{Aa}	32.50±0.500^{ABCa}
35th DPT	34.00±0.408^{Aa}	27.25±0.479^{Eb}	27.50±0.645^{DEb}	32.75±0.250^{Aa}	33.00±0.577^{ABa}
42nd DPT	34.25±0.250^{Aa}	27.75±0.479^{DEc}	27.00±0.408^{DEc}	32.75±0.750^{Aab}	32.25±0.479^{ABCb}
49th DPT	34.25±0.479^{Aa}	27.50±0.500^{DEb}	26.25±0.479^{EFb}	33.00±0.707^{Aa}	32.75±0.250^{ABCa}
60th DPT	34.50±0.500^{Aa}	27.25±0.479^{Eb}	25.00±0.707^{Fc}	33.25±0.479^{Aa}	32.75±0.750^{ABCa}
Total erythrocyte count (million/µl blood, Mean±SE)					
0 DPT	3.31±0.039^{Ga}	3.34±0.024^{Aa}	3.32±0.011^{Aa}	3.30±0.030^{Ga}	3.33±0.011^{Ca}
7th DPT	3.37±0.042^{Gb}	3.37±0.020^{Ab}	3.32±0.027^{Ab}	3.58±0.018^{EFa}	3.37±0.018^{Cb}
14th DPT	3.37±0.016^{Gb}	3.31±0.018^{Abc}	3.28±0.011^{Ac}	3.52±0.023^{Fa}	3.20±0.033^{Dd}
21st DPT	3.55±0.027^{Fa}	3.14±0.032^{Bb}	3.00±0.026^{Bc}	3.60±0.037^{EFa}	3.00±0.014^{Fc}
28th DPT	3.65±0.032^{Ea}	3.12±0.029^{Bb}	3.00±0.026^{Bc}	3.65±0.020^{Ea}	3.10±0.029^{Eb}
35th DPT	3.76±0.034^{Db}	3.00±0.035^{Cd}	2.90±0.035^{Cd}	3.90±0.029^{Da}	3.20±0.023^{Dc}
42nd DPT	3.88±0.040^{Cb}	2.92±0.039^{Dd}	2.91±0.029^{BCd}	4.00±0.025^{Ca}	3.70±0.017^{Bc}
49th DPT	4.00±0.031^{Bb}	2.92±0.035^{CDc}	2.70±0.020^{Dd}	4.10±0.021^{Ba}	4.00±0.026^{Ab}
60th DPT	4.22±0.038^{Aa}	2.50±0.029^{Ec}	2.15±0.052^{Ed}	4.25±0.018^{Aa}	4.01±0.007^{Ab}
Mean corpuscular hemoglobin (MCH) (pictogram/pg, (Mean±SE)					
0 DPT	28.61±0.037^{Aa}	28.64±0.022^{Aa}	28.65±0.026^{Aa}	28.64±0.089^{Aa}	28.61±0.011^{Aa}
7th DPT	28.72±0.013^{Aa}	28.66±0.400^{Aa}	28.61±0.819^{Aa}	28.70±0.410^{Aa}	28.60±0.403^{Aa}
14th DPT	28.91±0.011^{Aa}	28.48±0.823^{Aa}	28.43±0.001^{Aa}	28.95±0.410^{Aa}	28.71±0.012^{Aa}
21st DPT	28.95±0.013^{Aa}	28.26±0.095^{Ac}	27.63±0.031^{ABd}	29.00±0.104^{Aa}	28.74±0.026^{Aa}
28th DPT	29.20±0.028^{Aa}	27.94±0.007^{Ab}	27.25±0.022^{ABc}	29.06±0.077^{Aa}	28.88±0.342^{Aa}
35th DPT	29.48±0.359^{Aa}	27.78±0.639^{Aab}	26.99±0.879^{ABCb}	29.10±0.329^{Aab}	28.92±0.957^{Aab}
42nd DPT	29.71±1.082^{Aa}	27.49±0.540^{Aab}	26.48±0.491^{BCb}	29.19±0.940^{Aab}	29.02±0.917^{Aab}
49th DPT	29.76±1.012^{Aa}	27.32±1.022^{Aab}	25.86±0.741^{BCb}	29.33±0.682^{Aa}	29.14±0.994^{Aa}
60th DPT	29.98±0.534^{Aa}	27.91±0.516^{Aab}	25.27±0.723^{Cb}	29.50±0.855^{Aa}	29.19±0.560^{Aa}
Mean corpuscular hemoglobin concentration (MCHC, g/dl, Mean±SE)					
0 DPT	28.22±0.220^{Aa}	28.25±0.360^{Aa}	28.24±0.020^{Aa}	28.21±0.061^{Aa}	28.21±0.096^{Aa}
7th DPT	28.26±0.148^{Aa}	28.01±0.630^{ABa}	27.90±0.461^{Aa}	28.25±0.841^{Aa}	28.08±0.509^{Aa}
14th DPT	28.61±0.660^{Aa}	27.72±0.523^{ABCa}	27.50±0.652^{Aa}	28.00±0.559^{Aa}	28.00±0.256^{Aa}
21st DPT	28.99±0.842^{Aa}	27.67±0.719^{ABCa}	26.89±0.670^{ABa}	27.96±0.592^{Aa}	27.74±0.665^{Aa}
28th DPT	29.04±0.914^{Aa}	26.83±0.822^{ABCab}	26.40±0.883^{ABCb}	27.91±0.554^{Aab}	27.69±0.376^{Aab}
35th DPT	29.49±0.673^{Aa}	26.79±1.085^{ABCab}	25.01±1.205^{BCb}	27.62±0.642^{Aab}	27.44±0.526^{Aab}
42nd DPT	29.54±0.543^{Aa}	26.31±0.245^{ABCbc}	24.30±1.159^{Cc}	27.50±0.780^{Aab}	27.39±0.873^{Aab}
49th DPT	29.68±0.309^{Aa}	25.93±0.635^{BCab}	24.35±0.636^{Cb}	27.85±0.828^{Aa}	27.14±1.152^{Aa}
60th DPT	29..96±0.152^{Aa}	25.67±0.787^{Cbc}	24.80±0.592^{BCc}	27.80±1.165^{Aab}	27.05±0.662^{Aab}

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

Table 2:
Total and differential leucocytes count related parameters in different groups experiment birds at different time intervals.

Group DPT	Group I	Group II	Group III	Group IV	Group V
Total leucocyte count (TLC, x1000/ µl blood, Mean±SE)					
0 DPT	25.24±0.039^{Da}	25.28±0.046^{Aa}	25.24±0.027^{Aa}	25.24±0.045^{Ga}	25.28±0.044^{Ca}
7th DPT	25.60±0.064^{Ca}	25.10±0.040^{Bb}	25.00±0.091^{Bb}	25.50±0.078^{FGa}	25.10±0.037^{Db}
14th DPT	25.92±0.066^{Ba}	25.10±0.058^{Bc}	24.87±0.120^{Bd}	25.71±0.069^{EFa}	25.40±0.044^{Cb}
21st DPT	25.80±0.054^{Ba}	25.09±0.042^{Bc}	24.20±0.037^{Cd}	25.90±0.041^{EFa}	25.60±0.049^{Bb}
28th DPT	25.90±0.076^{Bab}	24.90±0.046^{Bc}	24.20±0.031^{Cd}	25.70±0.120^{DEb}	26.00±0.079^{Aa}
35th DPT	26.00±0.091^{Ba}	24.60±0.078^{Cb}	23.43±0.077^{Dc}	26.20±0.073^{Ca}	25.98±0.082^{Aa}
42nd DPT	26.50±0.037^{Aa}	24.50±0.151^{CDc}	23.90±0.091^{Ed}	26.10±0.058^{CDb}	26.07±0.031^{Ab}
49th DPT	26.50±0.058^{Aa}	24.31±0.066^{Dc}	22.80±0.091^{Fd}	26.62±0.129^{Ba}	26.10±0.058^{Ab}
60th DPT	26.70±0.089^{Aa}	24.10±0.061^{Ec}	22.00±0.082^{Gd}	26.90±0.043^{Aa}	26.10±0.056^{Ab}
Heterophil (percentage, Mean±SE)					
0 DPT	25.75±0.854^{Aa}	25.50±0.289^{Ca}	25.50±0.957^{Ea}	25.50±0.866^{Aa}	25.75±0.750^{Aa}
7th DPT	26.00±0.577^{Aa}	26.75±1.548^{BCa}	27.00±0.913^{DEa}	25.50±1.658^{Aa}	25.50±1.756^{Aa}
14th DPT	26.50±0.957^{Aa}	27.00±1.225^{ABCa}	27.75±0.947^{DEa}	26.25±1.315^{Aa}	26.5±1.041^{Aa}
21st DPT	26.75±0.629^{Aa}	27.50±1.893^{ABCa}	30.00±1.472^{CDa}	27.00±1.225^{Aa}	26.50±1.500^{Aa}
28th DPT	26.50±0.500^{Ab}	28.00±0.408^{ABCb}	31.25±1.750^{BCDa}	27.50±1.190^{Ab}	27.25±0.750^{Ab}
35th DPT	27.00±0.816^{Ab}	28.75±1.109^{ABCb}	33.50±1.190^{ABCa}	27.50±1.658^{Ab}	27.50±0.957^{Ab}
42nd DPT	27.75±0.946^{Ab}	29.25±1.493^{ABCb}	34.25±1.031^{ABCa}	28.00±1.472^{Ab}	27.50±1.555^{Ab}
49th DPT	27.25±1.652^{Ab}	30.50±0.866^{ABb}	35.50±1.708^{ABa}	28.75±1.493^{Ab}	28.75±1.436^{Ab}
60th DPT	28.00±1.080^{Ab}	30.75±0.750^{Ab}	36.00±1.826^{Aa}	28.75±1.750^{Ab}	29.25±1.181^{Ab}
Lymphocyte (percentage)					
0 DPT	60.25±0.250^{Aa}	60.00±0.000^{Aa}	60.00±0.408^{Aa}	60.25±0.250^{Aa}	60.25±0.479^{Aa}
7th DPT	60.50±0.866^{Aa}	60.00±0.000^{Aa}	60.00±1.414^{Aa}	60.00±1.472^{Aa}	60.00±0.707^{Aa}
14th DPT	60.75±0.250^{Aa}	59.75±1.250^{Aa}	59.00±1.472^{ABa}	60.00±1.225^{Aa}	59.00±0.707^{Aa}
21st DPT	60.00±0.816^{Aa}	59.50±0.645^{Aa}	58.50±2.179^{ABCa}	60.75±0.479^{Aa}	59.00±0.707^{Aa}
28th DPT	61.25±1.181^{Aa}	59.25±0.750^{Aa}	58.25±0.750^{ABCa}	60.00±1.225^{Aa}	59.00±2.614^{Aa}
35th DPT	61.50±0.957^{Aa}	59.00±0.913^{Aa}	56.75±0.750^{ABCa}	61.00±2.273^{Aa}	59.50±2.466^{Aa}
42nd DPT	61.75±1.315^{Aa}	58.50±1.936^{Aab}	56.25±0.946^{ABCb}	61.50±0.866^{Aab}	59.75±2.462^{Aab}
49th DPT	62.25±1.548^{Aa}	58.00±0.816^{Abc}	55.00±1.633^{BCc}	61.50±1.190^{Aab}	59.50±0.289^{Aab}
60th DPT	62.25±0.854^{Aa}	57.00±1.472^{Abc}	54.50±1.258^{Cc}	62.00±1.780^{Aa}	61.00±1.581^{Aab}

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 and C) indicate significant (P<0.05) difference between days within a particular group.

higher values than group V followed by groups II and III values.

On DLC, the mean values of heterophil in groups I, IV and V showed no significant variation from 0 to 60th DPT. In groups II and III, there was a significant increase in the values from 0 to 60th DPT. When these values were compared between different groups at different time intervals, no significant difference could be recorded between any group from 0 to 21st DPT. From 28th to 60th DPT, the values in groups I, II, IV and V did not show any significant variation but these were significantly lower as compared to the values in group III.

As shown in Table 2, the lymphocyte percentage in groups I, II, IV and V, the values showed no significant difference from 0 to 60th DPT. In group III, the lymphocyte count showed a significant increase from 0 to 60th DPT. When these values were compared between different groups at different time intervals, no significant difference could be noted between any group from 0 to 35th DPT. At 42nd DPT, group I showed significantly higher values than group III but showed insignificant variation with the values in groups II, IV and V. At 49th DPT, group I values were significantly higher than the values in groups II and III but showed insignificant differences with groups IV and V values. At 60th DPT, groups I and IV values did not differ significantly but showed significantly higher values when compared with groups II and III.

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 (Table 2).

DISCUSSION

The present study revealed a significant decrease in hemoglobin (Hb), packed cell volume (PCV), total erythrocyte count (TEC), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and total leukocyte count, whereas insignificant variation observed in mean corpuscular volume (MCV) in

groups II and III. In groups II and III, a significant increase in heterophil count was recorded with significant decrease in lymphocyte count. No significant variation was observed in eosinophil, basophil and monocyte count in any group. These results are in agreement with previous findings by Donavick (1966) in cow, Julian (1971), Stone and Soares (1976) in Japanese quail, Szymezak *et al*. (1983) and Khan *et al*. (2008) in mice, Helmy *et al*., (2000); MuGahi *et al.* (2003) and Kamruzzaman (2006) in rats. Decreased values of Hb, PCV, TEC, MCH and MCHC might be due to decreased life span of erythrocytes (Julian, 1971), increased fragility of erythrocytes (Donavick, 1966) and inhibitory effect of lead on erythrocyte enzymes i.e. GA3PD & G6PD (Stone and Soares 1976). Anuradha (2007) found that lead induced anemia, results from shortening of erythrocyte life span and an inhibition of hemoglobin synthesis. Lead acts on heme synthesis via its inhibitory effect of amino levulinic acid dehydogenase (ALAD), the enzyme involved in the final step of heme synthesis pathway. Lead also inhibit the conversion of coproporphyrinogen III to protoporphyrin IX leading to reduction in hemoglobin production (Klassen, 2001). Progressive destruction of RBCs due to binding of lead with RBCs, leading to increase fragility and destruction, could be another reason for decrease hematological values (Rous, 2000). Analysis of total leucocyte count and differential leucocyte count revealed leucopenia and lymphopenia in higher dose group. This might be due to direct toxic action of lead on leucopoiesis in lymphoid organs. Decrease in TLC is directly related with either their decreased production from the germinal center of lymphoid organs or increased lysis due to presence of lead in the body (Kumar *et al.,* 1998). The Hb, PCV, TEC, MCH, MCHC, TEC and DLC values were within the normal range in groups IV and V as compared to groups II and III and this illustrated the ameliorative effects of WSRP on dietary lead exposure. *Withania somnifera* have profound effects on the hematopoietic system and enhanced erythropoiesis. The major activity of *Withania somnifera* may be the stimulation of stem cell proliferation (Kuttan, 1996; Ziauddin *et al*., 1996 and Gautam *et al*., 2004). It was concluded from the present study that administration of WSRP at 1 % levels in 250 ppm and 400 ppm lead treated chickens have ameliorative effects on the these hematological parameters.

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Research Article

PARACETAMOL INDUCED ALTERATIONS IN HEMATOLOGICAL AND RENAL FUNCTIONS AND THEIR AMELIORATION BY *PICRORHIZA KURROA* IN WLH CHICKS

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ABSTRACT

This study was designed to evaluate the effect of *Picrorhiza kurroa* root (PKR) powder on hematological and renal function in paracetamol treated WLH chicks. Thirty chicks of either sex were divided into five groups of 6 chicks each. Group I was served as control. Chicks were administered paracetamol @50 mg/kg body weight i/m for 7 days in groups II, III, IV and V. Silymarin(100mg/kg, p/o) was orally administered for 14 days in chicks of group III. Chicks of group IV, V were orally administered for 14 days with 200 ppm and 400 ppm PKR in feed . Plasma creatinine, bilirubin and urea concentration were significantly ($P<0.05$) increased in PCM group II as compared to control after 7 days of treatment whereas in group III and V their values were significantly($P<0.05$) less than PCM group II. After 14 days treatment plasma creatinine, bilirubin and urea in groups III, IV and V were decreased significantly ($P<0.05$) as compared PCM group II and were close to normal values. A significant decrease in Hb, PCV, TEC, TLC, total proteins and blood glucose values in PCM treated groups were observed after 7 days treatment which were restored towards normalcy in silymarin and both PKR dose levels after 14 days. It is concluded from the above study that PCM@ 50mg/kg, i/m for 7 days altered hematological and renal function of chicks. *Pikrorhiza kurroa* root powder@ 200ppm and 400 ppm in diet for 14 days revealed ameliorative effect of *Picrorhiza kurroa* against PCM induced toxicity at par with silymarin@ 100mg/kg, p.o for 14 days in WLH chicks

Key words: Hematology, paracetamol, *Picrorhiza kurroa,* renal dysfunction, WLH chicks.

INTRODUCTION

Liver dysfunction is a major health problem that challenges not only health care professionals but also the pharmaceutical industry and drug regulatory agencies. Paracetamol is a COX inhibitor NSAID commonly used as an analgesic and antipyretic drug in man and animals. An overdose or prolonged administration of this drug can, however, cause severe hepatic damage (Thomas, 1993). Initially paracetamol is metabolized by cytochrome P-450 including CYP2E1, 1A2, 3A4 and 2A6 (Chen *et al*., 1998) to the reactive hepatotoxic metabolite N-acetyl-p-benzoquinone imine (NAPQI). Hepatic disorder is likely to cause hematological and renal dysfunction which may further aggravated by the available synthetic chemical drugs used to treat liver disorders. Hence, herbal drugs have become increasingly popular and are used widely. Among these *Picrorhiza kurroa* have immense potential and is traditionally used for liver disorders. *Picrorhiza kurroa* (of the family *scrophulariaceae*), also known as Kutki or Katuki, is a perennial herb used in Ayurveda. *It* grows in the Himalaya region on rocky places within 3500-4800m above sea level. The present study was carried out to evaluate ameliorative activity of *Picrorhiza kurroa* root powder against paracetamol induced alterations in hematobiochemical profile in white leg horn chicks (WLH).

MATERIALS AND METHODS

Experimental animals

White leg horn chicks of either sex, of 2 month old age, weighing between 500 to 600 gm, were procured from Instructional Poultry Farm (IPF), of the university. The animals were kept in cages and acclimatized for one week under environmentally controlled conditions with free access to feed and water. All the experimental animals were kept under constant observation during the entire course of the study and maintained as per the norms of guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). The project was duly approved by the IAEC before start of the experiment.

Experimental design

Thirty chicks of either sex were divided into five groups of 6 chicks each. Group I served as control . Chicks of Group II, III, IV, V were administered by paracetamol @50 mg/kg body weight i/m for 7 days, Group III chicks were orally administered for 14 days by silymarin(100mg/kg p/o) and Group IV,V chicks were orally administered with PKR in feed @ 200 ppm and 400 ppm for 14 days (Table 1).

Table1:

Groups	Treatment	Duration
1	Normal feed only	For 14 days
2	Paracetamol (50mg/kg,i/m)	Daily for Ist to 7th day
3	Paracetamol(50mg/kg,i/m) + Silymarin (100mg/kg)	Daily for Ist to 7th day Daily for 14 days
4	Paracetamol (50mg/kg,i/m) + PKR(200ppm) in feed	Daily for Ist to 7th day Daily for 1st -14th day
5	Paracetamol (50mg/kg,i/m) + PKR(400ppm)in feed	Daily for Ist to 7th day Daily for 1st -14th day

Collection of blood samples

5 ml blood was collected on 7th and 14th day of study from the wing vein of each white leg horn chick of which 4 ml was collected in heparin containing centrifuge tubes and 1 ml was collected in EDTA containing eppendrof tubes (1 ml). Plasma was separated by centrifugation at 3000 rpm for 20 minutes of heparin containing centrifuge tubes and was subjected to biochemical analysis .Blood collected in EDTA containing eppendrof tubes was used for hematology.

Haematological examination

Blood collected in EDTA containing eppendrofs were used for haematology. TEC was determined by method described by Natt and Harrick (1952) and TLC ,Hb , PCV was determined by the method as described by Jain (1986).

Assessment of renal functions

Total bilirubin, total protein, blood glucose, blood urea, creatinine were analysed spectrophotometrically by diagnostic kits manufactured by Erba Mannheim Germany and Autospan . Diagnostics kits from ERBA Mannheim, Germany and Autospan were used for biochemical analysis of plasma.

Statistical analysis

Total variation, present in a group was determined by two- way analysis of variance and Student's *t*-test was used for determining significance of difference at 5 % level of significance in various haemotological and biochemical parameters (Woolson, 1987).

RESULTS AND DISCUSSION

The present study was undertaken to determine the alteration in hematological and renal function and amelioration by *Picrorhiza kurroa* root powder (PKR) against PCM induced toxicity in poultry chicks model by determining plasma analytes and haematological parameters. As given in the Table 2 after 7 days, plasma creatinine, bilirubin and urea concentration significantly($P<0.05$) increased in PCM treated group II as compared to control indicating hepatorenal damage due to PCM. In group III, IV and V there was a significant($P<0.05$) decrease in the level of plasma creatinine, bilirubin and urea concentration as compared to PCM treated group II indicating protective activity of PKR which was more or less at par with silymarin. Plasma creatinine, bilirubin and urea concentration in group V was significantly($P<0.05$) lower as compared to group IV which might be due to the dose dependent effect of PKR and after 14th day of treatment plasma creatinine, bilirubin and urea concentration of groups II,III,IV and V further declined, however, were significantly higher than control. Plasma creatinine, bilirubin and urea concentration of group III came closer to control which could be due to liver regeneration whereas group IV and V values was declined but not as much as of group III showing comparatively slower recovery by PKR in comparing to silymarin.

Elevation in the level of plasma bilirubin in group II is the indicator of hepatic damage whereas elevation in the level of plasma creatinine and urea is the indicator of renal damage as in group IV and group V there was significant decline in their levels as compared to the group II which indicated that *Picrorhiza kurroa* had ameliorative effect . Bilirubin is the catabolic product of haemoglobin produced within the reticuloendothelial system, released in unconjugated form which enters into the liver, converted to conjugated forms bilirubin mono and diglucuronides by the enzyme UDP-glucuronyltransferase (Mauro *et al.*, 2006) . The serum bilirubin levels more than 17ìmol/L suggest liver diseases and above 24ìmol/L indicate abnormal laboratory liver tests (Wong *et al.,* 2004). Jaundice occurs when bilirubin becomes visible within the sclera, skin, and mucous membranes at a blood concentration of around 40 ìmol/L (Beckingham and Ryder., 2001).

The liver produces urea in the urea cycle as a waste product of the digestion of protein. Normal human adult blood should contain between 6 to 20 mg of urea nitrogen per 100 ml (6–20 mg/dL) of blood. Blood urea nitrogen (BUN) is used as an indicatior for

Table 2 : Effect of administration of PKR in diet on BUN , Blood glucose, Total protein, bilirubin and cretinine in plasma at 7th and 14th day against PCM induced hepatotoxicity in WLH chicks (n=6).

Groups	Treatments	BUN (mg/dl)		Blood glucose (mg/100ml)		Total protein (g/dl)		Bilirubin(mg/dl)		Creatinine(mg/dl)	
		7th day	14th day	7th day	14th day	7th day	14th day	7th day	14th day	7th day	14th day
I	Control	31.3+ 0.65	32.6+ 0.70	137.1+ 0.66	141.7+ 0.77	4.49+ 0.16	4.81+ 0.05	0.38+ 0.08	0.40+ 0.10	0.87+ 0.089	0.88+ 0.22
II	PCM(50mg/Kg.i/m)	$45.3+ 0.43^{a}$	$40.2+ 0.72^{Aa}$	$109.7+ 0.48^{a}$	$124.2+ 0.78^{aA}$	$3.27+ 0.23^{a}$	$4.20+ 0.23^{aA}$	$0.88+ 0.14^{a}$	$0.68+ 0.04^{aA}$	$1.24+ 0.11^{a}$	$1.20+ 0.16^{aA}$
III	PCM(50mg/Kg.i/m) +Silymarin(100mg/Kg) p.o	$34.4+ 0.42^{b}$	$33.9+ 0.61^{b}$	$137.7+ 0.59^{b}$	$138.4+ 1.21^{b}$	$4.32+ 0.05^{b}$	$4.79+ 0.21^{b}$	$0.47+ 0.10^{b}$	$0.49+ 0.16^{b}$	$0.91+ 0.16^{b}$	$0.92+ 0.12^{b}$
IV	PCM(50mg/Kg.i/m) +PKR(200ppm)in feed	$42.6+ 0.59^{ac}$	$38.6+ 0.54^{acA}$	$112.6+ 0.65^{ac}$	$131.7+0.60^{abcA}$	$3.69+ 0.24^{bc}$	$4.62+0.21^{acA}$	$0.80+ 0.12^{ac}$	$0.57+ 0.08^{abA}$	$1.17+ 0.22^{ac}$	$1.02+ 0.08^{abcA}$
V	PCM(50mg/Kg.i/m)+ KR(400ppm)in feed	$37.7+ 0.63^{bd}$	$35.1+ 0.68^{ab}$	$131.1+ 0.60^{abd}$	$134.6+ 0.60^{ab}$	$4.17+ 0.15^{abd}$	$4.77+ 0.19^{bdA}$	$0.54+ 0.14^{abd}$	$0.49+ 0.11^{aA}$	$0.98+ 0.22^{bd}$	$0.97+ 0.09^{ab}$

Note:All values represent Mean ± SEM (n=6). PCM administered for 1st to 7th day whereas silymarin and PKR for 1st to 14th day of the study.

Table 3
Effect of administration of PKR in diet on total erythrocytes count (TEC),TLC,PCV% and Hb% in blood at 7th and 14th day against PCM induced hepatotoxicity in WLH chicks (n=6).

Groups	Treatments	TEC(/ul)		TLC(10^3/ul)		PCV%		Hb%	
		7th day	14th day	7th day	14th day	7th day	14th day	7th day	14th day
I	Control	2.8×10^6+ 118300	2.9×10^6+124900	33+0.92	34+ 0.33	33+ 0.96	33+ 1.15	9.5 + 0.29	9.6+0.27
II	PCM(50mg/Kg,i/m)	2.3×10^6+ 106500^{a}	2.5×10^6+133300^{aA}	27+0.49^{a}	29+ 0.45^{Aa}	24+ 1.2^{a}	26+ 1.7^{a}	7.6+ 0.23^{a}	7.8+0.23^{a}
III	PCM(50mg/Kg,i/m)+ Silymarin(100mg/Kg)p.o	2.7×10^6+107800^{b}	2.8×10^6+424900^{b}	32+1.12^{b}	33+1.11^{b}	34+ 0.9^{b}	34+ 0.5^{b}	9.1+0.24^{b}	9.4+0.16^{bA}
IV	PCM(50mg/Kg,i/m)+ PKR(200ppm)in feedc	2.5×10^6+133300^{a}	2.6×10^6+414300^{a}	30+1.23^{b}	30+0.69^{b}	31+ 0.9^{b}	33+ 1.3^{b}	8.2+0.21^{abc}	8.5+0.20^{bcA}
V	PCM(50mg/Kg,i/m)+ PKR(400ppm)in feed	2.8×10^6+118300^{bd}	2.8×10^6+418500^{b}	32+0.72^{bcd}	32+0.91^{bd}	33+ 1.15^{b}	33+ 1.3^{b}	8.7+ 0.24^{bcd}	9.1+0.17^{bcdA}

All values represent Mean ± SEM (n=6). PCM administered for 1st to 7th day whereas silymarin and PKR for 1st to 14th day of the study .

kidney function. Normal ranges 1.8-7.1 mmol/L. The main cause of an increase in BUN includes kidney disorders, high protein diet, decrease in glomerular filtration rate (GFR) and blood volume (hypovolemia), congestive heart failure, gastrointestinal hemorrhage, fever and increased catabolism. Yadavand Khandelwal *(*2006) reported the similar effect of *Picrorhiza kurroa* extract @12 mg/Kg p.o for 4 weeks in cadmium intoxicated rats on BUN, creatinine. Our findings of *Picrorhiza kurroa ameliorative effect* on elevated bilirubin values are in agreement with the study carried by Chander *et al (*1990).They reported that administration of picroliv, a standardized fraction of alcoholic extent of *Picrorhiza kurroa* (Scrophulariaceae) (3-12 mg/kg/dayfor two weeks) simultaneously with *P. berghei* infection showed significant decrease in bilirubin concentration in *Mastomys natalensis*.

After treatment with 50mg/Kg of PCM for 7 days, a significant($P<0.05$) decrease in Hb, PCV, TEC, TLC ,total proteins and blood glucose values occurred in group II as compared to control as given in Table 3 indicating haemotoxic and hepatotoxic effect of paracetamol. Hb, PCV, TEC, TLC ,total proteins and blood glucose values of group III were closer to control that showed that silymarin was efficient in recuperating the toxic effect of paracetamol . Hb, PCV, TEC, TLC, total proteins and blood glucose values of group V was significantly($P<0.05$) more as compared to group IV which indicated that PKR produced dose dependent ameliorating efficiency after 7 days treatment. Data obtained after 14th day of treatment reveled that Hb, PCV, TEC, TLC ,total proteins and blood glucose values of group III and group V came close to control group showing the ameliorative efficiency of silymarin and PKR at 400 ppm whereas Hb, PCV, TEC, TLC ,total proteins and blood glucose values of PCM treated group II also increased as compared to at 7th day which may be due to recuperation of haematic and hepatic tissue. Decrease in Hb, PCV, TEC, TLC, total proteins and blood glucose values occurred in group II as compared to control indicated that paracetamol is both haemotoxic as well as hepatotoxic whereas elevation in Hb, PCV, TEC, TLC, total proteins and blood glucose values in group IV and group V amelioration against the toxicity induced by paracetamol. Our findings were in agreements with other reports on ameliorative potential of *Picrorhiza kurroa against induced hematinic and hepatorenal dysfunction.* Husain *et al. (*2009) reported that 100-200mg/kg of a water extract of *Picrorhiza kurroa* over the course of 14 days in diabetic rats (streptozotocin induced) was able to improve blood glucose and insulin levels in a dose-dependent manner. Turaskar *et al.* (2013) administered *Picrorhiza kurroa* extract at (100 mg/kg/day and 200 mg/kg/day up to 4 weeks) to the rats intoxicated with phenylhydrazine. They were reported to have significant increase in values of Hb, PCV, TEC and TLC. It was concluded from this study that paracetamol ((50mg/kg,i/m; daily for 7 day) altered hematological and renal functions were ameliorated by oral administration of PKR (200 and 400ppm in feed daily for 14 day in WLH chicks.

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Research Article

PREPARATION AND STANDARDIZATION OF ETHANOLIC EXTRACT OF STEM OF *ENTADA PURSAETHA* PLANT BY HPTLC

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ABSTRACT

Entada pursaetha is a plant possessing excellent documented medicinal properties in various diseases including hepatotoxicity, osteoarthritis, colitis, gastric ulcers etc. In this study, 85% ethanolic extract of stem of *Entada pursaetha* plant was prepared with a yield of 8.4%. This extract was standardized by HPTLC in the solvent system of toluene: ethyl acetate (1:1). The finger print profile of PSE showed 13 peaks at Rf 0.03, 0.15, 0.18, 0.23, 0.27, 0.32, 0.37, 0.50, 0.58, 0.66, 0.71, 0.89 and 0.96. Start position, start height, maximum position and maximum height, end position, end height and area under each curve were documented from the finger print profile of the ethanolic extract. TLC photos of extract developed in solvent system under UV 254 nm, UV 366 nm and after derivatisation were also documented. This standardization and profiling will provide easy and unique identification to the extract of this plant and will help researchers in correctly identifying this plant of immense pharmacological importance.

Key words: *Entada pursaetha*, ethanolic extract, standardization, HPTLC.

INTRODUCTION

Entada pursaetha DC (Mimosae) is an endangered woody liana of family- Fabaceae (Pea) which is most commonly found in forests at low and medium altitudes. In India, it is sparsely distributed in the subtropical evergreen forests of Odisha, Karnataka, Tamil Nadu, Kerala. It has various regional names like Bidhanta (Sanskrit), Elephant Creeper (English) and Ganape Kayi (Kannada). It has various synonyms like *E. rheedii*, *E. phaseoloides*, *E. scandens* (Gamble, 1928).

Entada pursaetha (Elephant creeper) extract was found to possess significant biological activities and pharmacological effects, including antioxidant, anti-inflammatory and modulation of immune system (Chopra *et al.,* 1958). Previous research work, has demonstrated the protective effect of alcoholic extract of *Entada pursaetha* against CCl4-induced hepatotoxicity in rats (Gupta *et al.*, 2014) and against DSS-induced colitis in mice (Gupta *et al.*, 2015). Similarly, *Entada pusaetha* has shown mitigating effect in monosodium iodoacetate-induced osteoarthritis (Rashmi *et al.*, 2015). Entadamide A and entadamide B were isolated from *E. phaseoloides* and it was also found that these compounds inhibits 5-lipooxigenase and hence have anti-inflammatory activity (Ikegami *et al.*, 1989).In view of its clinical values, statndardization of HPTLC method for preparation and analysis was done in this study.

MATERIALS AND METHODS

Drugs and chemicals

Silica gel 60 F 254 was purchased from Merck & Co., Kenilworth, New Jersey, (USA). Toluene, ethyl acetate, ethanol, sulphuric acid, vanillin and all other reagents used in the study were of analytical grade and procured form Sisco Research Laboratories, Mumbai, India and Sigma-Aldrich, St. Louis (USA).

Collection of plant materials

The stems of *E.pursaetha* were obtained from the jungles of Bhawanipatna, District- Kalahandi, Odisha. The specimen was botanically identified by Dr. B.N.Pandey, Department of Botany, Bareilly College, Bareilly (India). A voucher specimen ID/EP-2010 is maintained the Division of Pharmacology and Toxicology, Indian Veterinary Research Institute, Bareilly (India) for ready reference.

Preparation of alcoholic extract of *Entada pursaetha* (PSE)

The stems collected from the fully mature plant were shade dried, powdered and then extracted with 85% ethanol under reflux. The ethanolic extract of stem was concentrated to a semi-solid mass under reduced pressure and made free from any solvent.

Standardization of PSE

Preparation of sample solution for HPTLC

1.013 g of PSE was treated with 15 ml of distilled ethanol and kept overnight. Then the sample was boiled for 10 minutes and filtered. The filtrate was made in to 15 ml.

HPTLC methodology

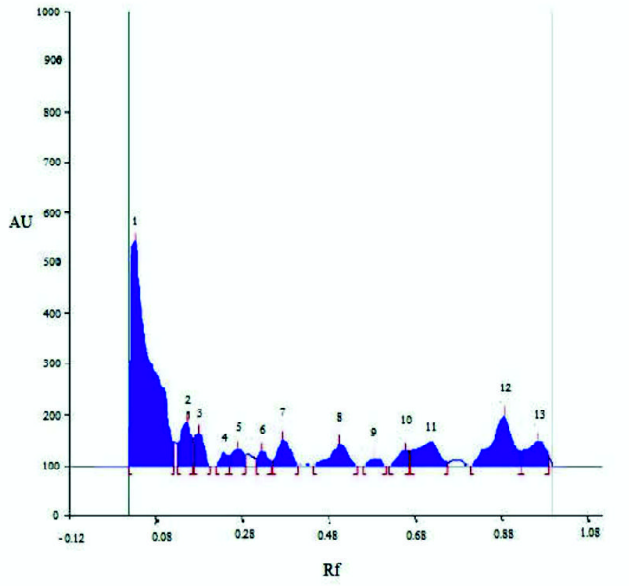
5, 10 and 15 µl of solution of PSE were applied separately on two different aluminium plates precoated with silica gel 60 F 254 (Merck) of 0.2 mm thickness using Linomat IV (CAMAG, Muttenz, Switzerland) applicator. One plate was developed using the solvent system of toluene : ethyl acetate (5:1.5, v/v) and other with toluene : ethyl acetate (1:1, v/v) up to the height of

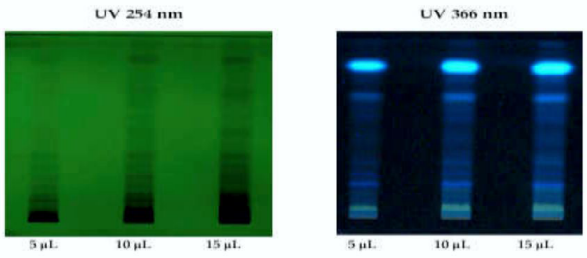
8 cm from the point of application of sample solution. Then the plates were scanned using the scanner 030618 equipped with win CATS version 1.4.4.6337 software. UV 254 nm was selected as scanning wavelength and deuterium lamp was used.

Derivatisation and photo documentation

After scanning, the plates were observed under UV 254 nm and 366 nm in the CAMAG Visualizer_1510111 and their images were documented. Then the plates were dipped in vanillin-sulphuric acid reagent (1g vanillin dissolved in 95:5 mixture of ethanol and sulphuric acid) and heated at 105°C in air circulated oven till the development of coloured spots. Then the plate was photo documented in the visible light.

Figure 1: The finger print profile of ethanolic extract of stem of *Entada pursaetha* developed in solvent system of toluene: ethyl acetate (1:1)





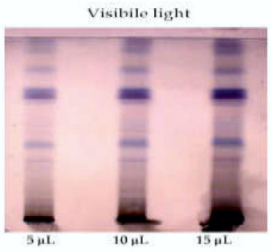


Fig.2 : TLC photo of PSE developed in solevent system of toluene: ethyl acetate (1:1) under UV 254 nm, UV 366 nm and after derivatization

RESULTS

Result of standardization of PSE by HPTLC

The finger print profile of PSE developed in the solvent system of toluene : ethyl acetate (1:1) showed 13 peaks at Rf 0.03, 0.15, 0.18, 0.23, 0.27, 0.32, 0.37, 0.50, 0.58, 0.66, 0.71, 0.89 and 0.96. Peak 1 at Rf 0.03 has the highest percent area (i.e. 52.79%) under it followed by peak 12 at Rf 0.89 (i.e. 11.93%). Under UV wavelength of 254 nm peak at Rf 0.12 exhibited dark green colour whereas peaks at Rf 0.37 and 0.89 exhibited green colour. Under UV wavelength of 366 nm peaks at Rf 0.07, 0.19, 0.26, 0.32, 0.68 and 0.86 exhibited blue colour. After derivatisation, peak at Rf 0.33 exhibited Pale violet colour whereas peaks at Rf 0.42, 0.68, 0.72, 0.83 and 0.93 exhibited violet colour.

The finger print profile of PSE developed in the solvent system of toluene: ethyl acetate (1:1) is illustrated in detail in Fig. 1 and values are given in Table 1. The TLC photos of PSE under UV 254 nm, UV 366 mn and after derivatization are shown in Fig. 2.

Table 1: The finger print profile of ethanolic extract of stem of *Entada pursaetha* plant developed in solvent system of toluene: ethyl acetate (1: 1)

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	0.01 Rf	0.0 AU	0.03 Rf	443.5 AU	44.58 %	0.12 Rf	43.3 AU	19002.9 AU	52.79 %
2	0.13 Rf	43.0 AU	0.15 Rf	85.7 AU	8.62 %	0.16 Rf	52.9 AU	2043.6 AU	5.68 %
3	0.16 Rf	53.1 AU	0.18 Rf	63.2 AU	6.36 %	0.20 Rf	0.1 AU	1224.4 AU	3.40 %
4	0.22 Rf	0.1 AU	0.23 Rf	24.8 AU	2.50 %	0.25 Rf	18.0 AU	392.0 AU	1.09 %
5	0.25 Rf	18.1 AU	0.27 Rf	30.7 AU	3.08 %	0.29 Rf	19.3 AU	792.3 AU	2.20 %
6	0.31 Rf	14.4 AU	0.32 Rf	25.0 AU	2.52 %	0.34 Rf	7.7 AU	548.5 AU	1.52 %
7	0.34 Rf	7.8 AU	0.37 Rf	49.7 AU	5.00 %	0.41 Rf	1.9 AU	1341.1 AU	3.73 %
8	0.44 Rf	0.9 AU	0.50 Rf	41.2 AU	4.14 %	0.54 Rf	0.3 AU	1440.6 AU	4.00 %
9	0.56 Rf	0.3 AU	0.58 Rf	13.7 AU	1.38 %	0.61 Rf	0.1 AU	338.3 AU	0.94 %
10	0.62 Rf	0.6 AU	0.66 Rf	26.6 AU	2.68 %	0.66 Rf	26.3 AU	602.6 AU	1.67 %
11	0.67 Rf	26.1 AU	0.71 Rf	45.4 AU	4.56 %	0.75 Rf	6.1 AU	2115.6 AU	5.88 %
12	0.81 Rf	0.2 AU	0.89 Rf	97.5 AU	9.80 %	0.92 Rf	24.5 AU	4293.1 AU	11.93 %
13	0.93 Rf	24.6 AU	0.96 Rf	47.5 AU	4.78 %	0.99 Rf	18.1 AU	1860.6 AU	5.17 %

Table 2 : Colour and Rf values of TLC of PSE developed in solevent system of toluene: ethyl acetate (1:1) under UV 254 nm, UV 366 nm and after derivatization.

S. No.	UV 254 nm		UV 366 nm		With spray reagent	
	Colour	Rf	Colour	Rf	Colour	Rf
1	Dark green	0.12	Blue	0.07	Pale violet	0.33
2	Green	0.37	Blue	0.19	Violet	0.42
3	-	-	Blue	0.26	Violet	0.68
4	-	-	Blue	0.32	Violet	0.72
5	Green	0.89	Blue	0.68	Violet	0.83
6	-	-	Blue	0.86	Violet	0.93

Their colour of the spot and Rf values are detailed in Table 2.

DISCUSSION

Entada pursaetha has saponin, fixed oil, 18%; traces of an alkaloid, sapogenin and oleanolic acid. The occurrence of oealeanic acid, echinocystic acid, and entagenic acid glycosides has been reported (Okada *et al.,* 1988). Five new triterpenoid saponins, pursaethosides A-E (1-5), were isolated from the n-BuOH extract of the seed kernels of *Entada pursaetha* along with the known phaseoloidin. On phytochemical screening, PSE revealed the presence of triterpenes, flavanoids, tannins, saponins as major compounds.

GC-MS analysis of ethanolic extract of this plant has shown presence of 1,2, Benzenedicarboxylic acid, diisooctyl ester (69.52%), Benzeneacetic acid, 2,5-dihydroxy- (syn: Homogentisic acid) (8.12%), n-Hexadecanoic acid (4.48%) Oleic acid (4.39%) Azulene, 1,4-dimethyl 1-7- (1-methylethyl)- (3.86%) and undecanoic acid (2.46%).(Kalpana Devi *et al,* 2012).

Active constituents present in seed of *E. pursaetha* are alfa-amyrin, oleanolic acid, entagenic acid, gallic acid, quercetin, beta-setosterol, Memercaptane (Samant and Pant, 2006).

Phytochemical screening of *Entada pursaetha* revealed the presence of triterpenes, saponins, tannins, flavanoids alkaloids and oleogenic acid. Ethanol (85%) is one of the most preferred solvents for the extraction of crude plant material, as almost all the constituents in the plant material are liable to get dissolved in 85% ethanol. The yield of PSE was 8.4% of the whole stem. The 13 peaks of finger print profile of PSE shows that, thirteen different types of chemical constituents are present in this plant extract.

Findings of this research work will provide unique finger print profile of this plant. This will help future researchers to identify the plant at chromatographic level as the genus “Entada”has various species namely *E. monostachya, E. Africana, E. abyssinica, E. chrysostachys, E. gigos, E. polystachya* etc. More or less all these species have similar morphological features and for identification purpose, can leave the researcher perplexed. This simple and cost effective technique can help researchers in identifying the plant based on the unique finger print profiling and Rf values developed from HPTLC.

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Short Communication

PHARMACOKINETIC AND BIOEQUIVALENCE STUDY OF BUPARVAQUONE IN SHEEP

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ABSTRACT

The present study was undertaken to evaluate the pharmacokinetics and bioequivalence of two formulations buparvaquone (Theilex® and Butalex®) @ 2.5 mg/kg body weight in sheep. The plasma concentration of buparvaquone was determined by High Performance Liquid Chromatography (HPLC). The decay in plasma concentration of drug was biexponential in sheep. The C_{max} value of 0.08 µg/ml was obtained at T_{max} of 1.52 h in sheep following i.m administration of Theilex®. C_{max} value of 0.12 µg/ml was obtained at T_{max} of 1.78 h in sheep, following i.m administration of Butalex®. The elimination half life ($\beta t_{1/2}$), volume of distribution (V1_F) and AUC were calculated as 36.25 h, 13.70 L/kg, and 2.24 µg.h/ml in sheep following i.m. administration of Theilex®.The elimination half life ($\beta t_{1/2}$), volume of distribution (V1_F) and AUC were calculated as 47.33h, 9.783 L/kg· 3.77µg.h/ml in sheep following i.m administration of Butalex®. Bioequivalence of Theilex® with respect to Butalex® in sheep was 65.45% following i.m administration.

Key words: Buparvaquone, sheep, pharmacokinetic and bioequivalence study.

Buparvaquone is used for therapy and prophylaxis for theileriasis. Its activity has been tested against *Theileria annulata*, *Theileria parva* and *Theileria sergenti* in lab and field trials. Buparvaquone is reported to have activity against cutaneous and visceral leishmaniasis (Croft *et al.,* 1992). 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).

The study was conducted in four adult, indigenous, female Muzzaffarnagari sheep (1.5-2.5 yrs in age, weighing 23±2 kg).The animals were reared under standard managemental and husbandry conditions as per the guidelines of CPCSEA, they were maintained on standard ration and provided water *ad libitum*. In this study, Theilex® was taken as test formulation and Butalex® as reference formulation and were administered as a single dose (2.5 mg/kg body weight) intramuscularly (i.m.) in gluteal muscles. An intervening wash out period of one month was given before administration of other formulation. Blood samples were collected from jugular vein in heparinized microcentrifuge tube at time intervals of 0, 0.25, 0.5, 1, 2, 4, 8, 12, 24, 48, 72 h. Drug extraction 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 supernatant was buffered with 0.5 ml of 0.05 M KH_2PO_4 and extraction was done using solid phase extraction (SPE) procedure. Elute obtained from SPE was evaporated, reconstituted in mobile phase and then filtered through millipore 0.22 µm membrane filter. An aliquot of 20 µL of the sample obtained was injected into HPLC system for analysis. 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 0.012 µg/ml, respectively. The pharmacokinetic analysis of the plasma concentration obtained following i.m. administration in this study was done by a pharmacokinetic software, Chromatopak. Bioequivalence of both formulations was calculated as area under curve (AUC) i.m. Theilex® / area under curve (AUC) i.m. Butalex®.

A two-compartment model adequately described the plasma concentration-time profile of Theilex® and Butalex® in sheep following single dose (2.5 mg/kg body weight) i.m. administration in this study. Peak plasma concentration was observed at 1 and 2 h post i.m administration of Theilex® and Butalex®, respectively (Fig.1).

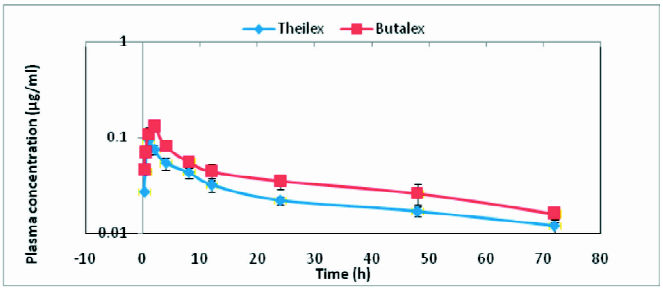

Fig 1:
Semilogarithmic plot of Theilex® and Butalex® concentrations in plasma versus time following i.m. administration (2.5mg/kg body weight) in sheep (n=4).

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

		Mean± S.E.	
Parameters	**Units**	**Theilex®**	**Butalex®**
V1_F	L/kg	13.70±1.11	9.78±1.30
V2_F	L/kg	41.36±7.91	32.90±5.12
$\alpha t_{½}$	h	0.86±0.08	1.21±0.17
$\beta t_{½}$	h	36.25±3.77	47.34±5.52
C_{max}	µg/ml	0.08±0.01	0.12±0.01
T_{max}	h	1.52±0.12	1.78±0.12
AUC	µg. h /ml	2.24±0.18	3.78±0.70

V1_F = Volume of distribution of central compartment; V2_F=Volume of distribution of peripheral compartment; $\alpha t_{½}$ = Distribution half-life; $\beta t_{½}$= Elimination half-life; C_{max}= Peak plasma concentration; T_{max}= maximum time required to attain peak plasma concentration; AUC= Area under curve.

Pharmacokinetic values describing the disposition kinetics of Theilex® and Butalex® following single dose (2.5mg/kg)i.m. administration in sheep are presented in Table1.The values of C_{max} were 0.08 and 0.12 µg/ml and T_{max} were 1.52 and 1.78 h following i.m administration of Theilex® and Butalex® respectively. The mean elimination half -lives were 36.25 and 47.34 h in sheep following i.m route of administration of drug while these findings vary from the half- life (26.44h) reported by Kinabo and Bogan (1988) following i.m administration in cattle calves and significantly different (9.13h) in rabbits (Venkatesh *et al.,* 2008) and (4.0h) in rats (Pinjari*et al*., 2008) following i.v administration. Volume of distribution was 13.70 and 9.783 L/kg in sheep following i.m administration of Theilex® and Butalex®, respectively. 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 (Venkatesh *et al.,* 2008) is equivalent to that determined in the present study. The area under the concentration time curve characterizes the relative availability of drug in the body (Dudley, 1991). The area under curve (AUC) was 2.24 and 3.78µg.h/ml 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 this study. Bioequivalence of Theilex® with respect to Butalex® in sheep was 65.45% following i.m. administration.

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Short communication

ANTI-NOCICEPTIVE ACTIVITY OF METHANOLIC EXTRACT OF *MURRAYA KOENIGII* L. LEAVES IN MALE WISTAR RATS

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ABSTRACT

In the present study anti-nociceptive activity of methanolic extract of Murraya koenigii L. (Curry patta) is evaluated in male wistar rats. Anti-nociceptive activity was evaluated by hot plate, tail immersion and tail clip tests. Methanolic extract of Murraya koenigii leaves (MEMKL) was administered @ 300mg/kg & 600mg/kg b wt orally in all these tests. MEMKL @ 300mg/kg and 600mg/kg b wt to all the pain induced models showed significant antinociceptive activity. However, increase was higher at high doese 600mg/kg but was lower than analgin.Thus the results indicate antinociceptive activity of MEMKL and support ethnomedical use of this plant in different painful conditions.

Key words: Hot plate test, Tail immersion test, Tail clip test

Murraya koenigii (Rutaceae), a medicinally important herb of Indian origin, has been used for centuries in the Ayurvedic system of medicine. Leaves, fruits, roots and bark of this plant are a rich source of carbazole alkaloids. These alkaloids have been reported for their various pharmacological activities such as antitumor, antiviral, anti-inflammatory, antidiarrhoeal, diuretic, stomachic, febrifuge and analgesic (Gupta *et al.,* 2011). In the present study anti-nociceptive activity of methanolic extract of *Murraya koenigii* leaves was evaluated in rats.

Plant material

Leaves of *Murraya Koenigii* were collected locally. The leaves were collected, shade dried and ground to obtain a fine powder. The powder was used for the preparation of lyophilized methanolic extract (MEMKL) and kept at 4^0C.

Experimental animals

A total of 24 male Albino Wistar rats were used. The rats were housed under the standard environmental conditions and fed with standard pellet diet and water *ad libitum.* The rats were divided into four groups with six rats in each group. The group I served as control and received 0.5 ml of normal saline orally. GroupII served as reference standard group to which analgin (Novalgin, Aventis pharma) was given @ 200mg/kg b wt intraperitoneally. The groups III and IV received MEMKL @ 300mg/kg and 600mg/kg body weight respectively orally. Analgesic activity was evaluated by hot plate test; tail immersion test and tail clip test (Ghosh, 2005).

In this test hot plate was maintained at 55±1^{0}C.The time taken to cause a discomfort reaction (licking paws or jumping) was recorded as response latency or reaction time. Before administration of the test compound or the standard, the normal reaction time was determined. The animals are submitted to the same testing procedure after 30, 60 and eventually 120 min after administration of the drug and test compound. For each individual animal the reaction time was recorded. A cut-off time of 30 seconds was followed to avoid any thermal injury to the paws.

In Tail immersion test, tail of rat upto5cm was dipped into hot water maintained at 55±1^{0}C . Time taken by rat to flip its tail clearly out of water was taken as reaction time. The reaction time in seconds before administration of the drug was recorded for all the rats from each group. Reaction time in seconds after administration of drugs was recorded at an interval of 30, 60 and 120min.

In tail clip test, clamp is applied at the distance of 1.5cm to the base of the tail and the time taken by the rat to remove the clip is recorded as the reaction time. The reaction time in seconds before administration of the drug was recorded for all the rats from each group. Reaction time in seconds after administration of drugs was recorded at an interval of 30, 60 and 120min.

Statistical analysis was carried out using ANOVA followed by Dunnet's test. A 'p' Value < 0.05 was considered to be significant and 'p' Value < 0.01 was considered to be highly significant.

The results of our findings are presented in Table1. In all the three models used for testing anti-nociceptive activity analgin significantly ($p<0.01$) increased the reaction time after 30min of administration except in tail clip test, where reaction time is increased at 60 min. MEMKL @300mg/kg body weight significantly ($p<0.01$) increased the reaction time in tail immersion test at 120min of administration. MEMKL @ 600mg/kg body weight significantly ($p<0.01$) increased the reaction time in all the three tests after 120 min of administration. Significant ($p<0.05$) increase in reaction time was observed by MEMKL @ 600mg/kg body weight at 60min in tail immersion and tail clip tests.

Table 1:
Anti-nociceptive activity of MEMKL by hot plate method, Tail immersion method and Tail clip method in rats.

Group	Dose (mg/kg)	Reaction time in seconds (Hot plate method)				Reaction time in seconds (Tail immersion method)				Reaction time in seconds (Tail clip method)			
		Pretreatment	30min	60min	120min	Pretreatment	30min	60min	120min	Pretreatment	30 min	60 min	120 min
Control	0.5ml (Normal saline)	5.92± 0.76	5.43± 0.75	5.36± 0.73	5.60± 0.33	6.11 ± 0.63	6.34 ± 0.64	6.15 ± 0.93	6.25± 0.89	6.24± 0.86	6.30 ± 0.74	6.10± 0.59	6.09 ± 0.95
Analgin	200	5.42± 0.87	9.17± 0.31**	14.37± 0.55 **	10.02± 0.53**	5.93 ± 0.56	11.18 ± 0.40**	13.70± 0.75**	15.13± 0.89**	8.10± 0.74	7.04 ± 0.65	10.25± 0.79**	16.35 ± 0.43**
MEMKL	300	5.09 ± 0.42	4.19± 0.46	5.03 ± 0.36	5.28± 0.43	5.8 ± 0.94	4.14 ± 0.76	7.03 ± 0.59	11.08± 0.65**	6.77± 0.51	6.70± 0.90	6.61± 0.40	8.05± 0.47
MEMKL	600	5.41 ± 0.48	6.02 ± 0.26	7.31± 0.63	8.13 ± 0.73**	5.00 ± 0.73	7.76 ± 0.82	9.40 ± 0.67*	14.67± 0.80**	6.30± 0.81	5.76 ± 0.31	8.98± 0.83*	12.98 ± 0.85 **

Data was analyzed by ANOVA Test followed by Dunnet's *post hoc* test. Each value is Mean ± SEM; n=6, *P<0.05, **P<0.01 when compared with pretreated group.

NSAIDs are the most commonly used anti-inflammatory, antipyretic, analgesic drugs.They act primarily on peripheral pain mechanisms but also in CNS to raise pain threshold. Most NSAIDs block prostaglandin synthesis by inhibiting COX-1 and COX-2 nonselectively, but now some selective COX-2 inhibitors have been developed. Of the common toxicities caused by NSAIDs due to inhibition of prostaglandin synthesis, gastric mucosal damage is most troublesome. (Tripathi, 2008). In the present study it was found that methanolic extracts of *Murraya koenigii* leaves @ 300 and 600mg/kg body weight to pain induced models resulted in significant increase in reaction time of rats. However, increase was higher at 600mg/kg but lower when compared with analgin. Our studies, are in agreement with the study, in which it was found that the methanolic extract of *Murraya koengii* leaves showed significant ($P < 0.001$) reduction in the carrageenan-induced paw edema and analgesic activity, evidenced by increase in the reaction time by Eddy's hot plate method and percentage increase in pain in formalin test. The methanolic extract showed anti-inflammatory and analgesic effect in dose dependent manner when compared with the control and standard drug, diclofenac sodium (10mg/kg, p.o) (Gupta *et al.,* 2010). In one of the study it was found that the petroleum ether extract of *Murraya koenigii* leaves @100 and 300mg/kg p.o has antinociceptive activity in mice during acute as well as chronic administration (Patil *et al.,* 2012). A significant increase in the reaction time indicated that the analgesic effect by *Murraya koenigii* leaves also elucidates the involvement of central mechanism in analgesic action. Analgesic effect mediated through central mechanism indicates the involvement of endogenous opioid peptides and biogenic amines like 5HT (Bensemana and Gascon, 1978). Hence it could be concluded that MEMKL has good efficacy as an analgesic. The potential of MEMKL as an additive analgesic to conventional drugs may further be explored.

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