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Review Article

TURMERIC: A REVIEW ON ITS PHARMACOLOGY

S.H. DAR[1], S.P. SINGH[2] , G.K. CHOUDHARY[4], SUDHIR KUMAR[2] AND DISHA PANT[3]

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ABSTRACT

Turmeric (*Curcuma longa)* is a rhizomatous herbaceous perennial plant of the Zingiberaceae family, and has been traditionally used in indigenous medicine and as a spice since antiquity. It has possesses antirheumatic, hepatoprotective, antidiabetic, neuroprotective, nephroprotective, hypoglycemic, and cardioprotective activities. The most important active principles of turmeric are a group of compounds called curcuminoids which include diferuloylmethane, demethoxycurcumin, and bisdemethoxycurcumin. It follows efficient first-pass metabolism and some degree of intestinal metabolism. Glucuronidation and sulfation of curcumin are major metabolic reaction for its poor systemic availability when administered via the oral route. The metabolites were characterized mainly as glucuronides of tetrahydrocurcumin and hexahydrocurcumin. Curcumin targets various protein kinases, antiapoptotic proteins, interleukins and other vital cellular pathways to produce their pharmacological actions. Curcumin is well-tolerated and has beneficial effects for dyspepsia, peptic ulcer, inflammatory bowel disease, rheumatoid arthritis, osteoarthritis, uveitis, orbital pseudotumor and pancreatic cancer. however the most common side effects are nausea and diarrhea.

Kew words: Pharmacology, therapeutic uses, *Curcuma longa*, curcumin, turmeric

INTRODUCTION

Turmeric has been traditionally used in indigenous medicine and as a spice since ancient times. Extracts isolated from the rhizome of turmeric have been utilized as anti-inflammatory agents in Ayurvedic and Traditional Chinese Medicine (TCM) for centuries. During the course of *in vivo* studies assessing turmeric's antiarthritic effects, curcuminoid-containing turmeric extracts also prevent osteoclastogenesis and peri-articular bone destruction in a model of rheumatoid arthritis. Curcumin is a yellow phenolic compound present naturally in various types of herbs, especially in *Curcuma longa* Linn (turmeric). Curcumin was first extracted in an impure form by Vogel and Pelletier (1815). It acts as a natural antioxidant, and has exhibited a number of pharmacological activities such as anti-microbial, anti-inflammatory, anti-Alzheimer, and anti-cancer, in preclinical as well as clinical studies. Moreover, it has been seen to possess antirheumatic, hepatoprotective, antidiabetic, neuroprotective, nephroprotective, hypoglycemic, and cardioprotectiveactivities. It also suppresses thrombosis, and provides protection against myocardial infarction (Sou*,* 2012). Curcumin has a molecular weight of 368.37 g/mol. This compound is an insoluble drug candidate which is conventionally limited to oral administration. A promising advantage of oral turmeric is that it displays minimal side effects in clinical applications as a drug, however, bioavailability of turmeric may be limited by its low bioavailability due to efficient first pass metabolism, poor gastrointestinal absorption, rapid elimination and poor aqueous solubility.

BOTANICAL CHARACTERISTICS

Turmeric (*Curcuma longa)* is a rhizomatous herbaceous perennial plant of the ginger family, Zingiberaceae. It is native to southwest India and grows in climatic conditions having temperatures between 20 and 30 °C (68 and 86 °F) with a considerable amount of annual rainfall. Plants are perennial which grow up to a height of 1 m with highly branched stems and yellow to orange colored cylindrical shape aromatic rhizomes. Leaves, divided into leaf sheath, petiole, and leaf blade, are usually 76 to 115 cm long. Terminally on the false stem is a 12- to 20-cm-long inflorescence stem containing many flowers. Plants are gathered annually for their rhizomes and propagated from some of those rhizomes in the following season. When not used fresh, the rhizomes are boiled for about 30–45 minutes and then dried in hot ovens, after which they are ground into a deep-orange-yellow powder which is commonly used as a spice in Indian subcontinents for dyeing and to impart color to mustard condiments.

PHYTOCONSTITUENTS

Turmeric was first extracted in an impure form by Vogel (Vogel *et al.,* 1815). The most important chemical components of turmeric are a group of compounds called curcuminoids, which include turmeric (diferuloylmethane), demethoxycurcumin, and bisdemethoxycurcumin.. There is variations in turmeric content in the different lines of the species *Curcuma longa* (1–3189 mg/100g). Chemically, turmeric is a diary heptanoid. Turmeric and the other two curcuminoids – desmethoxycurcumin and bis-desmethoxycurcumin – are natural phenols which are responsible for the yellow

colour of turmeric. Turmeric can exist in several tautomeric forms, including a 1,3-diketo form and two equivalent enol forms. The enol form is more energetically stable in the solid phase and in organic solvents, while in water the 1,3-diketo dominates (Manalova *et al.*, 2014; Priyadarshani, 2014).

There is a considerable amount of literature about the health benefits of curcuminoids, especially curcumin. They have been reported to have vast medicinal value and potential. Chemically, curcumin is (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione). Numerous reports suggest that this natural compound possesses various therapeutic benefit such as anti-tumour, anti-inflammatory and anti-oxidant activities. However, clinical reports have shown low bioavailability of conventional oral curcumin. Therefore, the development of an efficient drug delivery system for curcumin is of considerable interest. Indeed, recent studies have demonstrated that a curcumin delivery system based on nanoscience and nanotechnology increased the therapeutic potential of this compound. Specifically, several nano-sized carriers such as phospholipid vesicles (liposomes) (Karewicz *et al.,* 2011; Karewicz *et al.,* 2013), micelles (Song *et al.,* 2011), solid lipid nanoparticles, polymeric nanoparticles, emulsions, proteins (Esmaili *et al.,* 2011) and other molecular complexes have been developed for the efficient delivery of curcumin. Although curcumin itself is not a patentable compound, these formulations and the nanotechnology used to enhance the bioavailability of turmeric may lead to a patentable breakthrough for medical applications (Sou, 2012). It acts as a natural antioxidant (Lim *et al.*, 2001), and has exhibited a number of pharmacological activities such as anti-microbial, anti-inflammatory (Sandur *et al.,* 2007), anti-Alzheimer (Yang *et al.,* 2005; Hamaguchi *et al.,* 2010) and anti-cancer, in preclinical as well as clinical studies (Liu and Hong, 2006; Kim *et al.,* 2011). Moreover, it has been shown to possess antirheumatic, hepatoprotective, antidiabetic, neuroprotective, nephroprotective, hypoglycemic and cardioprotective activities (Aggarwal, 2010). It also suppresses thrombosis and provides protection against myocardial infarction (Naksuriya *et al.,* 2014).Many technologies have been developed and applied to develop efficient delivery system for turmeric including the recent works on the design and development of nano-sized delivery systems for curcumin including liposomes, polymeric nanoparticles and micelles, conjugates, peptide carriers, cyclodextrins, solid dispersions, lipid nanoparticles and emulsions (Naksuriya *et al.,* 2014).

Effect of turmeric on health are generally centered upon an orange-yellow colored, lipophilic polyphenol substance called “curcumin,” which is acquired from the rhizomes of the herb. Curcumin has recently been explored to have antioxidant, anti-inflammatory, anticancer effects which revealed its important role in prevention and treatment of various illnesses ranging notably from cancer to autoimmune, neurological, cardiovascular diseases and diabetese. Furthermore, it is aimed to increase the biological activity and physiological effects of the curcumin on the body by synthesizing curcumin analogues.

PHARMACOKINETICS

Curcuma spp. contains turmerin, essential oils and curcuminoids including curcumin. Curcumin is regarded as the most biologically active constituent of the spice turmeric and it comprises 2–8% of most turmeric preparations. Preclinical data from animal models and phase I clinical studies performed with human volunteers and patients with cancer have demonstrated low systemic bioavailability following oral dosing. Efficient first-pass metabolism and some degree of intestinal metabolism, particularly glucuronidation and sulfation of curcumin, might explain its poor systemic availability when administered via the oral route. A daily oral dose of 3.6 g of curcumin is compatible with detectable levels of the parent compound in colorectal tissue from patients with cancer which might be sufficient to exert pharmacological activity. There appears to be negligible distribution of the parent drug to hepatic or other tissues beyond the gastrointestinal tract. Therefore, a promising advantage of oral curcuminincludes its minimal side effects in clinical applications as a drug. Low bioavailability of curcumin is due to low aqueous solubility, low absorption and rapid rate of elimination (Shishodia *et al.*, 2005; Goel *et al.,* 2008).

Curcumin undergoes a rapid and efficient metabolism that severely curtails the availability of parent compound in the biophase. Absorption of oral curcumin was 60% and urinary agent-derived species were characterized as glucuronide and sulfate conjugates. When curcumin bioavailability was investigated using a 3H-radiolabeled agent, the majority of the oral dose was excreted in the feces and one-third was excreted unchanged (Ravindranath and Chandrasekhara, 1982). Intravenous and intraperitoneal administration of curcumin in rats resulted in large quantities of curcumin and metabolites in bile (Ravindranath and Chandrasekhara, 1981).

High pressure liquid chromatography (HPLC) analysis of plasma from rats given oral curcumin demonstrated substantial levels of Curcumin glucuronide and curcuminsulfate, small quantities of hexahydrocurcumin, hexahydrocurcuminol and hexahydrocurcumin glucuronide with negligible amounts of curcumin (Ireson *et al.,* 2001). Efficient first-pass metabolism and some degree of intestinal metabolism, particularly glucuronidation and sulfation of curcumin, might explain its poor systemic

availability when administered via the oral route (Sharma *et al.,* 2007).

Enhanced bioavailability of curcumin in the near future is likely to bring the promising natural product to the forefront of therapeutic agents for treatment of human disease. To improve the bioavailability of curcumin, numerous approaches have been undertaken. These approaches involve, first, the use of adjuvant like piperine that interferes with glucuronidation; second, the use of liposomal curcumin; third, thecurcumin nanoparticles; fourth, the use of curcumin phospholipid complex; and fifth, the use of structural analogues of curcumin (e.g., EF-24) (Anand *et al.,* 2007).

Age did not affect ATP concentrations and MMP in dissociated brain cells. After damaging cells with nitrosative stress, dissociated brain cells from old mice had a higher MMP than cells from young animals and were therefore more resistant. Furthermore, this effect was enhanced by Curcumin (Kocher *et al.*, 2015).

Murray and Casero (2017) surveyed that in human cancer cell lines, curcumin has been shown to reduced ornithine decarboxylase (ODC) activity, a rate-limiting enzyme in polyamine biosynthesis that is often upregulated in cancer and other swiftly proliferating tissues. Numerous studies have proven that pretreatment with curcumin can inhibit carcinogen-induced ODC activity and tumor occurrence in rodent tumerogenesis models targeting various organs.

Increased bioavailability of curcumin was verified when the same was orally administered mixed with piperine. Intestinal absorption of curcumin was relatively higher when administered concomitantly with piperine, and it stayed for significantly longer duration in the body tissues (Toden and Goel, 2017).

Ingesting curcumin by itself does not lead to the associated health benefits due to its poor bioavailability, which appears primarily due to poor absorption, rapid metabolism, and rapid elimination. There are several components that can increase bioavailability. For example, piperine, the major active component of black pepper when combined in a complex with curcumin, has been shown to increase bioavailability by 2000%. Curcumin combined with enhancing agents provides multiple health benefits (Hewlings and Kalman, 2017).

PHARMACODYNAMICS

Turmeric possesses wide-ranging anti-inflammatory and anticancer properties. Many of these biological activities can be attributed to its potent antioxidant capacity at neutral and acidic pH such as inhibition of cell signalling pathways at multiple levels, diverse effects on cellular enzymes, and effects on cell adhesion and angiogenesis. In particular, ability of turmeric to alter gene transcription and induce apoptosis in preclinical models advocates its potential utility in cancer chemoprevention and chemotherapy. Curcumin produces its pharmacological action by affecting various pathways(Sharma, Steward and Gesher, 2007):

1. Inhibition and /or downregulation of various protein kinases such as MAPK, c-Jun NH2-terminal kinases (JNK), PKA, PKC, SRC tyrosine kinase, IKBα kinase, growth factor receptor protein tyrosine kinases**.**
2. Targeting antiapoptic proteins sich as Induction of cytochrome-c release, activation of caspase-3 and caspase-9, downregulation of Bcl-2 and BclX2
3. **Downregulation of** proinflammatory proteins such as COX-2, 5-LOX, and iNOS
4. Down regulation of cytokines and growth factors like TNF, IL-6, IL-8, IL-12, and fibroblast growth factor-2**.**
5. Suppression of transcription factors viz.NF-KB, STAT3, Egr-1, AP-1, and peroxisome proliferators-activated factor (PPAR-β), activation of β-catenin
6. **By inhibition of** CYP1A1**, upregulation of oxidant enzyme systems**

PHARMACOLOGICAL EFFECTS

Anticancer activity

Oral administration of turmeric prevents cancer in the colon, skin, stomach, liver, lung, duodenum, soft palate, and breasts of rodents (Volate *et al.,* 2005).

Topical application of curcumin has been shown to inhibit chemical carcinogenesis of the skin (Li *et al.,* 2002; Conney, 2003). Studies so far have also demonstrated no attenuation of chemically induced prostate carcinogenesisin rats (Imaida *et al.,* 2001). Numerous reports, however, suggest that curcumin augmented the cytotoxicity of anticancer drugs such as paclitaxel in cells *in vitro* observations, however, data is lacking at present to confirm anticarcinogenic potential of turmeric in vivo models (Aggarwal *et al.,* 2005; Bava *et al.,* 2005). Inhibition of iNOS activity by curcumin at low concentration might represent a mechanism of intervention during carcinogenesis, the activity of curcumin and have might considerable implications in cancer chemoprevention (Chan *et al.,* 1998).

Anti-inflammatory effects

Curcumin caused a significant anti-inflammatory with inflammatory bowel disease (Holt *et al.,* 2005). Wojcik *et al.* (2018) reviewed curcumin (CUR; diferuloylmethane), a well-known polyphenol derived from the rhizomes of turmeric *Curcuma longa*, has attracted a great deal of attention as a natural compound with beneficial antidiabetic and anticancer properties due to its antioxidative and anti-inflammatory actions. The overproduction of nitric oxide (NO) was potently inhibited following treatment with curcumin and its three metabolites. In addition, curcumin and tetrahydrocurcumin significantly inhibited the release of prominent cytokines, including tumor necrosis factor α (TNF α) and interleukin 6 (IL 6) (Zhao *et al,* 2015).

Given below is a list of a number of or which the role of turmeric has been investigated for the prevention and treatment of disorders of various systems.:

Digestive system

Turmeric has been shown to fortify the gastro-intestinal system, serving the stomach with digestive acids by enhancing the gastric wall mucus as well as increasing absorption within the intestine and advancement intestinal flora. It prevents pancreatic cancer, oral cancer, colorectal cancer, nematode infestation, hepatic disorders, gastric ulcer, pancreatitis, ulcerative colitis, inflammatory bowel disease**.**

Cardiovascular system

Turmeric defends from a stroke or rather its main compound curcumin improves heart health by reducing the amount of cholesterol and plaque that has built up in your arteries as well as decrease the amount of inflammation around heart and arteries (Aggarwal *et al.*, 2009).

Reproductive system

Turmeric might reduce the risk of cancer and other malignant diseases in the reproductive system. *C. longa* produces protective effect on the reproductive organs by anti-inflammatory, anti-apoptotic and antioxidant activities in normal cells and also showed pro-apoptotic effects in the malignant cells. Therefore, different effects of *C. longa* are dependent on the doses and the type of cells used in various models studied (Yoshida *et al.*, 2009).

Neuro-endocrine system

Turmeric has major effect on thyroid gland as it reduces the inflammation by inhibiting the production of pro-inflammatory chemicals, antioxidant action and have endocrine balancing properties. It regulates the immune system and also helps in increasing the testosterone level. It prevents certain diseases and disorders like diabetes mellitus, depression, fatigue, epilepsy, Alzheimer's disease, Parkinson's disease (Xia *et al.*, (2007).

Skin and associated tissues

Turmeric helps in reducing acne and any resulting scars. The antiflammatory qualities can target pores and calm the skin. It prevents skin cancer, wound healing, fungal infections and bacterial infections (Sumiyoshi and Kimora, 2009). A specific bone marrow-derived (BMSC) sheet that was induced by the traditional Chinese medicine curcumin (CS-C) and wound repair was enhanced due to its immunomodulatory effects (Yang *et al.,* 2018).

Excretory system

Helps in regulating the blood pressure and has antiflammatory effect on kidneys thus helps in protecting kidney failure also prevents kidney cancer (**Deshpande *et al.*, 2003).**

Immune system

Turmeric has anti-carcinogenic properties, and all of these benefits are entirely because of an active compound curcumin. It helps in boosting immune system by helping body defence system. It also enhances the function of various T cells and cytokines (Jagetia *et al.*, 2007).

Skeletal system

On the basis of traditional usage, dietary supplements containing turmeric rhizome and turmeric extracts are also being used in the western human world for the treatment and prevention of arthritis including rheumatoid arthritis (Funk *et al.,* 2006; Funk *et al.*, 2009)). Curcumin reduced the osteoclast number and increased the osteoblast count suggesting the potential effect of curcumin for prevention of osteoporosis (Gu *et al.,*2012; Hussain, 2018).

Heo *et al.* (2014) conducted a study to with the aim to develop a therapeutic agent for osteoporosis based on the utilization of gold nanoparticles (GNPs) and confirm their effect both in vitro and *in vivo*. Li *et al.* (2015) investigated bone microarchitecture of the proximal tibia in glucocorticoid-induced osteoporosis (GIOP) in mice, and the underlying molecular mechanisms of curcumin in dexamethasone induced osteoporosis. Importantly, curcumin reversed induced trabecular deleterious effects and stimulated bone remodelling.

Chen *et al. (*2016) reported the the protective effects of curcumin on the bones of rats with dexamethasone induced osteoporosis. Wang *et al.* (2016) demonstrated that curcumin treatment enhanced the osteoblast differentiation of human adipose-derived mesenchymal stem cells. Rohanizadeh *et al.* (2016) showed that curcumin has raised a considerable interest in medicine owing to its negligible toxicity and multiple therapeutic actions including anti-cancer, anti-inflammatory and anti-microbial activities. Among the various molecular targets of curcumin, some are involved in bone remodeling, which strongly suggested that curcumin could affect the skeletal system.

Antiinflammatory action

Liu *et al.* (2018) performed self-assembly study of curcumin nanoparticles (CNPs) was done to improve bioavailability. The CNPs were enclosed into genetically modified for responding to the matrix metalloproteinases (MMPs) that are usually over expressed at diabetic nonhealing wound sites. The GMs containing CNPs were loaded into the thermos-sensitive hydrogel and were finally proved for the capacity of specially induced drug release at the wound bed, which promoted the efficacy in healing the standardized skin wounds in streptozotocin-induced diabetic mice.

THERAPEUTIC USES

Curcumin has beneficial effects for dyspepsia, peptic ulcer, inflammatory bowel disease, rheumatoid

arthritis, osteoarthritis, uveitis, orbital pseudotumor and pancreatic cancer. Curcumin is well-tolerated however the most common side effects are nausea and diarrhea. Theoretical interactions exist due to effects on metabolic enzymes and transport proteins, but clinical reports do not support any meaningful interactions. Nonetheless, caution, especially with chemotherapy agents, is advised. Late phase clinical trials are still needed to confirm most beneficial effects (Asher *et al.*, 2013). It prevents certain diseases and disorders like diabetes mellitus, depression, fatigue, epilepsy, Alzheimer's disease, Parkinson's disease (Xia *et al.*, (2007).

Safety evaluation

Dietary consumption of turmeric up to 1.5 g per person per day, equating to a probable maximum of 150 mg of curcumin daily, are not associated with adverse effects in humans (Eigner and Sholz, 1999). Doses up to 5 g/kg were administered orally did not demonstrate toxicity in rats (Wahlstrom and Blennow, 1978). No adverse effects were observed in rats, dogs, or monkeys at doses of up to 3.5 g/kg administered up to 3 months in duration (NCI, DCPC, 1996). 2% dietary curcumin, approximately 1.2 g/kg, did not produce toxicity in rats (Sharma *et al.,* 2001) or at 0.2% of the diet in mice (approximately 300 mg/kg) (Perkins *et al.,* 2002).

CONCLUSION

Curcumin is a yellow phenolic compound present naturally in various types of herbs, especially in *Curcuma longa* Linn (turmeric). Extracts isolated from the rhizome of turmeric have been utilized as anti-inflammatory agents in Ayurvedic and Traditional Chinese Medicine (TCM) for centuries. Curcuminoid-containing turmeric extracts have shown potential also prevent osteoclastogenesis and peri-articular bone destruction in a model of rheumatoid arthritis. It acts as a natural antioxidant, and has exhibited a number of pharmacological activities such as anti-microbial, anti-inflammatory, anti-Alzheimer, and anti-cancer, in preclinical as well as clinical studies. Moreover, it has been seen to possess antirheumatic, hepatoprotective, antidiabetic, neuroprotective, nephroprotective, hypoglycemic, and cardioprotective activities. It also suppresses thrombosis, and provides protection against myocardial infarction It is beyond doubt a wonderful herbal house hold remedy for prevention of various ailments of man and animals.. Many of its therapeutic values are supposed to be disclosed in future as well.

REFERENCES

Aggarwal, B. B. and Harikumar, K. B. (2009).Potential therapeutic effects of curcumin, the anti-inflammatory agent, against neurodegenerative, cardiovascular,pulmonary, metabolic, autoimmune and neoplastic diseases. *The International Journal of Biochemistry & Cell Biology*, **41(1)**, 40-59.

Aggarwal, B. B. (2010).Targeting inflammation-induced obesity and metabolic diseases by curcumin and other nutraceuticals. *Annu Rev Nutr*. **30:**173-99.

Anand, P., Kunnumakkara, A. B., Newman, R. A. and Aggarwal, B. B. 2007. Bioavailability of curcumin: problems and promises. *Molecular Pharmaceutics*, **4(6):** 807-818

Asher, G. N. and Spelman, K. (2013). Clinical utility of curcumin extract. *Altern.Ther.Health Med.*, *19*(2), 20-2.

Bava, S. V., Puliappadamba, V. T., Deepti A., Nair, A., Karunagaran, D. and Anto, R. J. (2005). Sensitization of taxol-induced apoptosis by curcumin involves down-regulation of nuclear factor-kappa B and the serine/threonine kinase Akt and is independent of tubulin polymerization. *J. Biol. Chem.* **280**: 6301–6308.

Chan, M. M., Huang, H. I., Fenton, M. R., Fong, D. (1998).*In vivo* inhibition of nitric oxide synthase gene expression by curcumin, a cancer preventive natural product with anti-inflammatory properties. *Biochem. Pharmacol.* **55**: 1955–1962.

Chen, Z., Xue, J., Shen, T., Mu, S. and Fu, Q. (2016). Curcumin alleviates glucocorticoid-induced osteoporosis through the regulation of the Wntsignaling pathway. *Internat. J. Mol. Med.* **37(2):** 329-338.

Conney, A. H. (2003). Enzyme induction and dietary chemicals as approaches to cancer chemoprevention: The seventh DeWitt S. Goodman Lecture. *Cancer Res.* **63**: 7005–7031.

Dar, S. H. (2018). Evaluation of Turmeric Nanoformulation in Heparin Induced Osteoporosis in rat model. M.Sc. Thesis (Molecular Biology and Biotechnology) submitted to G.B. University of Agriculture and Technology, Pantnagar, Uttarakhand, India**.**

Deshpande, U. R., Joseph, L. J. and Samuel, A. M. (2003). Hepatobiliary clearance of labelled mebrofenin in normal and D-galactosamineHCl-induced hepatitis rats and the protective effect of turmeric extract. *Indian J. Physiol. Pharmacol*. **47:** 332-336.

Esmaili, M., Ghaffari, S. M., Moosavi-Movahedi, Z., Atri, M. S., Sharifizadeh, A. and Farhadi, M. (2011). Beta casein-micelle as a nanovehicle for solubility enhancement of curcumin; food industry application. *LWT Food Sci Technol.* **44:** 2166-72.

Funk, J.L., Frye, J.B., Oyarzo, J.N., Kuscuoglu, N., Wilson, J., McCaffrey, G., Stafford, G., Chen, G., Lantz, R.C., Jolad, S.D. and Sólyom, A.M.

(2006). Efficacy and mechanism of action of turmeric supplements in the treatment of experimental arthritis. *Arthritis & Rheumatism*. **54 (11):**.3452-3464.

Funk, J.L., Frye, J.B., Oyarzo, J.N., Zhang, H. and Timmermann, B.N. (2009). Anti-arthritic effects and toxicity of the essential oils of turmeric (Curcuma longa L.). *Journal of Agricultural and Food Chemistry*. ***58*(2):** 842-849.

Goel, A., Kunnumakkara, A. B. and Aggarwal, B. B. (2008).Curcumin as "Curecumin": From kitchen to clinic. *Biochem.Pharmacol*. **75:** 787-809.

Gu, Q., Cai, Y., Huang, C., Shi, Q. and Yang, H. (2012). Curcumin increases rat mesenchymal stem cell osteoblast differentiation but inhibits adipocyte differentiation. *Pharmacognosy Magazine*. ***8*(31):** 202.

Hamaguchi, T., Ono, K. and Yamada, M. (2010). Review: Curcumin and Alzheimer's disease. *CNS Neurosci Ther*.**16:** 285-97.

Heo, D.N., Ko, W.K., Moon, H.J., Kim, H.J., Lee, S.J., Lee, J.B., Bae, M.S., Yi, J.K., Hwang, Y.S., Bang, J.B. and Kim, E.C. (2014). Inhibition of osteoclast differentiation by gold nanoparticles functionalized with cyclodextrincurcumin complexes. *ACS nano*. **8(12):** 12049-12062.

Hewlings, S. J. And Kalman, D. S. (2017). Curcumin: A Review of Its' Effects on Human Health. *Foods.* **6(10):** 92.

Holt, P. R., Katz, S. and Kirshoff, R. (2005). Curcumin therapy in inflammatory bowel disease: A pilot study. *Dig Dis Sci.* **11**: 2191–2193.

Imaida, K., Tamano, S., Kato, K., Ikeda, Y., Asamoto, M., Takahashi, S., Nir, Z., Murakoshi, M., Nishino, H. and Shirai, T. (2001). Lack of chemopreventive effects of lycopene and curcumin on experimental rat prostate carcinogenesis.*Rcinogenesis.* **22**: 467– 472.

Ireson, C., Orr, S. and Jones, D. J. (2001). Characterization of metabolites of the chemopreventive agent curcumin in human and rat hepatocytes and in the rat in vivo and evaluation of their ability to inhibit phorbol ester-induced prostaglandin E2 production.*Cancer Res.* **61:** 1058–64.

Jagetia, G. C. and Aggarwal, B. B. (2007)."Spicing up" of the immune system by curcumin. *Journal of Clinical Immunology*, *27*(1), 19-35.

Karewicz, A., Bielska, D., Gzyl-Malcher, B., Kepczynski, M., Lach, R. and Nowakowska, M. (2011). Interaction of curcumin with lipid monolayers and liposomal bilayers.*Colloids Surf B Biointerfaces.***88**: 231-9.

Karewicz, A., Bielska, D., Loboda, A., Gzyl-Malcher, B., Bednar, J. and Jozkowicz, A. (2013). Curcumin-containing liposomes stabilized by thin layers of chitosan de rivatives.*Colloid Surf. B.***109:** 307-16.

Kim, T. H., Jiang, H. H., Youn and Y. S. (2011). Preparation and characterization of water-soluble albumin-bound curcumin nanoparticles with improved antitumor activity.*Int. J. Pharm*. **403:** 285-91.

Kocher, A., Hagl, S., Schiborr, C., Eckert, G. P. and Frank, J. (2015). Concentrations of total curcuminoids in plasma, but not liver and kidney, are higher in 18-than in 3-months old mice. *NFS. Journal*. ***1:*** 3-8.

Li, G., Bu, J., Zhu, Y., Xiao, X., Liang, Z. and Zhang, R. (2015).Curcumin improves bone micro-architecture in glucocorticoid-induced secondary osteoporosis mice through the activation of microRNA-365 via regulating MMP-9. *International Journal of Clinical and Experimental Pathology*. **8(12):** 15684.

Li, N., Chen, X. and Liao, J. (2002). Inhibtion of 7, 12-dimethylbenz[a]anthracene (DMBA)- induced oral carcinogenesis in hamsters by tea and curcumin. *Carcinogenesis* **23**: 1307–1313.

Lim, G. P., Chu, T., Yang, F., Beech, W., Frautschy, S. A. and Cole, G. M. (2001).The curry spice curcumin reduces oxidative damage and amyloid pathology in an Alzheimer transgenic mouse. *J. Neurosci*. **21:** 8370-7.

Liu, J., Chen, Z., Wang, J., Li, R., Li, T., Chang, M. and Wang, Y. (2018). Encapsulation of curcumin nanoparticles with MMP9-responsive and thermos-sensitive hydrogel improves diabetic wound healing. *ACS Appl. Materials & Interfaces.* **10(19):**16315-16326.

Liu, Y. and Hong, X. (2006). Effect of three different curcumin pigments on the proliferation of vascular smooth muscle cells by ox-LDL and the expression of LDL-R.*ZhongguoZhong Yao ZaZhi.* **31:**500-3.

Manolova, Y., Deneva, V. and Antonov, L. (2014). The effect of the water on the curcumintautomerism: A quantitative approach. *Spectrochimica Act a.***132A (1):** 815–820.

Murray-Stewart, T. and Casero, R. A. (2017). Regulation of Polyamine Metabolism by Curcumin for Cancer Prevention and Therapy. *Medical Sciences*, **5(4):** 38.

Naksuriya, O., Okonogi, S., Schiffelers, R. M. and Hennink, W. E. (2014). Curcuminnano-formulations: a review of pharmaceutical properties and preclinical studies and clinical data related to cancer treatment. *Biomaterials*.**35(10):** 3365-3383.

NCI, DCPC. (1996). Clinical development plan: Curcumin.

*J. Cell Biochem.***26S**: 72–85.

Perkins, S., Verschoyle, R. D., Hill, K. A., Parveen, I., Threadgill, M. D., Sharma, R. A., Williams, M. L., Steward, W. P. and Gescher, A. J. (2002). Chemopreventive efficacy and pharmacokinetics of curcumin in the Min/+ mouse, a model of familial adenomatous polyposis. *Cancer Epidemiol Biomarkers Prev.* **11**: 535–540.

Priyadarsini, K. I. (2014). "The chemistry of curcumin: from extraction to therapeutic agent". *Molecules* **19 (12):** 20091–112.

Ravindranath, V. and Chandrasekhara, N. (1981). *In vitro* studies on the intestinal absorption of curcumin in rats. *Toxicology.* **20:**251–257.

Ravindranath, V. and Chandrasekhara, N. (1982). Metabolism of curcumin-studies with [3H] curcumin. *Toxicology.* **22:** 337–344.

Rohanizadeh, R., Deng, Y. and Verron, E. (2016). Therapeutic actions of curcumin in bone disorders. *Bone Key Reports*. *5*.

Sandur, S. K., Pandey, M. K., Sung, B., Ahn, K. S., Murakami, A. and Sethi, G. 2007. Curcumin, demethoxycurcumin, bisdemethoxycurcumin, tetrahydrocurcumin and turmerones differentially regulate anti-inflammatory and anti-proliferative responses through a ROS-independent mechanism. *Carcinogenesis.* **28:** 1765-73.

Sharma, R. A., Gescher, A. J. and Steward, W. P. (2005). Curcumin: The story so far. *Eur. J. Cancer.* **41:** 1955-68.

Sharma, R. A., McLelland, H. R. and Hill, K. A. (2001). Pharmacodynamic and pharmacokinetic study of oral Curcuma extract in patients with colorectal cancer. *Clin. Cancer. Res.* **7:** 1894-900.

Sharma, R. A., Steward, W. P. and Gescher, A. J. (2007). Pharmacokinetics and pharmacodynamics of curcumin. *The molecular targets and therapeutic uses of curcumin in health and disease* (pp. 453-470). Springer, Boston, MA.

Shishodia, S., Sethi, G. and Aggarwal, B. B. (2005). Curcumin: Getting back to the roots. *Ann NY Acad. Sci.* **1056:** 206-17.

Sumiyoshi, M. and Kimura, Y. (2009). Effects of a turmeric extract (Curcuma longa) on chronic ultraviolet B irradiation-induced skin damage in melanin-possessing hairless mice. *Phytomedicine*, *16*(12), 1137-1143.

Song, L., Shen, Y., Hou, J., Lei, L., Guo, S. and Qian, C. (2011). Polymeric micelles for parenteral delivery of curcumin: preparation, characterization and in vitro evaluation. *Colloid Surf A.* **390:** 25-32.

Sou, K. (2012). Curcumin towards nanomedicine. *PNM Recent Patents on Nanomedicine,* ***2*****(2):** 133-145.

Toden, S. and Goel, A. (2017). The Holy Grail of Curcumin and its Efficacy in Various Diseases: Is Bioavailability Truly a Big Concern. *Journal of Restorative Medicine*, **6(1):** 27-36.

Vogel, H. and Pelletier, J. (1815). Curcumin-biological and medicinal properties, *Journal de Pharmacie*. **I:**289.

Volate S. R., Davenport, D. M., Muga, S. J. and Wargovich, M. 2005. Modulation of aberrant crypt foci and apoptosis by dietary herbal supplements (quercetin, curcumin, silymarin, ginseng and rutin). *Carcinogenesis.* **26**: 1450–1456.

Wang, N., Wang, F., Gao, Y., Yin, P., Pan, C., Liu, W. and Wang, J. (2016). Curcumin protects human adipose-derived mesenchymal stem cells against oxidative stress-induced inhibition of osteogenesis. *Journal of Pharmacological Sciences*, ***132*****(3):** 192-200.

Wojcik, M., Krawczyk, M., Wojcik, P., Cypryk, K. and Wozniak, L. A. (2018). Molecular Mechanisms Underlying Curcumin-Mediated Therapeutic Effects in Type 2 Diabetes and Cancer. *Oxidative Medicine and Cellular Longevity*. *2018*.

Xia, X., Cheng, G., Pan, Y., Xia, Z. H. and Kong, L. D. (2007). Behavioral, neurochemical and neuroendocrine effects of the ethanolic extract from Curcuma longa L. in the mouse forced swimming test. *J. Ethnopharmacol.* **110(2):** 356-363.

Yang, F., Lim, G. P. and Begum., A. N. (2005). Curcumin inhibits formation of amyloid beta oligomers and fibrils, binds plaques and reduces amyloid *in vivo*. *J. Biol. Chem*. **280:** 5892-901.

Yang, Z., He, C., He, J., Chu, J., Liu, H. and Deng, X. (2018). Curcumin-mediated bone marrow mesenchymal stem cell sheets create a favorable immune microenvironment for adult full-thickness cutaneous wound healing. *Stem Cell Res. Therapy*. **9(1):** 21.

Yoshida, S., Hiyoshi, K., Ichinose, T., Takano, H., Oshio, S., Sugawara, I. and Shibamoto, T. (2009). Effect of nanoparticles on the male reproductive system of mice. *International Journal of Andrology*, **32(4)**, 337-342.

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Research Article

PHARMACOKINETICS OF TOLTRAZURIL FOLLOWING SINGLE DOSE ADMINISTRATION IN POULTRY

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ABSTRACT

The present study was undertaken to investigate the pharmacokinetics of Toltrazuril in poultry following single dose (7mg.kg^{-1}) oral administration. The concentration of Toltrazuril in plasma was analysed by High Performance Liquid Chromatography (HPLC). The maximum plasma concentration (C_{max}) of 3.383 µg.ml^{-1} was obtained. The time to reach C_{max} (T_{max}) was 0.28 days (6.72 h) post administration. The volume of distribution (Vd), elimination half life (Beta_HL) and area under curve (AUC) were calculated as 1908.93 ml.kg^{-1}, 2.30 days, 10.57 day.µg.ml^{-1} , respectively. It can be concluded from the study that a priming dose of 7.0 mg.kg^{-1} with a maintenance dose of 2.5 mg.kg^{-1} at every 14 day is recommended in poultry.

Key words- pharmacokinetics , toltrazuril, high performance liquid chromatography.

INTRODUCTION

Toltrazuril [TZR, 1-methyl-3-{3-methyl-4-[4 (trifluoromethylthio) phenoxy]phenyl}-1,3,5-triazine-2,4,6 (1H,3H,5H)-trione], is a triazine-based drug that is widely used in the prevention and treatment of coccidiosis in poultry and mammals. Toltrazuril after absorption is rapidly converted to the short-lived intermediary metabolite toltrazuril sulfoxide (TZR-SO), and then metabolized to the reactive toltrazuril sulfone (TZR-SO_2) (Lim *et.al.* 2010). Efficacy of a toltrazuril metabolite, toltrazuril sulfone has been reported for inhibiting merozoite production of *Sarcocystis neurona* in cell cultures (Lindsay *et al.,* 2000). Toltrazuril is active against both sexual and asexual stages of coccidia by inhibiting nuclear division of schizonts, microgamonts and the wall-forming bodies of macrogamonts. Toltrazuril interferes with the enzymes of the respiratory chain of coccidian parasite and also inhibits the pyrimidine synthesis (Mundt *et al.,* 2006). Toltrazuril has shown to be effective *in vivo* against *Eimeria* species in avians, intestinal and hepatic coccidiosis in rabbits and *in vitro* against *Toxoplasma gondii.* (Peters & Geeroms, 1986; Chapman, 1987; Ricketts & Pfefferkorn, 1993; Reynaud *et al.*, 1999).

MATERIALS AND METHODS

The pharmacokinetics of Toltrazuril was studied in Rhode Island Red (RIR) birds aged 3-4 months, weighing 1.5±0.5 kg. All birds were procured from Instructional Poultry Farm (IPF) of the University, kept under cage system in the animal house of the department. Birds had free access to clean fresh drinking water and standard poultry feed and were reared as per the guidelines of Institutional Animal Ethics Committee. Birds were acclimatized to the new environment for one month before the commencement of experiment and observed regularly.

Toltrazuril (Coxuril® 2.5% w/v oral solution, M/s Montajat Vet Pharmaceutical Ltd.) was administered as a single oral dose of 7mg.kg^{-1}. Blood samples were collected from six poultry birds in heparinized tubes through an i.v. cannula placed in wing vein at 0, 30 mins, 1, 2, 4, 6, 8, 12 hours and 1, 2, 3, 4, 5, 6, 7, 8 days daily upto 42 days after drug administration. Plasma was separated and stored at -20° C till analysis.

Drug extraction from plasma samples was carried out as per the method described by Guo *et al.* (2007) with slight modification. Deproteinization of plasma sample was carried out by adding 2 ml of HPLC grade acetonitrile in 0.5ml of separated plasma followed by vortex mixing at high speed for 1 min and subsequent centrifugation at 3000 rpm for 10 min. The clear supernatant was collected in a microcentrifuge tube and evaporated. The dried eluate was reconstituted in 0.25 ml of mobile phase and filtered through millipore 0.22 µm cellulose acetate membrane filter. An aliquot of 20 µl of the sample thus obtained was injected into HPLC system for analysis by UV-VIS detector (Shimadzu corporation, Kyoto, Japan).

The estimation of Toltrazuril in plasma and tissue was done by HPLC as per the method described by Lim *et al.* (2010). An isocratic mobile phase consisted of water (30%) and acetonitrile (70%). Flow rate was kept 0.6 ml.min^{-1} and elution was monitored at 25^{0}C with a UV detection at 254 nm.

The standards for Toltrazuril were made by dissolving 1 mg of pure Toltrazuril (Sigma Aldrich Ltd.) in acetonitrile. Further dilutions were made from this stock solution in acetonitrile in the concentrations of 10.0, 5.0, 2.5, 1, 0.5, 0.25, 0.1, 0.05 and 0.025µg.ml^{-1}.

The limit of quantification (LOQ) and limit of detection (LOD) of toltrazuril was 0.025 µg.ml^{-1}.

The pharmacokinetic analysis was done by pharmacokinetic software WinNonlin 5.3. The pharmacokinetic parameters were analyzed by nonparametric Wilcoxon's rank sum test (Steel and Torrie, 1960).

RESULTS AND DISCUSSION

The plasma concentration of 3.796±0.594 µg.ml^{-1} was attained at 6 h post administration which decreased slowly to a minimum of 0.037±0.004 µg.ml^{-1} at 11days, and could not be detected thereafter. A two-compartment model adequately (r= 0.968) described the plasma concentration-time profile of Toltrazuril in poultry following single dose administration at 7mg.kg^{-1}. The plasma concentration time plot and pharmacokinetic parameters of toltrazuril have been depicted in Figure 1 and Table 1.

The peak plasma concentration (C_{max}) of Toltrazuril in the present study was 3.383 µg.ml^{-1} which was close to C_{max} observed in pigs, 4.24 µg.ml^{-1}; (Lim *et al*, 2010) and horses, 4.5 µg.ml^{-1} (Tobin *et al.,* 1997). This difference in C_{max} values could be attributed to species difference.

The time required to reach maximum plasma concentration (T_{max}) in the present study was 0.28 days (6.72 h). This finding could be well corroborated with T_{max} (0.17days) observed by Lim *et al.* (2010) in broilers following single dose (10 mg.kg^{-1}) oral administration. However, higher values of T_{max} have been observed in rabbits (1.04 days) and pigs (0.5 days) following single dose oral administration by Kim *et al* (2010) and Lim *et al* (2010) respectively. The lower value of T_{max} in the present study could be attributed to fast absorption (absorption half life 0.06 days/1.44 h) of Toltrazuril from GIT.

The area under curve (AUC) value (10.57µg.ml^{-1}.day) of Toltrazuril in poultry in the present study can be compared with the finding (8.70 µg.ml^{-1}.day) of Lim *et al.* (2007) following single dose (10 mg.kg^{-1}) oral administration in broilers. However, a higher value of (104.94 µg.ml^{-1}.day) has been reported by Kim *et al.* (2010) in rabbits following single dose (10mg.kg^{-1}) oral administration. The lower value of AUC in the present study could be attributed to the difference in the formulation and poor dissolution of drug in GI fluids as highly lipophilic compounds are more difficult to dissolve in GI fluids (Houston *et al.,* 1974). The formulation used in this present study was also highly lipophillic.

The elimination rate constant of first phase (K_{10}) and second phase (β) were 0.362 and 0.308 day^{-1}, respectively, with an elimination half-life of first phase (K_{10}_HL) and second phase (β_HL) calculated as 1.923 and 2.304 days, respectively, which are in accordance with the value β_HL (2.02 days) reported by Lim *et al.* (2010) following single dose (10 mg.kg^{-1}) oral

Table 1:
Pharmacokinetic parameters of Toltrazuril in plasma following single dose (7mg.kg$^{-1)}$ oral administration in poultry (n=6).

Parameters	Units	Mean±S.E.
V1_F	ml/kg	1908.935±187.062
K01	1/day	18.340±5.371
K10	1/day	0.362±0.015
K12	1/day	0.107±0.030
K21	1/day	1.158±0.332
AUC	day.µg/ml	10.571±0.890
K01_HL	day	0.060±0.020
K10_HL	day	1.923±0.077
Alpha	1/day	1.320±0.338
Beta	1/day	0.308±0.022
Alpha_HL	day	0.698±0.154
Beta_HL	day	2.304±0.169
A	µg/ml	1.089±0.293
B	µg/ml	2.905±0.223
CL_F	ml/day/kg	696.653±83.548
V2_F	ml/kg	207.549±64.159
CLD2_F	ml/day/kg	220.933±74.973
T_{max}	day	0.281±0.066
C_{max}	µg/ml	3.383±0.310

V1_F: volume of distribution of central compartment; K01: First order absorption rate constant;K10: First order elimination rate constant;K12: first order rate constant of transfer of unbound drug from central to peripheral compartment;K21: rate constant of drug transfer from peripheral to central compartment; AUC: Total area under the curve; K01_HL: Absorption half life in one compartment model; K10_HL: Elimination half life in one compartment; Alpha_HL: Distribution half-life in two compartment model; Beta_HL: Elimination half-life in two compartment model; A: Coefficient of biexponential equation describing disposition curve; zero time intercept of plasma concentration in the distribution phase; α: First order rate constant; B: Zero time intercept of plasma concentration in the elimination phase; β: Regression coefficient for the elimination phase; V2_F: volume of distribution of peripheral compartment CL_F: Clearance from central compartment; T_{max}: Time to reach peak plasma concentration; Cmax Maximum plasma concentration; Regression coefficient for the distribution phase of the disposition curve; CLD2_F: Clearance from peripheral compartment.

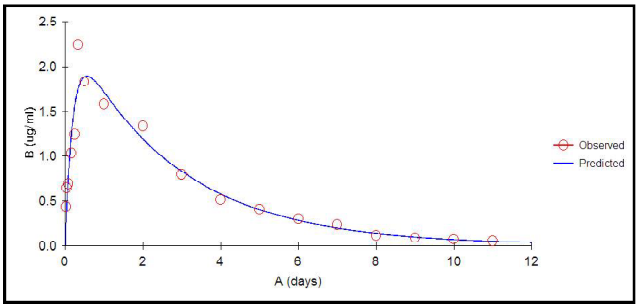

Fig 1:
Plasma concentration-time plot of observed concentration (mean) Vs predicted profile of Toltrazuril following single dose (7 mg.kg^{-1}) oral administration in poultry (n=6)

Table 2:
Dosage regimen of Toltrazuril for single dose oral administration in poultry

Desired therapeutic concentration (µg.ml^{-1})	Dose interval (days)	Priming dose (mg.kg^{-1})	Maintenance dose (mg.kg^{-1})	Minimum Steady state concentration (µg.ml^{-1})	Maximum steady state concentration (µg.ml^{-1})
4	7	2.92	1.483	18.87	38.97
4	14	6.78	2.502	11.2	44.16
4	21	15.74	6.620	1.77	24.2

administration in pigs. Kim *et al.* (2010) have calculated the value of β_HL (2.33 days) in rabbits similar to the value calculated in the present investigation. In contrast rapid elimination of Toltrazuril (β_HL 0.45 days) has been reported following single dose (40 $mg.kg^{-1}$) oral administration in broilers by Lim *et al.* (2007) which could be due to the difference in the formulation, pharmacokinetic model and health status of the experimental animals.

Dosage regimen was calculated with the therapeutic concentration of 4$\mu g.ml^{-1}$ (Ricketts and Pfefferkorn, 1993) The dosage regimen of Toltrazuril for single dose oral administration in poultry calculated on the basis of the pharmacokinetic parameters is presented in table 2. It is concluded that the plasma concentration of toltrazuril persist for 11days and a priming dose of 7 $mg.kg^{-1}$ with maintenance dose of 2.5 $mg.kg^{-1}$ of Toltrazuril is recommended at an interval of 14 days in poultry birds.

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REFERENCES

Chapman, H.D. (1987) Control of a line of *E. tenella*, partly resistant to monensin, by including toltrazuril discontinuously in the drinking water of chickens. *Journal of Comparative Pathology*, **97:** 21–27.

Guo, W., Li, W., Guo, G., Zhang, J., Zhou, B., Zhai, Y. and Wang C. (2007). Determination of atomoxetine in human plasma by a high performance liquid chromatographic method with ultraviolet detection using liquid-liquid extraction. *J Chromatogr B Analyt Technol Biomed Life Sci.* **854**: 128–134.

Houston, J.B., Upshall, D.G. and Bridges, J.W. (1974). A re-evaluation of the importance of partition coefficients in the gastrointestinal absorption of nutrients. *J. Pharmacol. Exp. Ther.* **189**: 244–254

Kim, M.S., Lim, J.H., Hwang, Y.H., Park, B.K., Song, I.B. and Yun, I. H. (2010). Plasma disposition of toltrazuril and its metabolites, toltrazuril sulfoxide and toltrazuril sulfone, in rabbits after oral administration. *Veterinary Parasitology* **169**: 51–56

Lim, J.H., Kim, M. S., Hwang Y. H., Song, I.B., Park, B. K. and Yun, H.I. (2010). Pharmacokinetics of Toltrazuril and Its Metabolites, Toltrazuril Sulfoxide and Toltrazuril Sulfone, after a Single Oral Administration to Pigs. *J. Vet. Med. Sci.* **72(8)**: 1085–1087.

Lim, J.H., Park, B.K., Kim, M.S., Hwang, Y.H., Song, I.B., Yun, H.I. (2007). Pharmacokinetics of toltrazuril after oral administrations in broilers. *J. Vet. Clin*. **24**: 308–311.

Lindsay, D.S. and Blagburn, B.L. (1995). Antiprotozoan drugs. In Veterinary Pharmacology and Therapeutics, 7th edn, Ed Adams, H.R., pp. 971–983. Iowa State University Press, Ames.

Mundt, H.C., Mundt-Wüstenberg, S., Daugschies, A. and Joachim, A. (2006). Efficacy of various anticoccidials against experimental porcine neonatal Isosporosis. *Parasitology research* **100:** 401 – 411.

Peters, J.E. & Geeroms, R. (1986) Efficacy of toltrazuril against intestinal and hepatic coccidiosis in rabbits. *Veterinary Parasitology*, **22:** 21.

Reynaud, M.C., Chauve, C.M., Gastellu, J. & Gounel, J.M. (1999). Administration of toltrazuril during experimental coccidiosis in mule ducks: comparison of the efficacy of a single administration at two different endogenous stages. *Veterinary Parasitology*, **81:** 265–274.

Ricketts,A.P. & Pfefferkorn, E.R. (1993). *Toxoplasma gondii*: susceptibility and development of resistance to anticoccidial drugs *in vitro*. *Antimicrobial Agents and Chemotheraphy*, **37**: 2358–2363.

Steel, R.G.D. and Torrie, J.H. (1960). Non parametric statistics. *In* : Principles and Procedures of Statistics, Mc Graw Hill Book Co. Inc., New York, pp : 407-415.

Tobin, T., Dirikolu, L., Harkins, J.D., Garnstrom, D.E., Carter, W., Lehner, F. and Trinder, P. (1997). Determination of Glucose in Blood Using Glucose Oxidase with an Alternative Oxygen Acceptor. *Ann. Clin. Biochem*. **6:** 24-25.

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Research Article

AMELIORATIVE EFFICACY OF *BUTEA MONOSPERMA* LEAF POWDER IN IMIDACLOPRID INTOXICATED JAPANESE QUAILS

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ABSTRACT

The present study was carried out to know the ameliorative efficacy of *Butea monosperma* in imidacloprid intoxicated Japanese quails. 75, two weeks old Japanese quails were divided into five groups of 15 birds in each. Group 1 served as control, while groups 2, 4 and 5 were treated with imidacloprid @ 50 mg /kg of feed for 28 days. In addition to imidacloprid, groups 4 and 5 also received *Butea monosperma* @ 2 g /kg of feed and 4 g /kg of feed respectively. Group 3 was maintained as *Butea monosperma* control @ 4 g /kg of feed. Significant decrease ($P<0.05$) was observed in Hb, PCV and TEC of T2 group and increase in T3, T4 and T5 group. Differences of MCV, MCH and MCHC were found to be statistically non significant between different treatment groups. The significant decrease was recorded in TLC in group T2 however, groups T4 and T5 showed progressive increase in TLC values suggesting ameliorative effect of plant. The significant decrease was recorded in absolute lymphocyte and increase in heterophil count whereas absolute eosinophil, monocyte and basophil count showed non significant differences.

Key words: *Butea monosperma*, imidacloprid, Japanese quails.

INTRODUCTION

Butea monosperma (Fabaceae) is also known as 'Flame of the forest'. The plant *B. monosperma is* used in treating various ailments. The leaves of *B. monosperma* is traditionally used as an anti-inflammatory, diuretic, antidiabetic, antimicrobial, appetizer, carminative, astringent and aphrodiasic (Muzumder *et al.,* 2011). It was found that these plants are found to posses polyphenolic constituents like flavonoids. Flavonoids are reported to have anti-inflammatory, anti-hepatotoxicity and antiulcer actions. They are potent antioxidants and have free radical scavenging abilities (Raj Narayana *et al.,* 2001). The present study was undertaken to know the ameliorative efficacy of *Butea monosperma* in imidacloprid intoxicated Japanese quails.

MATERIAL AND METHODS

The experimental trial was approved by the Institutional Animal Ethics Committee. Imidacloprid, Technical grade (97.20%) was procured from Krishi Rasayan Export Pvt. Ltd., Samba, Jammu, India. The leaves of *B. monosperma* plant were collected from local region of Akola, Mahrashtra, India, shade dried and mixed with the feed in a powdered form. The botanical identity was confirmed by the expert taxonomist Dr. S.P. Rothe, Professor and Head, Department of Botany, Shri Shivaji Science College, Akola (M.S.).Two weeks-old, seventy-five Japanese quails were randomly allocated to five equal groups, fifteen birds in each. Group 1 served as control, group 2 was treated with imidacloprid @ 50 mg/kg of feed, group 3 was treated with *B. monosperma* @ 4 g/kg of feed, groups 4 was treated with both imidacloprid @ 50 mg/kg of feed and *B. monosperma* @ 2 g/kg of feed in feed and group 5 was treated with imidacloprid @ 50 mg/kg of feed and *B. monosperma* @ 4 g/kg of feed in feed for a period of 28 days. At the end of 28th days of experimental period, blood samples from six birds of each group were collected in sterile test tubes and haematological parameters such as Hb, PCV, TEC, MCV, MCH, MCHC, TLC and absolute leucocyte count were estimated by the method of Benjamin (2001). The data was analyzed by applying simple CRD (Snedecor and Cochran, 1989).

RESULTS AND DISCUSSION

In the present study, significant decrease ($P<0.05$) in Hb, PCV and TEC (Table 1) in T2 group and increase in T3, T4 and T5 group birds was observed. Present result of decreased mean haemoglobin and PCV values are in accordance with Abbassy *et al.,* (2014). However, significantly higher haemoglobin value was observed in group T3 birds receiving *B. monosperma* alone @ 4 g/kg of feed.Similar findings of reversed haemoglobin level near to normal was reported by Sujith *et al.,* (2011) in alloxan diabetic rats administered with methanolic extract of *B. frondosa.* Decrease in the number or size of red blood cells also decrease the amount of space they occupy, resulting in a lower haematocrit (Quadir *et al.,* 2014). In the present study,

Table 1.
Effect on haematological parameters related to erythrocytes in different groups at the end of 4th week of experiment (Mean±S.E., n=6)

Groups	Hb(g/dL)	PCV(%)	TEC(10^6/cumm)	MCV(fL)	MCH(pg)	MCHC(g/dL)
T1	11.18^{ab}±0.138	38.25^{ab}±0.738	5.53^{ab}±0.19	69.47±2.102	20.37±0.856	29.30±0.696
T2	10.47^{c}±0.150	35.33^{c}±1.327	5.18^{b}±0.162	68.23±2.257	20.29±0.706	29.82±0.983
T3	11.73^{a}±0.201	40.00^{a}±0.577	5.80^{a}±0.071	69.05±1.702	20.25±0.454	29.34±0.308
T4	10.84^{bc}±0.236	36.07^{bc}±0.503	5.26^{b}±0.151	68.80±2.117	20.65±0.563	30.05±0.519
T5	11.01^{bc}±0.295	37.95^{ab}±0.402	5.78^{a}±0.093	65.70±0.585	19.10±0.785	29.05±1.055

Mean values with common alphabet as superscript do not differ significantly.

Table 2.
Effect on haematological parameters related to leucocytes in different groups at the end of 4th week of experiment (Mean±S.E., n=6)

Groups	TLC (10^3/cu mm)	Absolutelymphocyte	Absoluteheterophil	Absolute monocyte	Absoluteeosinophil	AbsoluteBasophil
T1	11.25^{ab}±0.383	6727.00^{ab}±324.437	4143.5^{b}±101.57	225.83±34.141	153.66±40.829	0±0
T2	10.32^{c}±0.182	4416.46^{d}±136.029	5545.28^{a}±209.557	171.43±21.253	172.31±35.379	17.83±17.833
T3	11.89^{a}±0.109	7277.90^{a}±223.566	4264.40^{b}±214.177	177.73±39.954	138.65±19.499	39.65±25.076
T4	10.82^{bc}±0.273	5163.81^{c}±240.822	5392.41^{a}±193.338	108.45±30.344	158.65±55.621	0±0
T5	11.02^{bc}±0.253	6213.01^{b}±193.422	4517.63^{b}±140.344	146.28±45.387	144.73±36.079	0±0

Mean values with common alphabet as superscript do not differ significantly

imidacloprid treated groups showed significant variability in Hb, PCV and TEC when compared with control. Reduction in haematological values was observed indicating anaemia in imidacloprid treated birds which may be due to erythrpoiesis, haemosynthesis and osmoregulatory dysfunction or due to increase in the rate of erythrocyte destruction in haematopoietic organ.

Differences of MCV, MCH and MCHC (Table 1) were found to be statistically non significant between different treatment groups. Present observation is supported by the reports of Preeti *et al.,* (2014) in male Swiss albino mice. Lower MCV values indicate decrease in size of erythrocyte suggesting possibility of iron deficiency anemia (Benjamin, 2001). It is thus concluded that imidacloprid during 4 weeks period slightly affect MCV, MCH and MCHC whereas *B. monosperma* could maintain the values. Birds treated with imidacloprid either alone or in combination with *B. monosperma* showed restored values of MCV, MCH and MCHC.

The significant decrease ($P<0.05$) was recorded in TLC (Table 2) in group T2 and highest value was recorded in T3 group fed *B. monosperma* @ 4 gm/kg of feed. However groups T4 and T5 showed progressive increase in TLC values suggesting ameliorative effect of plant. The significant decrease was recorded in absolute lymphocyte count and increase in heterophil count whereas absolute eosinophil, monocyte and basophil count showed non significant differences in control and treatment groups. Present finding of decrease in TLC count is in accordance with the previous observations reported by Balani *et al.,* (2011) in WLH cockerels. The decrease in TLC may be directly related with either their decreased production in the lymphoid organ or their increased lysis due to presence of imidacloprid in the bone marrow as evident from the microscopic picture of spleen observed during present study.

Hosen *et al.,* (2016) reported beneficial role of *B. monosperma* in restoring TLC count against arsenic induced toxicity in rats. Decreased lymphocyte count and increased values of neutrophil (%) during imidacloprid toxicity in present study is in agreement with Badgujar *et al.,* (2013). Decreased percent lymphocyte and TLC in imidacloprid treated group indicated a risk of lymphopenia and immunomodulation. It is thus concluded that, imidacloprid exposure leads to marked alterations in haematological parameters of Japanese quails and co-treatment with *Butea monosperma* moderately ameliorated the imidacloprid induced toxicity.

REFERENCES

Badgujar, P. C., Jain, S. K., Singh, A., Punia, J. S., Gupta, R. P. and Chandratre, G. A. (2013). Immunotoxic effects of imidacloprid following 28 days of oral exposure in BALB/c mice. *Environmental Toxicology and Pharmacology.* **3 (5)**: 408–418.

Balani, T., Agrawal, S. and Thaker, A. M. (2011). Hematological and biochemical changes due to short-term oral administration of imidacloprid. *Toxicol. Int.* **18(1)**: 2–4.

Benjamin, M. M. (2001). Outline of Veterinary Clinical Pathology, 3rd Edn. Kalyani Publisher, Ludhiana.

Hosen, S. M. I., Kobi, R., Das, D., Chowdhury, D. U. S., Alam, M. J., Rudra, B., Bakar, M. A,. Islam, S., Rahman, Z. and Al-Forkan, M. (2016). Protective effects of *Butea monosperma* against arsenic contaminated rice induced toxicity. *Canadian Journal of Pure and Applied Sciences*. **10 (2)**: 3873-3881.

Mazumder, P. M., Das, M. K., Das, S. and Das, S. (2011). *B. monosperma* (Lam) *Kuntze- A comprehensive review. International* Journal of Pharmaceutical Science and Nanotechnology. **4(2)**:1390-1393.

Preeti, V. K., Sikka, A. K. and Punia, J. S. (2014). Hematological and morphometric studies of imidacloprid through oral administration in Swiss albino mice. *Haryana Vet.* **53 (2)**: 144-147.

Qadir, S., Latif, A., Ali, M. and Iqbal, F. (2014). Effects of imidacloprid on the hematological and serum biochemical profile of labeo rohita. *Pakistan J. Zool.* **46(4)**: 1085-1090.

Raj Narayana, K., Sripal Reddy, M., Chaluvadi, M. R. and Krishna, D. R. (2001). Bioflavonoids Classification, Pharmacological, Biochemical effects and therapeutic potential. *Indian Journal of Pharmacology.* **33**: 2-16.

Snedecor, G. W. and Cochran, W. G. (1989). Statistical Methods, 8th Edn. Iowa Univ. Press, Iowa, USA.

Sujith, K., Darwin, C. R. and Roosewelt, C. (2011). Antidiabetic activity of methanolic extract of *Butea frondosa* leaves with its possible mechanism of action. *Asian Journal of Pharmaceutical and Clinical Research.* **4(3)**: 93-98.

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Research Article

ANTIMICROBIAL AND MUSCLE RELAXANT POTENTIAL OF LEAF EXTRACT OF SAUSAGE TREE

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ABSTRACT

The present study investigated the potential of different leaf extracts of Sausage tree (*Kigelia africana*) as a muscle relaxant in rodents and our aim was also to study *in-vitro* antibacterial activity of the various extracts from leaves of the plant. Different leaf extracts of *Kigelia africana* i.e. aqueous, alcoholic, acetone and chloroform were investigated for *in-vivo* muscle relaxant activity and *in-vitro* antibacterial activity at the dose rate of 50 mg/kg and 100 mg/kg in Wistar rats. For muscle relaxant activity rota-rod apparatus was used and fall-off time was noted. *In-vitro* antibacterial activity was assessed against clinical isolates of *E.coli* and *S. aureus* using agar well diffusion method. The results showed no significant antibacterial and skeletal muscle relaxant activity in any of the leaf extracts of the plant under study.

Key words: Antibacterial, Muscle relaxant, *Kigelia africana*

INTRODUCTION

From the time immemorial, plants have been widely used as curative agents for variety of ailments. Nature has served as a rich repository of medicinal plants for thousands of years and an impressive number of modern drugs have been isolated from natural sources, notably of plant origin. Medicinal plants have become the focus of many studies in terms of validation of their traditional use through the determination of their actual pharmacological effects. As the incidence of antimicrobial resistance towards the currently available chemotherapeutic agents has increased, antimicrobial research is geared towards the discovery and development of novel antibacterial agents.

Kigelia africana (Lam) Benth, (*Kigelia pinnata*) belongs to the family Bignoniaceae. Its common names include sausage tree (English); worsboom (Africa); pandoro (West Nigeria) (Aiyelola *et al* 2006). It is also known as Balamkheera in hindi and distibuted all over India but found abundantly in West Bengal. It is a tree growing up to 20 m tall or more. The bark is grey and smooth at first, peeling on older trees. It can be as thick as 6 mm on a 15 cm branch. The wood is pale brown or yellowish, undifferentiated and not prone to cracking (Roodt 1992). Flowers are produced in panicles; they are bell shaped (similar to those of the African tulip tree but darker and more waxy), orange to reddish or purplish green and about 10 cm wide. Individual flowers do not hang down, but are oriented horizon-tally (Joffe 2003).

Several parts of the plant are used for medicinal purposes by certain people. In Malawi during famine the seeds were roasted to eat. Baked fruits are used to ferment beer and boiled ones yield a red dye. The tonga women of Zambezi valley regularly apply cosmetic preparation of Kigelia fruits to their faces to ensure a blemish free complexion (Pooley 1993). In folk medicine, the fruits of plant are used as dressing for ulcers, purgative and to increase the flow of milk in lactating women (Oliver-Bever 1986). In South Africa, the root and unripe fruit is used as vermifuge and as a treatment for haemorrhoids and rheumatism (Irvine 1961). Most commonly traditional healers used it to treat a wide range of skin ailments like, fungal infections, boils, psoriasis and eczema. It also has internal application including the treatment in dysentery, ringworm, tape-worm, post-partum haemorrhage, malaria, diabetes, pneumonia and toothache (Gill 1992). In the light of above, the present study investigated the potential of different leaf extracts of Sausage tree (*Kigelia africana*) as a muscle relaxant in rodents and our aim was also to study *in-vitro* antibacterial activity of the various extracts from leaves of the plant.

MATERIAL AND METHODS

Plant material

Based on ethno pharmacological information, leaves were collected from campus of Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab. Plant was identified by the botanist of Collarative Ayurveda Research Centre, GADVASU, Ludhiana. Immediately after collection leaves were washed and dried under sunlight. The dried leaves were finely grounded into powder, weighed and kept for further analysis.

Extraction

Four different extracts were prepared i.e. aqueous, alcoholic, acetone and chloroform extracts. 100 g of powdered material was soaked in 1 litre of solvent and left for 24 h. Mixture was stirred at 30 min interval. Mixture was filtered with double layered muslin

cloth and re-filtered with Whatman filter paper no.1. The filtrate obtained was evaporated in oven at a temperature of 40°C. Powdered leaves yielded 10% aqueous, 2.7% alcoholic, 1.3% acetone and 2.3% chloroform extracts which were stored in air tight bottles and refrigerated at 4ºC prior to use.

Microbial strains

Strains of two bacteria, namely *Staphylococcus aureus and Escherichia coli* were obtained from the pure clinical isolates maintained in the Department of Microbiology, GADVASU, Ludhiana.

Preparation of the extract for antimicrobial assay

Plant extracts were dissolved in 100% dimethylsulphoxide (DMSO) for antimicrobial study. Concentration of aqueous, alcoholic, acetone and methanolic extracts was 500 mg/ml.

Culture Media and Chemicals

Absolute alcohol, Mueller Hinton agar medium (Merck Specialties Private Limited, Mumbai), nutrient broth (Merck Specialties Private Limited, Mumbai), brain heart infusion agar (HIMEDIA®) were commercially procured.

Preparation of medium

Inoculates of test organisms were prepared by growing each pure isolate in Nutrient broth for 18 hours at 37°C. The overnight broth culture was matched with McFarland turbidity standard to give an approximate 10^8cfu/ml. From this broth 0.2 ml was then used to seed a molten Mueller Hinton agar medium which has been allowed to cool to 45°C to obtain approximately 10^8 cfu/ml. The cultured agar was poured into sterile Petri dishes and used for analysis (Ibeh *et al* 2002).

Well diffusion method assay

The agar well diffusion method (Collins *et al* 1995) was followed to test the antibacterial activity of four extracts. The sterilized media was distributed into pre-sterilized petri plates at a depth of approximately 4 mm (15 ml), and allowed to solidify. The medium of each plate was surface inoculated with a suspension of respective microorganism (10^6-10^8cfu/ml), and the wells (8 mm) were cut using a sterile cork borer. The solution of various plant extract (50 µl) in corresponding solvent at concentration (500 mg/ml) was delivered into the wells. The extractive solvent i.e. DMSO (50 µl), and ampicillin (10 mg/ml) were maintained as negative and positive controls, respectively. The plates were incubated at 37º C for 24 h. Following incubation the diameter of inhibition zones was recorded in mm.

Muscle relaxant activity

The animals were trained to maintain balance for 40-60 sec on the rotarod rotating at a speed of 25 rpm. Only those rats which could balance themselves for 45-60 seconds were selected for study. Each rat was placed individually on the Rota rod, and time spent on Rota rod was noted. The Control group was treated with distilled water. In the positive control group diazepam @ 4 mg/kg was injected i.p as a standard drug. In group III, group V, group VII and group IX the aqueous, alcoholic, acetone and chloroform extracts were given respectively, @ 50 mg/kg orally whereas in group IV, group VI, group VIII and group X respectively these extracts were given @ 100 mg/kg orally. Fall off time was noted at 0 h, 1 h and 2 h respectively.

Design of experiment

50 Swiss albino rats (180-200 g) of either sex were kept at Small animal colony, GADVASU, Ludhiana were used. The experiments were approved by the Institutional Animal Ethics Committee (IAEC) vide reference no VMC/13/1786-1806 dated 4/4/13 and were conducted in accordance with ethical committee guidelines. The animals maintained under standard environmental condition had free access to standard rat feed pellets (Ashirwad industries, Mohali, Punjab) and water. Rats were divided into ten groups of five animals each. Group I served as control, group II as positive control and remaining groups served as test groups**.** Group III and IV were administrated with aqueous extract of concentration 50 mg/kg and 100 mg/kg orally, respectively. Group V and VI were administered with alcoholic extract having concentration of 50 mg/kg and 100 mg/kg, respectively. Similarly group VII and VIII with acetone extract and group IX and X with chloroform extract having concentrations of 50 mg/kg and 100 mg/kg, respectively.

RESULTS AND DISCUSSION

Antibacterial activity

In present study four different extracts of *Kigelia africana* i.e. aqueous, alcoholic, acetone and chloroform were used to study their antibacterial activity against clinical isolates of *Escherichia coli* and *Staphylococcus aureus* at dose rate of 500 mg/ml. Ampicillin was used as standard drug. DMSO was used as control. Results revealed no antibacterial activity of any extract as zone of inhibition was non significant as shown in Table 1.

Table 1:

Effect of different leaf extracts of *Kigelia Africana* on antibacterial activity against *E.coli* and *Staphylococcus aureus* (Measurements on Zone of inhibition (mm) inclusive of the width of the well).

Extract	Dose (concentration)	*E.coli*	*Staphylococcusaureus*
Control(DMSO)	100%	0	0
Standard (Ampicillin)	10 mg/ml	>14	>14
Aqueous	500 mg/ml	< 14	<14
Alcoholic	500 mg/ml	< 14	<14
Acetone	500 mg/ml	< 14	<14
Chloroform	500 mg/ml	< 14	<14

Table 2:
Effect of different leaf extracts of *Kigelia africana* on muscle relaxant activity in rats

Extract	Dose(mg/kg)	Fall off time(sec)		
		0 hr	1 hr	2 hr
Control(DW)	-	47.40±1.12^{a}	45.40±1.20^{a}	46.00±1.64^{c}
Standard(Diazepam)	4 mg/kg	48.20±0.97^{a}	6.40±0.60^{b}	5.80±0.37^{d}
Aqueous	50	49.20±1.24^{a}	48.20±0.73^{a}	50.00±0.45^{a}
Aqueous	100	47.20±1.01^{a}	46.60±1.20^{a}	48.20±0.66^{abc}
Alcoholic	50	49.00±0.89^{a}	48.40±0.68^{a}	49.00±0.89^{ab}
Alcoholic	100	47.20±1.24^{a}	46.00±0.63^{a}	47.60±0.50^{abc}
Acetone	50	46.80±0.97^{a}	47.80±1.31^{a}	47.00±0.63^{bc}
Acetone	100	46.40±1.36^{a}	47.60±1.02^{a}	47.00±1.41^{bc}
Chloroform	50	46.00±1.18^{a}	46.60±0.93^{a}	47.60±0.81^{abc}
Chloroform	100	45.40±1.63^{a}	47.00±1.92^{a}	45.80±0.86^{c}

Muscle relaxant activity

The effect of various extracts of *Kigelia africana* in different dose concentrations on muscle relaxant activity of animals belonging to the different groups are given in the Table 2 and fig 1. The control group was treated with distilled water. In the standard group diazepam @ 4 mg/kg was injected as a standard drug. In group III, group V, group VII and group IX the aqueous, alcoholic, acetone and chloroform extracts were given respectively, @ 50 mg/kg whereas in group IV, group VI, group VIII and group X respectively these extracts were given @ 100 mg/kg orally. Rats were placed on rotarod and fall off time was noted. In standard group there was significant decrease in fall off time after 1 h and remained upto 2 h after drug administration. In the control group there was no significant decrease in fall off time. All other groups showed non significant decrease in fall off time. Present study showed no muscle relaxant activity of *Kigelia africana* leaf extracts.

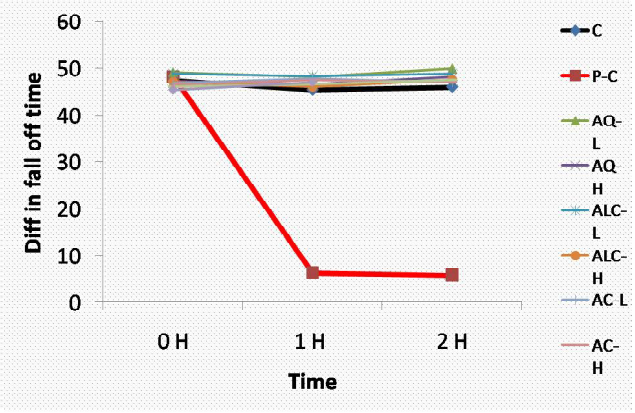

Fig 1: Effect of different leaf extracts of *Kigelia africana* on muscle relaxant activity in rats.

The study revealed non-significant zones of inhibition when compared to the control, depicting no antibacterial activity of any of the leaf extracts of *Kigelia africana* against clinical isolates of *E. coli* and *S. aureus* at dose rate of 500 mg/ml. Also no *in vivo* skeletal muscle relaxant activity was recorded in any of the extracts of the plant.

REFERENCES

Coates-Palgrave K.(1988). Trees of Southern Africa, edn. 2. Struik, Cape Town

Aiyelola AA and Bello O.A. (2006). Ethnobotanical potentials of common herbs in Nigeria:A case study of Enugu state. *Educational Research and Review* **1**: 16-22.

Roodt V 1992. Kigelia Africana in the shell Field Guide to the common Trees of the Okarango Delta and Moremi Game reserve. Gaborone, Botswana; shell Oil Botswana. Dewels: 582.1609883: 20-110.

Joffe P.Kigelia Africana (Lam) Benth, (2003). *Pretoria National Botanical Garden (www. plantzafrica. com)*.

Oliver-Bever B E P. (1986). *Medicinal Plants in Tropical West Africa*. pp 240-245, Cambridge University Press, New York.

Gill LS (1992).Ethnomedical uses of plants in Nigeria.pp.143, Uniben Press, Benin.

Pooley E (1993). The complete field guide to trees of Natal, Zululand & Transkei. pp 22-24, Natal Flora Publications Trust.

Irvine FR (1961). Woody Plants of Ghana with Special Reference to their Uses.pp 736-40, Oxford University Press, London.

Ibeh LN, Idu M and Obasuyi O. (2002). Studies on the Antimicrobial properties of Lepistemono-wariense leaf.*J. Med. Lab. Sci.* **2**: 55.

Collins GH, Lynes PM and Grange JM (1995). Microbiological Methods.7thedn.pp. 175–190, Butterwort – Heinemann Ltd, Britain.

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Research Article

ANTIDIABETIC EFFECT OF DISTILLED COW URINE IN ALLOXAN INDUCED DIABETIC WISTAR RATS

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ABSTRACT

The sacred Indian cow, *Bos indicus* known as "*Kamadhenu*" in Indian scripts, is believed to be a "mobile hospital" for the treatment of many diseases. Though Indian Ayurvedic literature cites many medicinal properties of cow urine, there are limited scientific evidences to support this. Earlier studies have revealed the presence of antioxidants and free radical scavengers in cow urine which might be responsible for the observed antidiabetic effects. Hence, the present study of the antidiabetic activity of distilled cow urine was undertaken.

The effect of cow urine distillate was studied *in vivo* in rats administered with Alloxan. Diabetes was induced by administration of single dose of Alloxan (120 mg/kg body wt., IP) dissolved in sterile normal saline. The antidiabetic effect of the cow urine distillate (three different doses) and a standard drug, Glibanclamide (0.25 mg/kg, PO.) was studied in these diabetic rats. The parameters used in the study included assessment of blood glucose levelsand biochemical parameters. The cow urine distillate produced a significant ($P<0.05$) reduction of the elevated blood glucose, serum cholesterol, serum triglycerides,AST, ALT levels when compared with the diabetic control and was comparable with standard drug. Diabetic animals treated with cow urine distillate showed significant lowering of blood glucose levels.

Key words : Antidiabetic, cow urine distillate, Alloxan, Glibenclamide

INTRODUCTION

Diabetes mellitus is a metabolic disease characterized by high blood glucose level resulting from defects in insulin secretion, insulin action or both.The metabolic disturbances contribute massively to most neurological, cardiovascular, retinal and renal diabetic complications and it is characterized by hyperglycemia and disturbances of carbohydrate, protein, and fat metabolism, secondary to an absolute or relative lack of the hormone insulin. The incidence of diabetes mellitus is on the rise in the world. Based on the World Health Organization (WHO) report, the number of diabetic patients is expected to increase from 171 million (in year 2000) to 366 million or more by the year 2030 (Wild *et al.,* 2004). As well, it is the most frequently found in dogs and cats. Estimates of the incidence of diabetes range as high as 1:66 (1.52%) for dogs and 1:800 for cats. Diabetes mellitus in animals has been frequently reviewed (Cotton *et al.,* 1971; Foster 1975; Ling *et al.,* 1977; Engerman and Kramer 1982). There is a role of genetic and environmental factors in feline and canine diabetes however type 2 diabetes is most common form of diabetes in cats. The management of diabetes involves both the non-pharmacological and pharmacological approaches.The non-pharmacological approach includes exercise, diet control and surgery, while the pharmacological approach includes the use of drugs such as insulin, and oral hypoglycemic agents.

In Ayurveda, cow urine (Gomutra) occupies a unique place and has been recognized as water of life or "Amrita" (Dhama *et al.,* 2005).In *Panchagavya Ghrita*, it is one of the most important ingredient. *Panchgavya,* a term used to describe a formulation constituted with five major substances like urine, milk, ghee, curd, and dung obtained from cow. All the five products possess medicinal properties and are used singly or in combination with some other herbs against many diseases (Achliya *et al.,* 2003).The most probable mechanism of action is that cow urine increases the glucose transport across cell membrane thus it increases peripheral glucose utilization. It increases glycogen synthesis from glucose and also increases insulin release from β-cells. Cow urine enhances sensitivity of insulin receptor, decreases insulin resistance. Various herbal metabolites are present in cow urine and they produce anti hyperglycemic effect (Sachdev *et al.,* 2012).There are so many claims regarding the use of cow urine. Out of these the most important claim is regarding its antidiabetic and antioxidant activity, but only few scientific literatures are available to support this claim. Hence, the present study was designed to validate the antidiabetic effect of distilled cow urine (*Gomutra Ark*) in Alloxan induced diabetic rats.

MATERIALS AND METHODS

Cow urine distillate preparation:

The first early morning voided urine of cow

(Khillar) was collected from the livestock farm of KNP College of Veterinary Science, Shirwal and immediately distilled, using a temperature-controlled distillation apparatus and then stored in air tight container for further use.

Alloxan

Alloxan monohydrate powder, CAS. No. ASA 1919, Batch No. RC/0810/0189 manufactured by Avra Synthesis Pvt. Ltd. Hyderabad, purchased from a local pharmacist was used to induce diabetes in Wistar rats. Alloxan, a chemical used in inducing diabetes acts mainly by the generation of reactive oxygen species (ROS) (Lenzen, 2008).

Induction of diabetes

For induction of diabetes alloxan monohydrate powder manufactured by Avra Synthesis Pvt. Ltd. Hyderabad, was procured and diabetes was induced in rats by the administration of single intra peritoneal dose of Alloxan monohydrate (120 mg/kg b.wt.). Three days after Alloxan injection (After 72 hrs. of Alloxan administration), blood samples were collected by retroorbital method. Blood glucose was measured by using strips and glucometer. Rats showing hyperglycemia with blood glucose level 200 mg/dl and above were considered as diabetic and recruited in the present study.

Selection of dose of cow urine

Rat dose was calculated from human dose (60 ml/day), multiplied by a factor 0.018 x 5 which is equal to 5.4 ml/kg b.wt. (First dose). The third dose selected was twice of first dose i.e. 10.8 ml/kg b.wt. Second dose is average of first & third dose i.e. 8.1 ml/kg b. wt. (Ghosh *et al.,* 2008).

Experimental animals

Apparently healthy Wistar rats weighing 200-250 gm of either sex procured from National Institute of Biosciences; Bhor. Dist.Pune-411051 approved by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) laboratory animal house and used for the present research work. All the rats were maintained in Central Laboratory Animal House (CLAH) by Department of Veterinary Pharmacology and Toxicology, KNP College of Veterinary Science, Shirwal, under laboratory conditions of temperature 22±2 °C and allowed free access to standard pellet feed and water *ad libitum*. The experimental protocol was approved by the Institutional Animal Ethical Committee (IAEC) Reg. No.309/CPCSEA of KNP College of Veterinary Science, Shirwal. After 72 hrs of alloxan administration, blood glucose levels were analyzed and 60 (diabetic) and 24 (nondiabetic) rats equals to 84 were employed in the present study. All the rats were divided into seven groups comprising 12 animals with equal sex ratio in each group.

Experimental groups and their treatments:

After overnight fasting, administration of a single dose of Alloxan monohydrate at dose of 120 mg/kg b.wt. (i/p) supplied by (Avra synthesis, Hyderabad) prepared in sterile saline to all group (group B, C, D, E and F) except group A which served as normal control and group G served as cow urine control was carried out to induce hyperglycemia. During this period, the animals were given free access to water.

After 72 hrs. of Alloxanadministration, fasting blood glucose levels of rats were checked by glucometer (GlucoOne BG-03- Dr.Morepen) The animals having blood glucose levels 200 mg/dl and above were separated and selected for studies. The details pertaining to grouping of animals and details of treatment given is depicted in Table 1.

Biochemical studies

The blood samples were collected on day 0 (72 hrs. of Alloxan administration), 14th and 28th day post treatment in clean, dried, sterilized and anticoagulant free test tubes. The blood samples were processed for separation of serum. The serum was analyzed for blood urea nitrogen (BUN), serum creatinine, serum alanine transaminase (ALT), serum aspartate transaminase (AST), Total cholesterol and triglycerides and blood glucose estimation was carried out from vials containing sodium fluoride. The biochemical estimations were carried out by using serum auto analyzer as per the protocol mentioned in the literature of respective kit at CIF, KNP College of Veterinary Science, Shirwal.

Statistical analysis

The data obtained for various parameters was statistically analyzed by using WASP 2statistical software Completely Randomized Design (C.R.D.). All the values in the text were expressed as mean ± SE(Snedecor and Cohran, 1994).

RESULTS AND DISCUSSION

Alloxan administration to experimental animals resulted in a significant ($P<0.05$) rise in blood glucose levels. The changes in blood glucose levels, before and after treatment with the test drug in Alloxan induced diabetic animals are shown in Table 2. Blood glucose levels of untreated diabetic rats were significantly higher. Diabetic animals treated with cow urine distillate showed significant lowering of blood glucose levels ($P<0.05$).

Blood glucose levels of all the groups after 72 hrs of alloxan administration (to groups C, D, E, F and G) is depicted in Table 2. Among all the groups, glucose values at day 0 were significantly higher in alloxan treated groups viz groups C, D, E, F and G after 72 hrs. of alloxan treatment when compared to normal control group (Group A). The values of glucose in group C (Diabetic control group) were remained higher throughout the experimental period. Whereas group D receiving

glibenclamide as standard treatment of diabetes showed significant reduction in glucose values as compared to group C throughout the experimental duration.

Among the cow urine treated groups the animals showed reduction in glucose values at 14th and 28th day of treatment. It was observed that cow urine treatment at the dose of 8.1 and 10.8 ml/kg were able to reduce the increased blood glucose levels when compared to diabetic group (Group C) and the values were comparable to that of standard treatment group. It is obvious that cow urine distillate was able to reduce increased blood glucose levels in a dose dependent manner.

Biochemical analysis

Serum cholesterol, triglycerides and other biochemical levels in all the groups of treated diabetic animals are given in Table 3. The cholesterol, triglyceride, BUN, creatinine,AST, ALT levels were significantly higher in the untreated diabetic rats when compared with the values in normal rats.

There was significant increase in the values of blood urea nitrogen, Creatinine triglycerides, cholesterol, AST and ALT of only alloxan treated group when compared normal control group throughout the experimental period. Whereas all the values in normal group were found within the normal range. Similarly biochemical values of group receiving standard antidiabetic drug glibenclamide was found to be nonsignificant and in normal range on day 28th of the experiment except AST and ALT levels and also showing significant difference than alloxan induced group.

Treatment groups receiving cow urine at different dose levels were showing ability to reduce values of BUN, creatinine, triglycerides, cholesterol, AST and ALT when compared to diabetic group receiving only alloxan. Among all the cow urine treated group, group receiving higher dose of cow urine (10.8ml/kg) had shown greatest recovery regarding biochemical parameters studied.

The treatment of distilled cow urine at variable dose levels in group E, F and G was not significant as group D to lower AST levels. However, as compared to group C (diabetic control), AST levels in groups treated with distilled cow urine (group D, E and F) were found to be significantly less suggestive of distilled cow urine treatment.

Distilled cow urine at variable dose levels in groups D, E and F was not significant to lower the ALT values as compared with group D (treated with Glibenclamide). However, as compared to group C (diabetic control), the ALT levels in groups treated with distilled cow urine (group D, E and F) were found to be significantly less suggestive of distilled cow urine treatment.

The treatment of distilled cow urine at variable dose levels in group D, E and F was significant to lower the BUN levels as compared with group D (treated with glibenclamide). However, as compared to group B (diabetic control), the BUN levels in groups treated with distilled cow urine (group D, E and F) were found to be significantly lowered level like glibenclamide. Benjamin (2001) stated that the prerenal causes such as dehydration and renal diseases increase the levels of the serum BUN(Nikkila, and Kekki, 1973) In the present study, increase of BUN levels in group B rats might be associated with increase in glomerular pressure due to hyperglycemic conditions, while in group C the diabetic rats fed with drug glibenclamide exhibited decrease BUN levels due to its hypoglycemic effect and nephroprotective effect.

Distilledcow urine when administered at variable dose levels in group D, E and F was not significant to lower the serum triglyceride values as compared with group D (Glibenclamide treated). However, as compared to group C (diabetic control), the serum triglyceride levels in groups treated with distilled cow urine (group D, E and F) were found to be significantly less suggestive to decrease triglyceride levels in diabetic group of distilled cow urine treatment.The observed hypolipidemic effect may be because of decrease of fatty acid synthesis because of distilled cow urine supplementation to the diabetic animals. Significant lowering of triglycerides is a desirable biochemical state for prevention of diabetes and its complications (Gururaja *et al.,* 2011).

The treatment of distilled cow urine at variable dose level in groups D, E and F was not significant to lower the serum total cholesterol values as compared with group D (treated with Glibenclamide). However, as compared to group C (diabetic control), the serum cholesterol levels in groups treated with distilled cow urine (group D, E and F) were found to be significantly less suggestive of distilled cow urine treatment.In this study, the marked increases in total cholesterol observed in Alloxan induced diabetic rats agree with the findings of Nikkila and Kekki (1973). (Gururaja *et al.,* 2011) also observed that the cholesterol levels were significantly higher in the untreated diabetic rats compared with the values in normal rats. The treated diabetic rats had lower levels of cholesterol compared with those in the untreated diabetic group. The treatment with cow urine distillate produced almost normal levels of cholesterol.

Serum creatinine values of animals treated with distilled cow urine at variable dose levels in group D, E and F was significant to lower the as compared with group D (treated with Glibenclamide). However, as compared to group C (diabetic control), the creatinine levels in groups treated with distilled cow urine (group D, E and F) were found to be significantly better suggestive of distilled cow urine treatment. Nagy and Amin, 2015) stated that kidney function markers like

Table 1:
Details of treatment groups

Group	Treatment	Dose
Group A (Normal control)	No treatment	Ad lib feed and water
Group B (cow urine control)	Cow urine distillate	10.8 ml/kg body weight for 28 days
Group C (Diabetes control)	Alloxan	Single dose 72 hrs before start of experiment at dose rate of 120mg/kg body weight
Group D (standard treatment)	Glibenclamide (after 72hrs of alloxan treatment IP 120mg/kg body weight)	0.25 mg/kg b. wt. p/o.
Group E (treatment 1)	Distilled cow urine (after 72hrs of alloxan treatment IP120mg/kg body weight)	5.4 ml/kg b. wt. p/o.
Group F (treatment 2)	Distilled cow urine (after 72hrs of alloxan treatment IP 120mg/kg body weight)	8.1 ml/kg b. wt. p/o.
Group G (treatment 3)	Distilled cow urine (after 72hrs of alloxan treatment IP120mg/kg body weight)	10.8 ml/kg b. wt. p/o.

Table 2.
Results of blood glucose levels (mg/dl) at different time points.

	Blood glucose levels in mg/dl		
Group	Day 0 (after 72hrs of alloxan administration)	Day 14th	Day 28th
Group A (Normal control)	96.83^{b}±6.25	96.92^{d}±6.05	97.08^{e}±5.25
Group B (cow urine control)	104.58^{b}±28.05	103.58^{d}±7.81	102.00^{e}±7.68
Group C (Diabetes control)	348.08^{a}±5.22	384.08^{a}±6.22	320.83^{a}±9.27
Group D (standard treatment)	312.50^{a}±17.79	192.92^{c}±16.33	118.92^{d}±3.00
Group E (treatment 1)	316.58^{a}±36.22	251.25^{b}±21.89	170.67^{b}±4.69
Group F (treatment 2)	313.83^{a}±30.61	206.17^{c}±16.06	162.42^{b}±2.53
Group G (treatment 3)	310.50^{a}±21.07	200.67^{c}±14.80	140.50^{c}±2.10

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

Table 3:
Groupwise biochemical observations on 28th day

Group	BUN (mg/dl)	Creatinine (mg/dl)	Triglycerides (mg/dl)	Cholesterol (mg/dl)	AST (U/L)	ALT (U/L)
Group A (Normal control)	16.78^{c}±0.18	0.33^{d}±0.34	75.70^{c}±0.92	71.24^{d}±1.20	136.90^{e}±10.58	36.40^{e}±0.8
Group B (cow urine control)	17.50^{bc}± 0.22	0.30^{d}±0.00	73.91^{c}±0.51	67.24^{d}±1.70	133.75^{e}±2.3	35.96^{e}±0.4
Group C (Diabetes control)	36.48^{a}±0.96	1.77^{a}±0.4	193.42^{a}±1.0	177.40^{a}±6.8	185.6^{a}±1.18	77.27^{a}±0.7
Group D (standard treatment)	17.18^{bc}±0.18	0.40^{c}±0.01	88.60^{c}±1.60	73.95^{cd}±0.76	144.67^{d}±1.13	42.95^{d}±0.6
Group E (treatment 1)	18.34^{b}±0.38	0.48^{b}±0.01	137.22±2.12	83.95^{b}±0.83	153.05^{b}±0.77	53.73±0.6
Group F (treatment 2)	18.27^{b}±0.16	0.46^{b}±0.02	131.35±2.91	81.90^{b}±0.62	152.62^{bc}±1.1	52.76^{bc}±1.2
Group G (treatment 3)	17.57^{bc} ±0.23	0.44^{bc}±0.01	124.83^{b}±2.71	78.17^{bc}±0.31	149.15^{c}±1.4	48.03^{cd}±0.5

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

urea and creatinine were elevated in the Alloxan induced diabetic rats when compared with the normal rats.

There was significant increase (P<0.05) in blood glucose in Alloxan induced diabetic rats group C as compared to normal control group A. On the contrary, the diabetic rats treated with distilled cow urine doses of 5.4 ml/kg, 8.1 ml/kg and 10.8 ml/kg b.wt. exhibited significant decrease (P<0.05) in the values of blood glucose as compared to diabetic control rats (group C). (Gururaja *et al.,* 2011) showed that diabetic animals treated with cow urine distillate revealed a significant reduction in the blood glucose level when compared with diabetic control groups at the end of the experimental period. Diabetic animals treated with cow urine distillate showed significant lowering of blood glucose levels.

The marked increase observed in serum triglycerides and cholesterol in untreated diabetic rats is in agreement with the findings of Nikkila&Kekki (1973). The treated diabetic rats had lower levels of cholesterol, triglycerides, BUN, creatinine in the untreated diabetic group. The treatment with cow urine distillate produced almost normal levels of cholesterol, triglyceride, BUN and creatinine. It can be concluded from the present study that cow urine distillate can become alternative to chemical drugs in the treatment of hyperglycemia and proven to be a restorative regimen for alterations caused due to diabetes.

REFERENCES

Achliya, G.S., Kotagale, N.R. Wadodkar,S.G. and Dorle, A.K. (2003) Hepatoprotective activity of *panchagavyaghrita* against carbontetrachloride induced hepatotoxicity in rats. *Indian J. Pharmacol.* **35 (5)**: 308-311.

Cotton, R. B., Cornelius, L.M. and Theran, P. (1971) "Diabetes mellitus in the dog: a clinicopathologic study." *Amer Vet. Med. Ass. J.*

Dhama, K., Rathore, R., Chauhan, R.S. and Tomar, S. (2005) Panchgavya (cowpathy): An Overview. *Int. J. Cow Sc.***1(1):** 1-15

Engerman, R.L. and Kramer, J.W. (1982) "Dogs with induced or spontaneous diabetes as models for the study of human diabetes mellitus." *Diabetes.* **31(1)** : 26-29.

Foster, S.J. (1975) "Diabetes mellitus A study of the disease in the dog and cat in Kent." *J. Small Animal Practice.* 16, no.**1 12**: 295-315.

Ghosh, S., Thomas, P., Gloria,Y., Girish,K. Ding An, Dake Qi, Ashraf, A. and Brian, R. (2005) "Cardiomyocyte apoptosis induced by short-term diabetes requires mitochondrial GSH depletion." *Am. J. of Physiol. Heart and Circulatory Physio.* **289 (2):** H 768-H 776.

Gururaja, M.P, Joshi, A.B., Joshi, H., Sathyanarayana, D., Subrahmanyam, E.V. and Chandrashekhar, K.S. (2011). Antidiabetic potential of cow urine in Streptozotocin induced diabetic rats. *Asian J. Tradit Med.* **6**: 8-13.

Lenzen, S. (2008) The Mechanisms of Alloxan and Streptozotocin induced Diabetes. Diabetologia. **51(2):** 216-226.

Ling, G.V., Lowenstine, L.J., Pulley, L.T., and Kaneko, J. J. (1977) "Diabetes mellitus in dogs: a review of initial evaluation, immediate and long term management, and outcome." *J. Am.Vet. Med. Asso.* **170 (5)** : 21-530.

Nagy, M.A., and Amin, K.A. (2015) "Biochemical and histopathological analysis of Cystoseiramyrica aqueous extract on Alloxan induced diabetic rats." *BioChemistry: An Indian J.* **9,(3)**81-91.

Nikkila, E.A. and Kekki,M. (1973) Plasma transport kinetics in diabetes mellitus. Metabolism: **22**.

Sachdev, D.O, D.D.Gosavi, K.J.Salwe (2012) Evaluation of antidiabetic, antioxidant effect and safety profile of gomutra ark in wistar albino rats. *Anc Sc. Life.* **31**: 84-9.

Snedecor, G.W. and Cohran W.G. (1994) Statistical method 8th edn. Oxfard and IBM publishing Co.New Delhi- 59. 527-537.

Wild, S., Roglic, G., Green, A., Sicree, R. and H. King (2004) Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. *Diabetes Care.* **27**: 1047-53.

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Research Article

IMMUNOTOXIC EFFECTS OF ACETAMIPRID FOLLOWING SUBCHRONIC EXPOSURE IN SWISS ALBINO MICE

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ABSTRACT

Acetamiprid, a neonicotinoid insecticide has been in extensive use worldwide for several years in agriculture and veterinary medicine. The objective of the present study was to evaluate immunotoxic effects of acetamiprid following subchronic exposure in male Swiss albino mice. Acetamiprid was administered i.p. daily at 4.6 and 2.3 mg/kg/day along with 3% gum acacia as negative control for 60 and 90 days. Specific parameters of humoral and cellular immune response including hemagglutinating antibody (HA) titer to sheep red blood cells (SRBC; T-dependent antigen) and delayed type hypersensitivity (DTH) response to SRBC was evaluated. Histopathology of spleen and footpad was also done. The result showed that acetamiprid suppressed both humoral and cell mediated immune response as evident from decrease in HA titer and decrease in DTH response. In 60 days treatment groups, significant decrease in HA titer and DTH response was only observed at 4.6 mg/kg/day. In 90 days treatment groups, significant decrease in HA titer and DTH response was observed at 4.6 and 2.3 mg/kg/day from control group. At these dose levels, there were prominent histopathological alterations in spleen and footpad sections. The results indicated that acetamiprid has immunosuppressive effects at both dose levels which could potentially be attributed to direct cytotoxic effects of acetamiprid against T cells and that long term exposure could be detrimental to the immune system.

Key words: Immunotoxicity, acetamiprid, Swiss albino mice, hemagglutinating antibody (HA) titer, delayed type hypersensitivity (DTH) response

INTRODUCTION

Pesticides are the chemical formulations increasingly used in agriculture, animal husbandry and public health operation to kill the insects, weeds and fungi and to get rid of insect transmitted diseases. The frequent and continuous use of pesticide has resulted in their widespread distribution in the environment. These pesticides are toxic not only to insects and pests but at different levels to animals and human beings (Mondal *et al.,* 2012). These agrochemicals, if not properly used, may pose serious hazards to human and animal health. Therefore, the present-day concern is with regard to their judicious and proper use, so that they can be applied safely with proper instructions and guidance to have minimum risk to human and animal health. The neonicotinoids, the newest major class of insecticides developed in the past three decades, have outstanding potency and systemic action for crop protection against piercing- sucking pests and they are highly effective for flea control on cats and dogs (Tomizawa and Casida, 2005). Several neonicotinoids are harmful to honeybees, either by direct contact or ingestion. This may lead to a reduction in pollination (Blacquiere and Smugghe, 2012). The neonicotinoids are related to nicotine in their structure and are potent selective agonists of the nicotinic acetylcholine receptor (nAChR) in both invertebrates and vertebrates (Bagri *et al.,* 2015). Acetamiprid (ACP), a member of the neonicotinoid insecticide family, is widely used in floriculture, on leafy vegetables, cole crops, citrus, cotton, ornamentals, and fruiting vegetables. The studies found ACP concentration in bee collected materials, i.e., pollen, bee bread, honey and beeswax (Genersch *et al.,* 2010). Widespread use of ACP is causing pesticide entry into the food chain, which in turn causes toxicity to man and animal. Information regarding selectivity of insecticide for judicial use may be generated only through properly designed laboratory and field studies.

Exposure of animals to residual concentration of pesticides can lead to immunosuppression either directly or with the participation of stress mechanisms neuroendocrine system (Kacmar *et al.,* 1999). The analysis of toxic properties of drugs and chemicals using in vivo mammalian systems (mice or rat) is of enormous value which reflects indirect toxic effects on humans because of their high degree of presumptive human relevance (Bagri *et al.,* 2013). Thus, the present investigation was undertaken to explore the impact of 60 and 90 days exposures of acetamiprid on the humoral and cell-mediated immune responses of Swiss albino mice.

MATERIALS AND METHODS

Experimental animals

Mice were housed in polystyrene cages (six/cage) with *ad libitum* access to standard pellet feed and filtered tap water. The room was maintained under a 12/12 h light–dark cycle, an ambient temperature of

20–25 æ%C, and a relative humidity of 45 (±15)%. All mice were housed for 1 week for acclimatization before start of experiment. All animal experiments were approved by Institutional Animal Ethics Committee (IAEC) of the University.

Chemicals

Technical grade acetamiprid was provided by Tropical Agrosystem (India) Pvt. Limited, Chennai. Dexamethasone, cyclophosphamide and Freund's complete adjuvant were purchased from Sigma (St. Louis, MO). Dulbecco's phosphate-buffered saline (DPBS; without calcium or magnesium) and hematoxylin and eosin (H&E) stains were procured from HiMedia Laboratories Pvt. Ltd. (Mumbai).

Doses and exposure schedules

The selection of two test doses of acetamiprid was based on the MTD for technical grade acetamiprid, (46 mg/kg b.wt.) (Karthikeyan *et al.,* 2012). Based on this initial value, two MTD-based test (toxic) doses were proposed for use: (i) 4.6 mg/kg (10% of MTD; high); (ii) 2.3 mg/kg (5% of MTD; low). Animals were divided into four groups (6mice/group); two groups treated with two different acetamiprid doses, one positive control group (received dexamethasone [DXM] or cyclophosphamide [CYP], depending on experiment), and one negative control group (gum acacia, vehicle control). Acetamiprid suspension was prepared in gum acacia vehicle. Mice in the two test groups were then administered intraperitoneally, 4.6 and 2.3 mg acetamiprid/kg in appropriate volumes. Animals in the test groups were administered acetamiprid daily for 60 and 90 days. The negative control mice received gum acacia (3% solution in distilled water) i.p. daily for 60 and 90 days. Positive control mice were administered CYP (55 mg/kg, i.p.) or DXM (10 mg/kg, i.p.) daily for 5 days, using fresh solutions of DXM (1 mg/ml) and CYP (5.5 mg/ml) prepared in sterile normal saline. Each acetamiprid suspension in gum acacia was also prepared fresh daily and thoroughly vortexed before administering i.p. All dosing were performed between 11:00 and 13:00 each day as far as possible; body weights were recorded daily prior to the time of dosing. Antigen, i.e. sheep red blood cells (SRBC), was injected in mice as a single injection 6–8 h after completion of one day acetamiprid dosing (specific days indicated below) in the experiments examining hemagglutination antibody titers and DTH response.

Non-functional assays

Body and organ weights

Mice from each group were weighed daily just before dosing and at the time of autopsy to record their mean body weights. At the end of the experimental trial (i.e. after 60 and 90 days), mice were euthanized by chloroform over-anesthetization. The spleen, liver and thymus were then excised, lightly blotted on tissue paper, and weighed; all data were expressed as relative organ weight.

Histopathology

The spleen and footpad tissues were placed in 10% buffered formalin. Thereafter, paraffin-embedded sections of these tissues were cut (5–6 mm thickness) and stained with H&E.

Functional assays

Hemagglutinating antibody (HA) titer

Fresh blood from healthy sheep (collected in sterile Alsever's solution) was washed (centrifuged for 800×*g* for 10 min at 4 æ%C) three times with sterile DPBS. The pelleted sheep red blood cells (SRBC) were then diluted to 1.5×109 cells/ml with DPBS for immunization. For evaluation of HA titer, separate sets of treated/control mice were immunized by an intraperitoneal (IP) injection of 0.3 ml of the SRBC suspension (4.5 $\times 10^8$ cells/ mouse) 10 days before completion of the treatment period (Elsabbagh and El-Tawil, 2001). At the end of the experimental period (day 61 and 91 for acetamiprid treated and negative control groups/day 5 for CYP-treated mice group), sera were prepared from peripheral blood samples from each immunized mouse and de-complemented (56 ^{0}C, 30 min). To prevent non-specific agglutination, a 1% (v/v) SRBC suspension was prepared in DPBS containing 1% (w/v) bovine serum albumin. The microtiter HA technique was then employed to determine serum antibody titer. Serial 2-fold dilution of each serum sample was made in 96-well U-bottom microtiter plates; an equal volume of 1% SRBC suspension was then added to each well and the plate was incubated for 2 h at room temperature. The reciprocal of the highest dilution yielding hemagglutination was taken as the antibody titer. Serum samples of mice from all four groups were tested again for confirmation of HA titer.

Delayed-type hypersensitivity (DTH) response

DTH response (using SRBC as antigen) was assessed as described by Hassan *et al.* (2004), with some modifications. Ten day before completion of the exposure period (or day 2 for DXM control group), mice were sensitized by a subcutaneous (SC) injection into their back with 50 µl of SRBC (10^0 cells) suspended in Freund's complete adjuvant (FCA). After 10 days, these sensitized mice were challenged (under light ketamine [100 mg/kg body weight, IP] anesthesia) by injecting 50 µl of SRBC (10^0 cells) into their right hind footpad. Swelling in the right hind footpad was measured using a pressure sensitive micrometer screw gauge (Mitutoyo, Kawasaki, Japan) 24 and 48 h post-challenge. After the final measurement, the mice were euthanized and footpad sections prepared for histopathological examination to evaluate cellular changes in DTH

response. In brief, the right footpad was isolated from each host, immediately placed in 10% buffered formalin, and then processed for histopathology in a manner similar to other organs as above.

Statistical analysis

The results are presented as mean ± S.E. The data were analyzed by one way ANOVA using SPSS statistics 17 software and the means were compared by employing Duncan's multiple comparisons as post hoc test. A p value of <0.05 was considered as statistically significant.

RESULTS

Organ weight

Liver, spleen and thymus were weighed and mean weight of mice from each group was recorded and expressed as relative weight for each organ. Table 1 presents relative weight of spleen, liver and thymus for each group of mice. There was no significant difference observed in organ weight of liver and spleen, whereas significant difference in thymus weight was observed at 4.6 mg/kg b.wt. administered for 60 and 90 days.

Hemagglutination antibody (HA) titer

Hemagglutination titer against SRBC, a T cell dependent antigen was expressed as $\log_2$ antibody titer and presented in Table 2. In 60 days treatment groups, significant decrease in HA titer was observed at higher dose level group (4.6 mg/kg/day) whereas in 90 days treatment groups, significant decrease in HA titer was observed at both lower and higher dose groups (4.6 and 2.3 mg/kg/day) from control group. The CYP treatment led to a near-complete abrogation of response.

Delayed-type hypersensitivity (DTH) response

DTH response to SRBC was characterized by intense local inflammatory reaction with erythema, edema, vesiculation, and swelling in the vehicle control mice (Fig. 1). In ACE-treated mice, the intensities of these symptoms/inflammatory reactions were inversely

Table 1:
Effect of Acetamiprid on relative organ weights (g/100g body weight) of mice

Experiment	Treatment (mg/kg, i.p.)	Liver	Spleen	Thymus
60days	Control (300)	4.88±0.39^{a}	0.48±0.04^{a}	0.14±0.01^{a}
	ACE (2.3)	4.94±0.16^{a}	0.46±0.03^{a}	0.16±0.01^{ab}
	ACE (4.6)	5.25±0.28^{a}	0.46±0.02^{a}	0.19±0.02^{b}
	CYP (50)	5.52±0.20^{a}	0.35±0.04^{b}	0.10±0.01^{c}
90days	Control (300)	5.57±0.58^{a}	0.62±0.06^{a}	0.14±0.01^{a}
	ACE (2.3)	5.93±0.36^{a}	0.59±0.04^{a}	0.12±0.01^{a}
	ACE (4.6)	6.11±0.30^{a}	0.54±0.05^{a}	0.12±0.01^{ab}
	CYP (50)	6.52±0.34^{a}	0.16±0.01^{b}	0.09±0.01^{b}

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

Table 2:
Effect of Acetamiprid on serum antibody titer against SRBC in Swiss albino mice

Experiment	Treatment (mg/kg, i.p.)	Log_2 Antibody Titre
60 days	Control (300)	5.83±0.40^{a}
	ACE (2.3)	4.83±0.40^{ab}
	ACE (4.6)	4.33±0.21^{bc}
	CYP (50)	3.50±0.43^{c}
90 days	Control (300)	7.50±0.43^{a}
	ACE (2.3)	6.00±0.36^{b}
	ACE (4.6)	5.67±0.33^{bc}
	CYP (50)	4.67±0.21^{c}

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

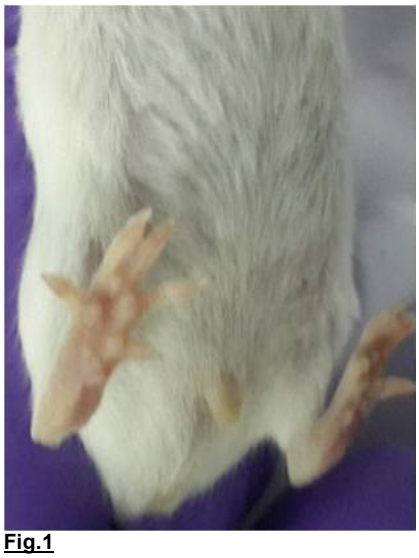

Fig.1
Vehicle control group showing severe swelling; DTH reaction (after 48 h) in right hind foot pad of Swiss albino mice.

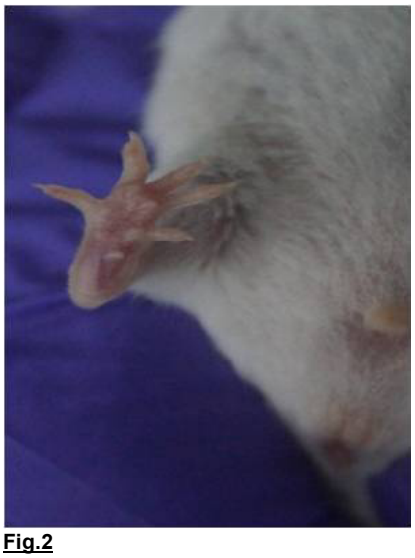

Fig.2
Acetamiprid treated showing less swelling; DTH reaction (after 48 h) in right hind foot pad of Swiss albino mice.

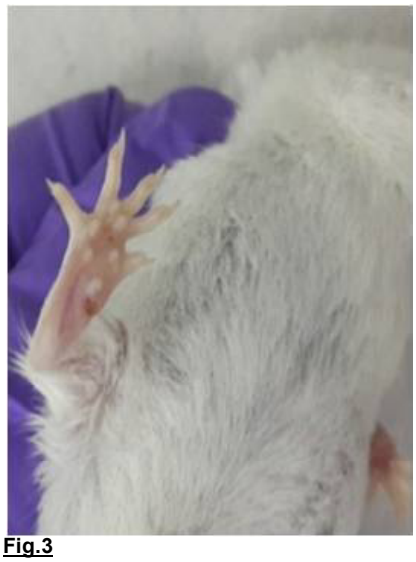

Fig.3
Dexamethasone treated showing no swelling DTH reaction (after 48 h) in right hind foot pad of Swiss albino mice

related to the dose of ACE administered, i.e. at high dose, only mild inflammatory reaction with mild edema and erythema was noted. Mice in the low dose group showed reactions nearly similar to those of the vehicle control (Fig. 2). The DXM-treated mice group showed only very mild inflammatory reactions, very mild edema, and negligible swelling (Fig. 3).

In evaluation of DTH response to SRBC, maximum increase in paw thickness (in mm) was measured after 24 hours and 48 hours of challenge and calculated as percent increase in paw thickness which is then expressed as % DTH response. The results are expressed as percent DTH response 24 and 48 hours later and presented in Table 3.

Histopathology

Histopathological examination of spleen revealed mild depletion in splenocytes and megakaryocytes in one month treated animals as compared to control group. Spleen parenchyma of three month treated animals showing severe degeneration of lymphocytes with vacuolation in white pulp and severity of depletion increased with increase in treatment level and treatment period (Fig.4 and 5).

Histopathological examination of foot pad was done for evaluating cellular changes in DTH response and revealed significant pathological alterations. Foot pad sections of vehicle control mice group showed intense reaction and large number of inflammatory cells such as macrophages, lymphocytes along with few neutrophils/polymorphonuclear cells present in dermis. Some of the mononuclear cells were also present in deep dermis portion of foot pad section. Foot pad of mice from lower toxic dose group exhibited moderate to good inflammatory reaction as compared to control. Cells such as macrophages and lymphocytes were present, but in less number than that of control indicating moderate suppression of DTH response. Foot pad sections of mice from higher toxic and dexamethasone treated dose group revealed very mild inflammatory reaction with very few macrophages and lymphocytes in dermis portion of foot

Table 3:
Effect of Acetamiprid on DTH response in Swiss albino mice

Experiment	Treatment (mg/kg, i.p.)	% DTH Response	
		24 hrs later	48 hrs later
60 days	Control (300)	36.48±1.42^{a}	26.95±2.03^{a}
	ACE (2.3)	33.02±1.90^{a}	18.99±3.58^{ab}
	ACE (4.6)	24.28±2.58^{b}	16.75±2.34^{bc}
	Dexamethasone	14.70±3.83^{c}	9.05±2.62^{c}
90 days	Control (300)	31.40±2.36^{a}	25.36±3.62^{a}
	ACE (2.3)	22.67±2.86^{b}	14.94±1.99^{b}
	ACE (4.6)	19.73±3.15^{b}	11.83±2.96^{b}
	Dexamethasone	10.28±2.23^{c}	7.18±1.11^{b}

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

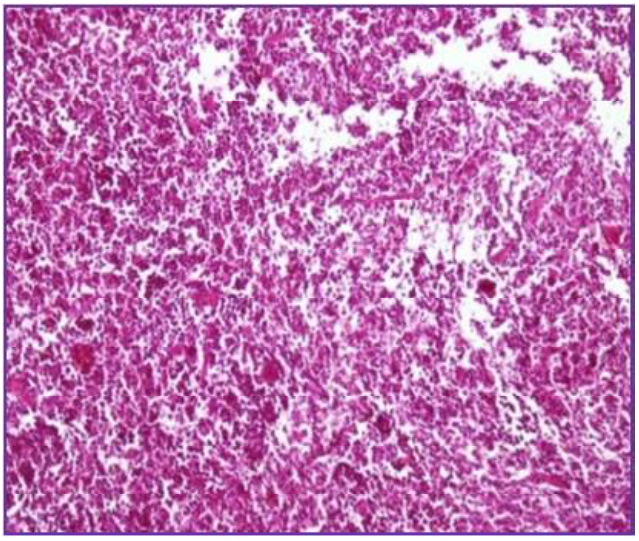

Fig.4
Spleen mild depletion of splenocyte and megakaryocytes.

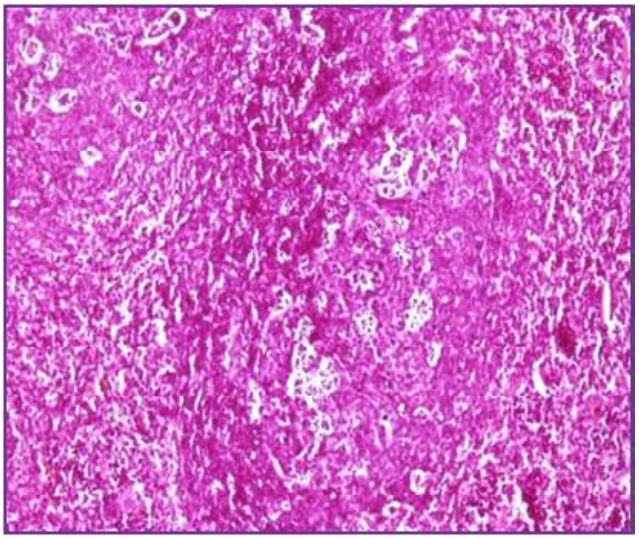

Fig.5
Spleen (3 month) spleen parenchyma showing degeneration of lymphocyte with vacuolation in white pulp

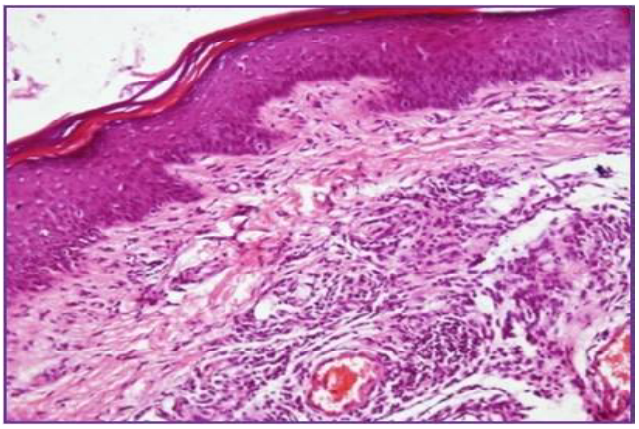

Fig.6
Footpad (control) severe infiltration of mononuclear cells and neutrophils in skin

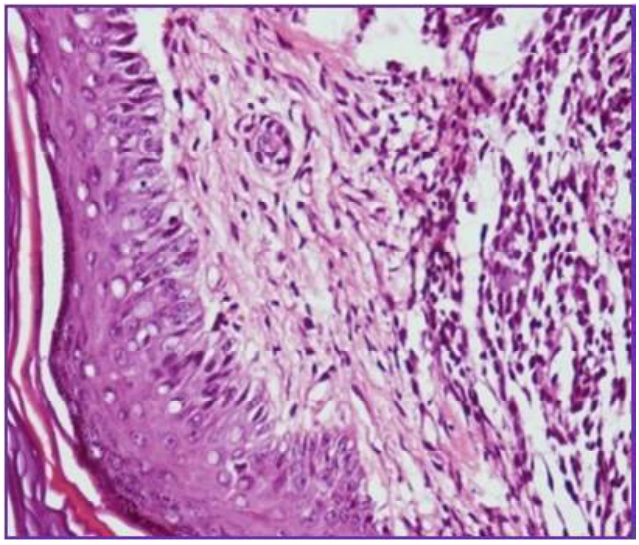

Fig. 7
Footpad (3 month) moderate infiltration of mononuclear cells and neutrophils in skin.

pad (intensity of reaction in dexamethasone treated mice group was still lower/milder than that of higher toxic dose group), suggesting marked suppression of DTH response (Fig.6 and 7).

DISCUSSION

The immunosuppressive effects of pesticides may also be associated with an increased cancer risk; as, an increase in the number of cases has been observed among agricultural workers (Sathiakumar *et al.,* 2011). Toxicological studies of acetamiprid are limited. Immunomodulatory effects of various xenobiotic classes, including polycyclic aromatic hydrocarbons, halogenatedaromatic hydrocarbons, heavy metals, organochlorine, organophosphorous, carbamates and other majority of pesticides, have been relatively well characterized (Blakley *et al.,* 1999; Luebke *et al.,* 2007), but not those of the neonicotinoid insecticides. Along with recent development in the area of immunotoxicology in the last decade, agrochemical compounds (such as insecticides) have been presented asimportant candidates for the testing of immunotoxic potential in order to determine 'no observable adverse effect levels' (NOAEL) (Thomas, 1998; Vohr and Ruhl-Fehlert, 2001). Despite overt use of acetamiprid in agriculture and veterinary medicine, there is little information about its immunotoxicity and its NOAEL in mice. Recently, a developmental immunotoxicity study in Wistar rats has shown age-related, dose-dependent developmental immunotoxic effects of imidacloprid (IMD) and clearly demonstrated developmental immunotoxicity as one of thepotential risk associated with IMD exposure at high doses (Gawade *et al.,* 2013). Specifically, other studies have shown IMD to be immunosuppressive in the rat (Gatne *et al.,* 2006) and the White leghorn cockerel (Siddiqui *et al.,* 2007) models. Available literature in these animal models shows IMD to be immunotoxic, but mechanisms involved therein have not been demonstrated. Efforts have been made in the present investigation (as far as possible) to evaluate parameters described by Jong and Loveren (2007), who defined parameters indicative of direct immunotoxicity into non-functional and functional assays. Also, cyclophosphamide treated mice group exhibited severely decreased antibody titer which was even significant at 1% level of significance. Kawani (2007) reported dose dependent decrease in antibody titer of imidacloprid treated groups when evaluating immunotoxic effect of imidacloprid in BALB/c mice. Gatne *et al.* (2006) also reported progressive and proportional decrease in haemagglutination antibody titer in Sprauge-Dawley rats treated with different doses of technical grade imidacloprid. Banerjee *et al.* (1996) reported significant influence of subchronic exposure of lindane on humoral immunity in mice. Lindane suppressed both primary and secondary antibody responses to SRBC, the effects being more pronounced on the secondary than primary response. Willtrout *et al.* (1978) found significant suppression of the humoral immune response following orally administered pesticides (carbaryl, DDT, parathion, chlordneform and ametryne) in mice at near lethal doses. The primary and secondary hemolysin titers against SRBCs were reduced to 62% and 63% of control values for cupravit and 76% and 76% of control values for pervicur (Elsabbagh and Tawil, 2001).

The effect of subchronic exposure to acetamiprid on cellular immune response in Swiss albino mice was assessed with the help of delayed type hypersensitivity (DTH) response to SRBC.

Delayed type hypersensitivity (DTH) response to SRBC was characterized by intense inflammatory reaction with erythema, edema, vesiculation, swelling in vehicle control mice group. There was progressive increase in skin thickness after injecting challenging dose of SRBC in all mice of vehicle control group maximum being after 24 hours. In acetamiprid treated mice groups, the gross symptoms or inflammatory reaction was inversely proportional to the doses of acetamiprid administered i.e. at higher dose mild inflammatory reaction was observed with mild edema, erythema and mild swelling, and the lower dose group showed reaction nearly similar to that shown by the mice of vehicle control group. Also, dexamethasone treated mice group showed very mild inflammatory reaction with very mild edema and negligible swelling was seen.

Maximum increase in paw thickness (in mm) was measured 24 hours and 48 hours after challenge

and calculated as percent increase in paw thickness which was then expressed as % DTH response. In 30 and 60 Days treatment groups significant decrease in DTH response was observed at higher dose level (4.6 mg/kg b.wt.) both in 24 hrs and 48 hrs later. In 90 days treatment groups significant decrease in DTH response was observed at both lower and higher dose level in 24 hrs and 48 hrs later, it was suppressed significantly as compared to control.

Histopathological evaluation of foot pad also revealed significant pathological or cellular alterations. Foot pad sections of vehicle control mice showed intense reaction and large number of inflammatory cells such as macrophages, lymphocytes along with few neutrophils/polymorphonuclear cells were present in dermis and in epidermis. Some of the mononuclear cells were also present in deep dermis portion. Foot pad sections of mice from lower dose group exhibited moderate to good inflammatory reaction as compared to control. Cells such as macrophages and lymphocytes were present, but in less number than that of control indicating moderate suppression of DTH response. Foot pad sections of mice from higher dose group and dexamethasone-treated group revealed very mild inflammatory reaction with very few macrophages and lymphocytes in dermis portion of foot pad (intensity of reaction in dexamethasone treated mice was milder than that of higher dose group), suggesting marked suppression of DTH response.

The finding of DTH response clearly indicated that acetamiprid had immunotoxic effects and at higher toxic dose i.e. 4.6 mg/kg/day, it suppressed cellular immune response. DTH response involves T effector cells (Danneberg, 1991) and its suppression at higher dose of acetamiprid suggests that acetamiprid has direct cytotoxic effect on T effector cells. Following interaction with a specific antigen, the effector cells are responsible for elaboration of lymphokines. In the DTH reaction, the primary lymphokine response involved appears to be responsible for the accumulation of mononuclear cell infiltrates, mononuclear cell interaction and increased vascular permeability that occur in the vicinity of stimulus (Luster *et al.,* 1982). Neishabouri *et al.* (2004) also reported suppression of DTH reaction evaluated against SRBC at highest and middle toxic dose (25 mg/kg/day and 2 mg/kg/day for 28 days) of diazinon in C57BL/6 mice. High doses of propoxur (10 mg/kg) also suppressed the DTH response 24 hours after secondary injection of antigen (SRBCs); however there was no significant change in DTH response after 48 hours of injection at this dose rate. Propoxur at lower doses (0.2 and 2 mg/kg) did not change DTH response after 24 and 48 hours of challenge (Hassan *et al.,* 2004).

Mondal *et al.* (2009) studied the effects of acetamiprid on immune system in female Wistar rats and revealed decrease in mean TLC and lymphocyte count with significant decrease in spleen weight and decrease in globulin. Acetamiprid also suppressed both CMI and antibody forming ability of lymphocytes. Devan *et al.* (2015) studied the subchronic effect of acetamiprid (20% SP-soluble powder) causes significant decreases in the lymphocyte proliferation as well as the macrophage function at the dose level of 110 mg/kg. The fungicides cupravit and pervicur were evaluated for immunotoxic potential and significantly inhibited DTH reaction to tuberculin revealing suppression of cell-mediated immune response (Elsabbagh and Tawil, 2001). Gatne *et al.* (2006) also reported that imidacloprid at 160 mg/kg/day decreased DTH response against SRBC in Sprague-Dawley rats which was statistically non-significant. There are two basic mechanisms by which xenobiotics may induce suppression of the immune system: (1) by direct action of the xenobiotics upon the lymphoid organs or cells involved in the immune response and (2) by indirect action of the xenobiotics on other organ or physiological systems, such as neuroendocrine interactions, metabolic activation of xenobiotics to toxic metabolites or hepatic modulation which then impact immune response (Ladics and Woolhiser, 2007).

Significant reduction in spleen cell cellularity in cyclophosphamide treated mice group was quite obvious, since it is known immunosuppressive agent which causes general reduction in lymphocytes as well as alterations in lymphocyte function. The mechanism of action of cyclophosphamide is indirect, that involves metabolic activation or bioactivation. Cyclophosphamide is a prototype member of a class of drugs known as alkylating agents. Upon entering the cell, the inactive non-cytotoxic drug is converted into phosphoramide mustard, a DNA alkylating agent that inhibits cell replication (Shand, 1979). Since spleen is important for immune functions, histological changes in spleen may reflect as dysfunction. Histopathological evaluation of spleen revealed dose-dependent depletion of lymphocytes in white pulp of spleen and confirms our findings of reduced relative weight of spleen. Thus, acetamiprid may induce cell death of splenocytes by tissue destruction. Kim *et al.* (2007) also reported that sub-chronic oral exposure with pirimiphos-methyl at 120 mg/kg/day for 28 days produced significant decrease in relative spleen weight and splenic cellularity in BALB/c mice.

Histopathological examination of spleen not only revealed depletion of lymphocytes, but also presence of neutrophils and reticulo-endothelial cells in white pulp at higher toxic dose of acetamiprid. This indicates tissue destruction and injury.

The present study clearly demonstrates

immunotoxicity as one of the potential risks associated with chronic exposure to ACP at high dose possibly leading to immunocomprised state in humans and caution should be taken to avoid direct or indirect exposure to ACP through residues and by occupational means. At the same time, frequent assessment of pesticide residues and further studies are warranted to better characterize these toxicities/mechanisms therein observed here.

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REFERENCES

Bagri P, Kumar V, Sikka A K and Punia J S. (2013). Preliminary acute toxicity study on imidacloprid in Swiss albino mice. *Vet. World* **6:** 955-59.

Bagri P, Kumar V and Sikka A K. (2015). An in vivo assay of the mutagenic potential of imidacloprid using sperm head abnormality test and dominant lethal test. *Drug and Chem. Toxicol.* **38**: 342-48.

Banerjee B D, Koner B C, Ray A and Pasha S T. (1996). Influence of subchronic exposure to lindane on humoral immunity in mice. *Indian J. Expertl. Bio.* **34**: 1109-13.

Blacquiere T and Smugghe G. (2012). Neonicotinoids in bees: A review on concentrations, side effects and risk assessment. *Ecotoxicol.* **21**: 973-92.

Blakley B, Brousseau P, Fournier M and Voccia I. (1999). Immunotoxicity of pesticides: a review. *Toxicol. and Indus. Health.* **15**: 119–32.

Danneberg A M. (1991). Delayed-type hypersensitivity and cell mediated immunity in the pathogenesis of tuberculosis. *Immunol. Today.* **12**: 228-33.

Devan R K S, Prabu P C and Panchapakesan S. (2015). Immunotoxicity assessment of sub-chronic oral administration of acetamiprid in Wistar rats. *Drug and Chem. Toxicol.* **38**: 328-36.

Elsabbagh H S and El-Tawil O S. (2001). Immunotoxicity of cupravit and pervicur fungicides in mice. *Pharmacol. Res.* **43**: 71–76.

Gatne M M, Ramesh Bhoir P S and Deore M D. (2006). Immunotoxicity studies of imidacloprid in rats. *Toxicol. Inter.* **13**: 82–84.

Gawade L, Dadarkar S S, Husain R and Gatne M. (2013). A detailed study of developmental immunotoxicity of imidacloprid in Wistar rats. *Food and Chem. Toxicol.* **51**: 61-70.

Genersch E, Von der Ohe W, Kaatz H, Schroeder A, Otten C, Buchler R, *et al.* (2010). The Geman bee monitoring project: A long term study to understand periodically high winter lossess of honey bee colonies. *Apidologie* **41**: 332-52.

Hassan Z M, Ostad S N, Minaee B, Narenjkar J, Azizi E and Neishabouri E Z. (2004). Evaluation of immunotoxicity induced by propoxure in C57BL/6 mice. *Inter. Immunopharmacol.* **4**: 1223–30.

Jong W H D and Loveren H V. (2007). Screening of xenobiotics for direct immunotoxicity in an animal study. *Methods* **41**: 3–8.

Kacmar P, Pistl J and Mikula I. (1999). Immunotoxicology and veterinary medicine. *Acta Vet. Brno* **68**: 57–79.

Karthikeyan V, Jain S K and Punia J S. (2014). Acute toxicity and neurobehavioural effects of acetamiprid in mice. *J. Vet. Pharmacol. Toxicol.***11:** 115-17.

Kawani H J. (2007). Subacute immunotoxicological studies of imidacloprid in mice and evaluation of the ameliorating potential of *Aspargasus racemosus* on it. M.V.Sc. thesis, Gujarat Agricultural University, Anand, India.

Kim H S, Eom J H, Cho H, Cho Y J, Kim J Y, Lee J K, Kim S and Park K L. (2007). Evaluation of immunotoxicity induced by pirimiphos-methyl in male Balb/c mice following exposure for 28 days. *J. Toxicol. Environ. Health. A.* **70**: 1278-87.

Ladics G S and Woolhiser M R. (2006). Mechanisms of Immunotoxicity. *In:* Immunotoxicology and Immunopharmacology, Luebke, R., House, R. and Kimber, I. (eds.), CRC Press, Boca Raton. pp. 49-62.

Luebke R, House R and Kimber I. (2007). Immunotoxicology and Immunopharmacology: Target Organ Toxicology Series, third ed. CRC Press, Boca Raton, FL.

Luster M I, Dean J H and Moore J A. (1982). Evaluation of immune functions in toxicology. *In:* Principle and methods of toxicology. Hayes A.W., ed. New York: Raven Press: pp. 561-86.

Mondal S, Ghosh R C, Mate M S and Karmakar D B. 2009. Effects of acetamiprid on immune system in female wistar rats. *Procee. Zool. Soci.* **62**: 109-17.

Mondal S, Ghosh R C and Mukhopadhyaya S K. (2012). Studies on the electrolytes and microelements in Wistar rat following multiple exposures to acetamiprid. *Toxicol. Indust. Health.* **28**: 422-27.

Neishabouri E Z, Hassan Z M, Azizi E and Ostad S N. (2004). Evaluation of immunotoxicity induced by diazinon in C57BL/6 mice. *Toxicol.* **196**: 173-79.

Sathiakumar N, MacLennan P A and Delzell J M E.

(2011). A review of epidemiologic studies of triazine herbicides and cancer. *Crit. Review in Toxicol.* **41**: 1–34.

Shand F L. (1979). The immunopharmacology of cyclophosphamide. *Inter. J. Immunopharmacol.* **1**: 165.

Siddiqui A, Choudhary M, Goriya H V, Bhavsar S V and Thaker A M. (2007). Evaluation of immunotoxic effect of short-term administration of quinalphos and imidacloprid in white leghorn cockerels. *Toxicol. Inter.* **14**: 15–19.

Thomas P T. (1998). Immunotoxicology hazard identification and risk assessment. Nutrition Reviews **56**: 1–6.

Tomizawa M and Casida J E. (2005). Neonicotinoid insecticide toxicology: mechanisms of selective action. *Annual Review of Pharmacol. Toxicol.* **45**: 247-68.

Vohr H W and Fehlert R C. (2001). Industry experience in the identification of the immunotoxic potential of agrochemicals. *Sc. of the Total Environ.* **270**: 123–33.

Willtrout R W, Ercegovich C D and Ceglowski W S. (1978). Humoral immunity in mice following oral administration of selected pesticides. *Bulletin of Environ. Contam. and Toxicol.* **20**: 423-31.

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Research Article

SPINOSAD: SUB-ACUTE GENOTOXICITY STUDIES IN MICE

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ABSTRACT

The genotoxicity studies of spinosad were conducted using two specific tests viz. MNT and CAs at two dose levels of spinosad administered daily for 28 days i.p. in Swiss albino male mice. Significant difference was observed in Polychromatic erythricyte (PCE) with MN, and total MN cells (PCEs+ NCEs) in spinosad treated group at higher dose level (58 mg/kg body weight) only. However, P/N ratio did not differ from that of saline treated group at any of the dose of spinosad. Spinosad treatment increased the frequency of chromosomal aberrations at higher dose only, though it was non-significant as compared to saline treated group. Spinosad produced mild genotoxic effects when administered @58 mg/kg body weight as revealed by Micronuclei test.

Keywords : Genotoxicity, chromosomal observations, micronuclei test, mice,

INTRODUCTION

Pesticides are the largest group of substances that are widely used in modern agricultural practices. Insecticides are also used in animal husbandry practices to control ticks, mites, fleas etc. As agriculture and animal husbandry are the backbone of livelihood of a large proportion of human population world over, particularly in developing countries, so a large proportion of human population is exposed to pesticides.

Bio-pesticides are typically produced by growing and concentrating naturally occurring organisms and/or their metabolites including bacteria and other microbes, fungi, nematodes, proteins etc. They are often considered to be important components of integrated pest management (IPM) programmesand have received much practical attention as substitutes to synthetic chemical plant protection products (PPPs).

The discovery and characterization of the soil actinomycete*Saccharopolysporaspinosa* represented a novel opportunity to develop a portfolio of progressive insect management tools (Sparks *et al.,* 1998). Spinosad has a unique mode of action that it causes excitation of the insect nervous system, leading to involuntary muscle contractions, prostration with tremors, and finally paralysis (Salgado, 1997; Salgado, 1998; Salgado *et al.,* 1998). These effects are consistent with the activation of nicotinic acetylcholine receptors by a mechanism that is clearly novel and unique among known insecticidal compounds. Spinosad also has effects on γ-amino butyric acid (GABA) receptor function that may contribute further to its insecticidal activity.

Its favourable mammalian and environmental profile, insect selectivity and IPM fit, unique mode of action and resistance management properties and outstanding efficacy are resulting in rapid registration and adoption by growers around the world documenting increased agricultural produce.

Till date, only preliminary toxicological information is available on spinosad and investigations in the literature show very few pharmacological and toxicological studies on spinosad with few reports on genotoxic effects. Hence, the proposed investigation was undertaken with an objective to evaluate the genotoxic potential of spinosad. The information obtained from this investigation will help in understanding the adverse effects of spinosad, if any, on exposure of animals and human beings and thereby will help in prevention and treatment of poisoning and assessing safety for its widespread use.

MATERIALS AND METHODS

The study was conducted on male Swiss albino mice. The formulation product of spinosad, Tracer (Spinosad 45% SC), Dow AgroSciences India Pvt. Ltd., procured from the local market in Hisar was used in this study.

Experimental animals

Swiss albino male mice weighing 20-25g were procured from Disease Free Small Animal House, LLR University of Veterinary and Animal Sciences (LUVAS), Hisar. The experimental animals were housed in polyacrylic cages, in groups of six animals per cage, in the Departmental Small Animal House. The animals were kept at room temperature with a natural light-dark cycle and provided with feed and tap water *ad libitum*. The animals were acclimatized to laboratory conditions for 3-4 days before the experiments were conducted. The prior approval of Institutional Animal Ethical Committee for the protocol of this study was obtained.

Chemicals and Reagents

May-Grunewald's stain, Giemsa stain, Hank's

Balanced Salt Solution (HBSS), Phosphate Buffer Saline (PBS) and Colchicine were procured from HiMedia Laboratories Pvt. Ltd. Mumbai, India. Xylene and potassium chloride were procured from CDH, New Delhi. methanol, acetic acid and glycerin were procured from Fisher Scientific Mumbai, India. Plastic wares such as centrifuge tubes (15 ml and 50 ml), microcentrifuge tubes (0.7 ml) and micro tips (2-200 µl and 200-1000 µl) were procured from Genetix.

METHODS

Dose selection

The doses selected for administration were derived from the MTD of spinosad. The genotoxic alterations induced by spinosad were studied in mice at two dose levels of 1/20th (5%) and 1/10th (10%) of MTD (MTD from preliminary studies was determined to be 580 mg/kg, intraperitoneally (i.p.) and thus the selected doses were 29 mg/kg and 58 mg/kg; i.p. of spinosad, respectively).

Experimental Design

Spinosad induced genotoxicity was assessed by micronucleus test (MNT) and chromosomal aberrations studies (CAs)

Each dose of the insecticide was administered daily to a group of 6 male mice i.p. for a period of 28 days. The results were compared with two control groups, one negative control group in which normal saline at a dose rate of 10 ml/kg body weight was administered daily to a group of 6 male mice i.p. for a period of 28 days and one positive control group received cyclophosphamide @ 55 mg/kg body weight, i.p. in MNT and CAs experiment for 5 days (from day 24th of drug administration, unless mentioned otherwise). The test and control groups of animals were sacrificed on day 29 of the experiment.

Evaluation of the genotoxic effect of spinosad in mice

Micronucleus test (MNT)

A micronucleus is an erratic (third) nucleus formed during the anaphase of mitosis or meiosis as a result of chromosomal aberrations. Micronuclei appear when the fragments of chromosomes lacking the centromeres are left out during cell division. Hence, MNT detects freshly induced structural chromosomal aberrations in the bone marrow of test animals, serving as a useful indicator of cryptogenic damage and prove to be a convenient, rapid and reliable method for screening of the chromosomal damage in vivo in mice. The occurrence of micronuclei in spinosad treated and control animals were assayed by the test described by Boller and Schmid (1970) and Heddle (1973). The procedure is described briefly in following steps:

Dissection and harvesting of bone marrow

Treatment of test and control groups of animals was done. At the end of treatment, mice were sacrificed under chloroform anesthesia. The femur bone from each animal was excised intact. The condyles epiphysis of each bone was severed and bone marrow from each animal was flushed and squeezed out and collected in centrifuge tube containing phosphate buffer saline (PBS) of pH 7.4. The bone marrow suspension was subjected to spin at 1000 rpm for 10 min and cell pellet was resuspended in fetal calf serum.

Slide preparation and staining

Cell pellet was resuspended and a small drop of the cell suspension was placed on a dry cleaned and non-greasy glass slide. A thin and uniform smear was prepared and air dried for 24 hours. The air dried slides were stained by May-Grunwald's (MG) and Giemsa stain according to the method of Schmid (1976). The slides were stained with stock MG stain (0.30%) for 5 min and then with diluted MG stain (0.15%) for 2 min. Slides were then rinsed twice in distilled water, counter stained with 20% Giemsa stain for 10 min followed by washing in distilled water and air dried. The slides were then cleared in xylene and screened.

Slides analysis and photomicrography

The slides with even spreading were selected at lower magnification (40X) and then analyzed for the presence of micronuclei under oil immersion objective at higher magnification (100X). The micronuclei are deeply stained round bodies of various sizes in various types of bone marrow cells which were identified as follows:

- Polymorphonuclear (PMN) cells were with deeply stained nucleus and light blue plasma.
- Polychromatic erythrocytes (PCE) were immature erythrocytes without nucleus and plasma stained bluish to purple.
- Normochromatic erythrocytes (NCE) were mature erythrocytes without nucleus and plasma stained yellow.
- Micronuclei were deeply stained round bodies of various sizes observed in PCE and NCE.

500 PCE and NCE cells per animal separately were screened for presence of micronuclei and subjected to photography using a digital camera.

Chromosomal aberrations studies (CAs)

The effect of spinosad on the chromosomes of bone marrow cells was studied as per the guidelines given by Ad-hoc committee on chromosomes methodologies in mutagen testing (1972). Sequential steps carried out in CAs were as follows:

Dissection and harvesting of bone marrow

Treated animals of test and control groups were injected with aqueous colchicine (300 µg/kg i.p.) two hours prior to their sacrifice. All mice were sacrificed

under chloroform anesthesia and their femur bones were excised intact. After severing condyles epiphysis, the bone marrow was flushed and squeezed out and collected in centrifuge tube containing HBSS. The bone marrow suspension was spinned at 1000 rpm for 15 min to obtain a packed button. The cell pellet was given hypotonic treatment by incubation in 5 ml of aqueous potassium chloride (75 µM) at 37ºC ± 0.5 for 20 min. Hypotonic treatment was terminated by addition of one ml of freshly prepared chilled fixative solution (Appendix I), followed by centrifugation at 1000 rpm for 10 min. The pellet was subjected for at least three washings with 5 ml chilled fixative so as to get a clear whitish cell pellet. Slides were prepared by splashing the drops on moist clean slides from a height of about one meter and fixed immediately by warming over spirit lamp for 2 seconds and stored.

Staining and analysis of slides

The dried slides were stained in buffered 2% Giemsa (pH 6.8) stain for 30 min followed by rinsing in distilled water and drying in incubator at 37ºC for 24 hours. The slides with good metaphase spreads were selected under low power (40X) and scored at 100X magnification under oil immersion. At least 100 well spread metaphase per animal were carefully observed at random for various types of chromosomal aberrations. The following types of chromosomal aberrations were recorded:

(A) Structural aberrations
- (a) Chromatid type aberration
  - (i) Chromatid gaps (CG)
  - (ii) Chromatid breaks (CB)
- (b) Chromosomal type aberrations
  - (i) Isochromatid gaps (ICG)

(B) Numerical aberrations
- (a) (i) Polyploidy (POLY)
- (b) (ii) Endoreduplication (ER)

Statistical analysis

Data were analysed by one-way ANOVA followed by Dunnet's multiple comparison test. Results were expressed as mean ± standard error of mean. The threshold of statistical significance was set at p d" 0.05. Statistical analysis was performed using Graph pad prism version 7.0

RESULTS

The results of investigation on genotoxic effects of Spinosad in mice are as follows:

Micronucleus Test (MNT)

Incidence of micronucleated erythrocytes in the bone marrow of mice

The effect of spinosad on micronucleus formation in normochromatic and polychromatic erythrocytes is presented in Table 1 and incidence (percent) of micronucleated polychromatic erythrocytes is depicted graphically in Fig. 1. For each animal 500 polychromatic erythrocytes (PCEs) and corresponding number of normochromatic erythrocytes (NCEs) were screened for the presence of micronuclei (Fig 1a). In control group mean value of incidence of micronucleated PCEs (MN-PCE) was 0.23 ± 0.06 while the mean value of incidence of micronucleated NCEs (MN-NCE) was 0.15 ± 0.05.

In treated groups, for the lower dose (29 mg/kg b.wt.) level, the mean value of incidence of micronucleated PCEs was 0.35 ± 0.04 while the mean value of incidence of micronucleated NCEs was 0.21 ± 0.08. For higher dose level (58 mg/kg b.wt.), the mean value of incidence of micronucleated PCEs was 0.88 ± 0.06 while the mean values of incidence of micronucleated NCEs was 0.32 ± 0.05. For cyclophosphamide treated group, the mean value of incidence of micronucleated PCEs was 3.02 ± 0.12 while the mean value of incidence of micronucleated NCEs was 2.22 ± 0.13.

The statistical analysis of data showed that

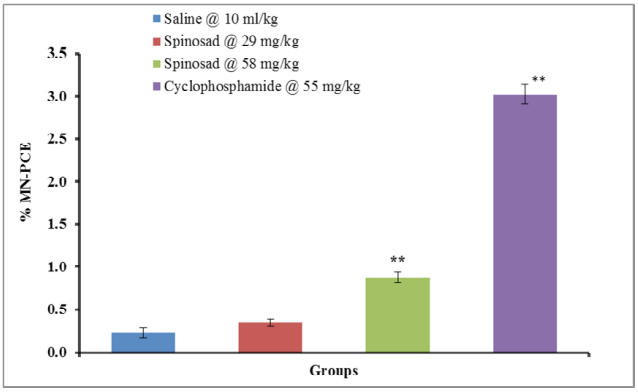


Fig 1:
Incidence (percent) of micronucleated polychromatic erythrocytes in the bone marrow in spinosad treated Swiss albino male mice

Table 1
Incidence (percent) of micronucleated erythrocytes in the bone marrow in spinosad treated Swiss albino male mice

Treatment	MN-PCE	MN-NCE	MN-(PCE + NCE)
Saline @ 10 ml/kg	0.23 ± 0.06	0.15 ± 0.05	0.19 ± 0.04
Spinosad @ 29 mg/kg	0.35 ± 0.04	0.21 ± 0.08	0.29 ± 0.04
Spinosad @ 58 mg/kg	0.88 ± 0.06**	0.32 ± 0.05	0.62 ± 0.05*
Cyclophosphamide @ 55 mg/kg	3.02 ± 0.12**	2.22 ± 0.13**	2.53 ± 0.12**

Data presented as Mean ± SE; (n = 6 mice per group)
* Values differ significantly from control (P≤0.05), ** Values differ significantly from control (P≤0.01)

spinosad treatment caused dose dependent increase in incidence of micronuclei formation in PCEs and NCEs. The incidence of micronucleated PCEs formation was significantly higher at dose of 58 mg/kg b.wt. The incidence of micronucleated NCEs at either dose of spinosad did not differ significantly from saline treated group. The incidence of micronucleated erythrocytes (PCEs + NCEs) was also significantly higher at dose level of 58 mg/kg b.wt from control group mice. In cyclophosphamide treated group the corresponding incidence was significantly higher as compared to saline treated group.

Effect on polychromatic to normochromatic erythrocytes (P/N) ratio

The effect of spinosad on the proliferation of PCEs and NCEs was determined by P/N ratio for which about 1000 erythrocytes (PCEs + NCEs) were counted from each animal. The results of P/N ratio are presented in Table 2 and depicted graphically in Fig 2. In control group mean value of per cent of PCEs was 53.72 ± 1.58 while in spinosad treated lower and higher dose group the values were 53.90 ± 0.76 and 53.30 ± 0.77 respectively. In cyclophosphamide treated group the mean value of per cent of PCEs was 39.85 ± 1.34. The corresponding mean values of per cent of NCEs in these groups were 46.28 ± 1.58, 46.10 ± 0.76, 46.70 ± 0.77 and 60.15 ± 1.34 respectively. No significant change in percent of PCEs or NCEs was observed in spinosad

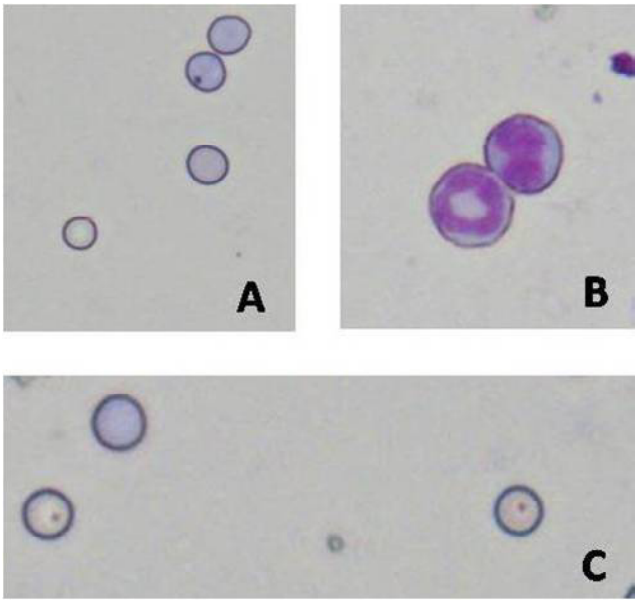


Fig 1a:
Microphotograph showing different type of bone marrow cells with or without the micronucleus (x400):

A: Polychromatic erythrocyte (PCE), polychromatic erythrocytes with micronucleus (MN-PCE) and normochromatic erythrocyte (NCE)
B: Polymorphonuclear cells (PMNC)
C: Normochromatic erythrocytes with micronucleus (MN-NCE) and polychromatic erythrocyte (PCE)

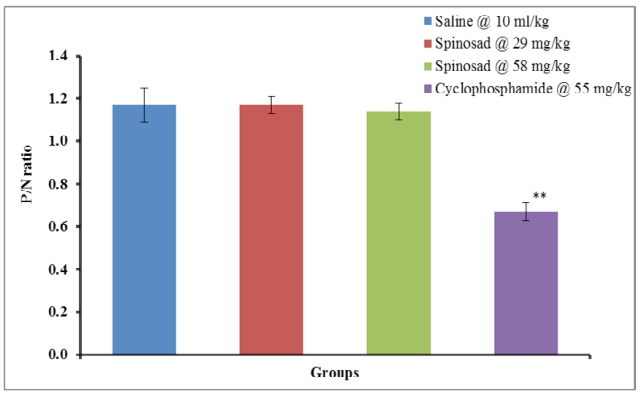


Fig 2:
Effect of spinosad on P/N ratio in Swiss albino male mice
Vertical bars over columns indicate standard error of mean
** Values differ significantly from control (P≤0.01)

Table 2:
Effect of spinosad on P/N ratio in Swiss albino male mice

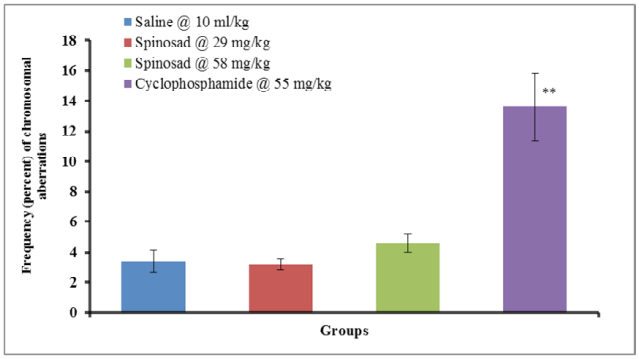
Treatment	PCE	NCE	P/N ratio
Saline @ 10 ml/kg	53.72 ± 1.58	46.28 ± 1.58	1.17 ± 0.08
Spinosad @ 29 mg/kg	53.90 ± 0.76	46.10 ± 0.76	1.17 ± 0.04
Spinosad @ 58 mg/kg	53.30 ± 0.77	46.70 ± 0.77	1.14 ± 0.04
Cyclophosphamide @ 55 mg/kg	39.85 ± 1.34**	60.15 ± 1.34**	0.67 ± 0.04**

Data presented as Mean ± SE; (n = 6 mice per group), **Values differ significantly from control (P≤ 0.01)

Table 3:
Effect of spinosad on frequency (percent) of chromosomal aberrations in metaphase cells of Swiss albino male mice

Treatment	No. of Metaphase cells with CAs	Chroma-tid Gap	Chroma-tid Break	Iso-Chroma-tid Gap	Endo Reduplication	Centromeric break
		CG	CB	ICG	ER	CB
Saline @ 10ml/kg	3.40 ± 0.75	1.80 ± 0.37	0.40 ± 0.24	0.80 ± 0.20	0.40 ± 0.24	0.00 ± 0.00
Spinosad @ 29mg/kg	3.20 ± 0.37	1.20 ± 0.20	0.60 ± 0.24	1.00 ± 0.32	0.20 ± 0.20	0.20 ± 0.20
Spinosad @ 58mg/kg	4.60 ± 0.60	2.00 ± 0.32	1.20 ± 0.20	1.00 ± 0.32	0.20 ± 0.20	0.20 ± 0.20
Cyclophosphamide @ 55 mg/kg	13.60 ± 2.23**	3.20 ± 0.49*	3.60 ± 1.60*	4.40 ± 2.04	1.60 ± 1.17	0.80 ± 0.80

Data presented as Mean ± SE; (n = 6 mice per group), * Values differ significantly from control (P≤0.05), ** Values differ significantly from control (P≤0.01)



Vertical bars over columns indicate standard error of mean, **Values differ significantly from control (P≤0.01)

Fig 3:
Frequency (percent) of chromosomal aberrations in metaphase cells of Swiss albino male mice treated with spinosad

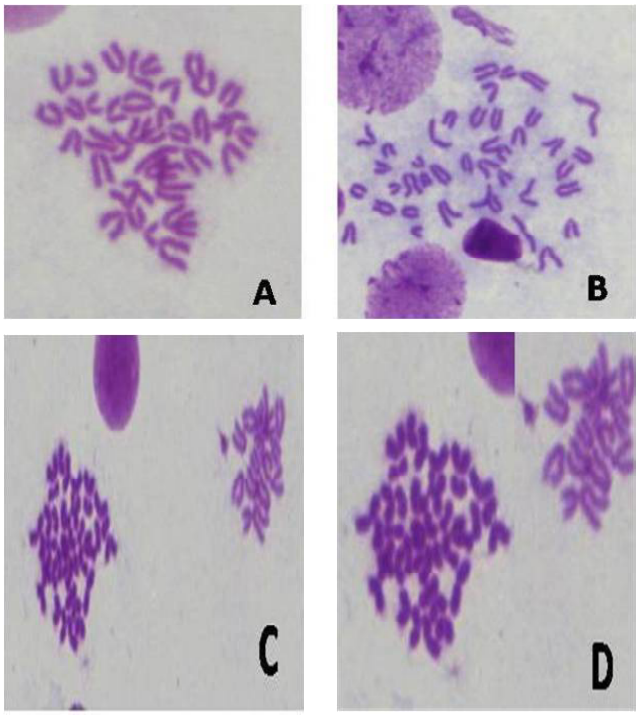


Fig 3a:
Microphotographs showing normal metaphase spreads and chromosomal abnormalities in bone marrow cells of Swiss albino male mice, stained with Giemsa stain (x 400):
A and B: Normal metaphase spreads in metaphase chromosomes
C and D: Endoreduplication characterized with compact appearance of metaphase chromosomes

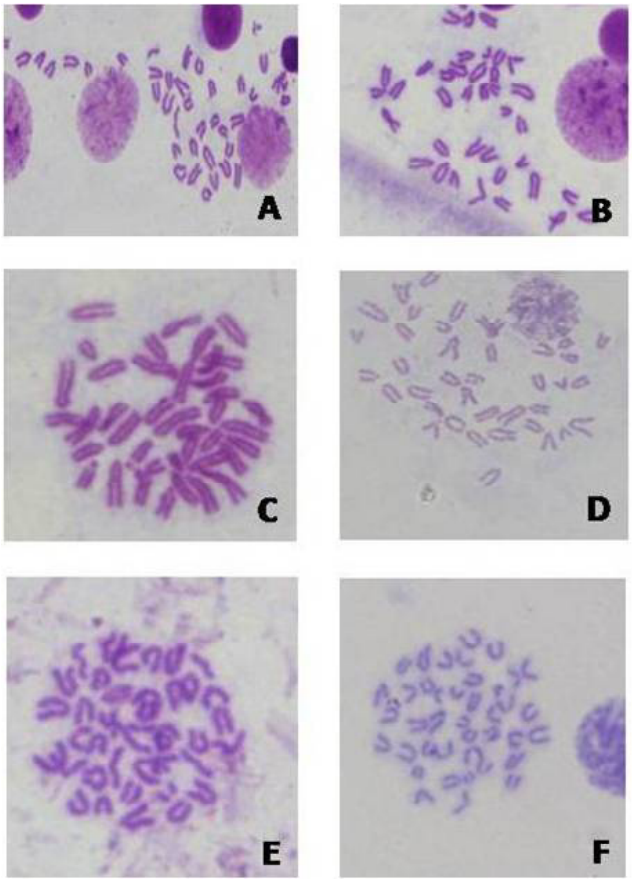


Fig 3b:
Microphotograph showing chromosomal abnormalities in bone marrow cells of mice stained with Giemsa stain (x 400):
A: Ring chromosomes; B and C: Isochromatid gap (ICG);
D: Chromatid gap (CG); E and F: Centromeric break

treated groups.

In control group the mean values of P/N ratio was 1.17 ± 0.08 while the mean values of P/N ratio for lower dose level group and higher dose level group were 1.17 ± 0.04 and 1.14 ± 0.04, respectively. In cyclophosphamide treated group mean value of P/N ratio was 0.67 ± 0.04. No significant change in P/N ratio was observed at any of the dose levels for spinosad. Cyclophosphamide treatment caused highly significant difference in the mean value of per cent of PCEs, NCEs and P/N ratio.

Chromosomal Aberrations (CAs) Studies:

About 100 metaphase cells per animal were observed to examine various types of chromosomal aberrations. Major aberrations observed were chromatid gap (CG), iso-chromatid gap (ICG) while other chromosomal anomalies (Endo Reduplication, Chromatid Break and Centromeric Break) were of lower frequency (Fig 3a-b). The results of chromosomal aberrations are represented in Table 3 and depicted graphically in Fig 3.

In saline treated group, the mean value of frequency (per cent) of chromosomal aberrations was 3.40 ± 0.75 while the mean values of frequency of chromosomal aberrations at lower dose level (29 mg/kg b.wt.) and higher dose level (58 mg/kg b.wt.) were 3.20 ± 0.37 and 4.60 ± 0.60 respectively. For cyclophosphamide treated group the mean value of frequency (per cent) of chromosomal aberrations was 13.60 ± 2.23.

Spinosad treatment increased the frequency of chromosomal aberrations at higher dose only, though it was non-significant. Cyclophosphamide treatment significantly increased the frequency of chromosomal aberrations as compared to saline treated group.

DISCUSSION

Biomarkers such as chromosomal aberrations (CA) analysis and micronucleus test are among the most extensively used markers of genotoxic effects of pesticides. CA is particularly dangerous to the cell, because the physical discontinuity of the chromosome may cause loss of genetic information and even cell death if a housekeeping gene is involved (Carbonell *et al.,* 1995). Also, CA may be used as an early warning signal for cancer development and it has been suggested that the detection of an increase in chromosome aberrations, related to an exposure to genotoxic agents, may be used to estimate cancer risk (Hagmar *et al.,* 1994). Additionally, to assess the degree to which tested doses cause chromosomal damage of developing red blood cells, the incidence of residual chromosome fragments (micronuclei) in polychromatic erythrocytes from femoral bone marrow has to be determined, as this is a well-established biomarker of chromosome breakage due to DNA damage (Schmid, 1976). Many studies have demonstrated the efficiency of the MN assay to detect DNA damage induced by pesticides.

The mode of action of spinosad is characterized by an excitation of the nervous system with activation of nicotinic acetylcholine receptors (nAChRs), along with effects on γ-amino butyric acid (GABA) receptor function, GABA-gated chloride channels and can elicit a small-amplitude Cl- current (Salgado, 1998). On the other hand, spinosad toxicity is consistent with altered phospholipid metabolism, resulting in cellular phospholipidosis. In support of this, chemical structure of spinosad (a hydrophobic ring structure with a hydrophilic side-chain containing a basic amine group) is comparable to other cationic amphiphilic drugs, which produce similar cellular toxicity (Halliwell, 1997). According to EPA (1997), spinosad has no mutagenic activity and the literature offers no additional data in this respect. Recently, Mansour *et al.* (2007) found that spinosad at 9.0; 37.38 mg a.i. kg^{-1} body weight reduced the total number of erythrocytes and inhibited serum ChE in male rats.

In genotoxicity study, MNT and CAs were used to evaluate effect of spinosad on gene system. The micronucleus test is an in vivo cytogenetic screening procedure for the detection of freshly induced structural chromosomal aberrations and for revealing the chromosomal loss due to partial impairment. A micronucleus is an erratic (third) nucleus that is formed during the anaphase of mitosis or meiosis as a result of chromosomal aberrations. Micronuclei originate from chromatin when the fragments of chromosomes lacking the centromeres are left out during cell division. In the course of telophase, this material is included into one or other daughter cells where it can either fuse with the main nucleus or form one or several secondary nuclei (Schmid, 1976).

Micronuclei frequency depends upon the rate of chromosomal breakage and the rate of cell division (Heddle *et al.,* 1984). Hence MNT that detects freshly induced structural chromosomal aberrations in the bone marrow cells of test animals, serving as a useful indicator of cryptogenic damage, have been used by several workers for the screening of mutagenic properties of various chemicals and substances (Tapisso *et al.,* 2009; Rasgele and Kaymak, 2010; Sekeroglu*et al.,* 2013). It proved to be a convenient, rapid and reliable method for screening of the chromosomal damage in vivo in mice. The other advantage that the young erythrocytes generally known as polychromatic erythrocytes (PCEs) have bluish to purple stained plasma up to 24 hours of their origin as compared to the mature normochromatic erythrocytes (NCE) which have yellow stained plasma. This is due to the presence of numerous ribosomes in PCEs that stains basophilic blue with May-Grunwald's stain (Rifkind, 1976). These ribosomes are dissolved during the maturation of immature PCEs into mature NCEs.

Three types of cells observed in MNT were polymorpho-nuclear cells, young polychromatic erythrocytes (PCE) and mature normochromatic erythrocytes (NCE). Micronuclei were deeply stained round bodies of various sizes observed in PCE and NCE. On the basis of these observations, the P/N ratio and frequency of micronucleated erythrocytes were calculated at two dose levels i.e. 58 mg/kg body weight (higher dose level) and 29 mg/kg body weight (lower dose level) of spinosad.

In the present study, micronucleus induction in the erythrocytes was observed at both dose levels of spinosad. The incidence of micronucleated PCEs formation was significantly higher at dose of 58 mg/kg b.wt. The incidence of micronucleated NCEs at either dose of spinosad did not differ significantly from saline treated group. The incidence of micronucleated erythrocytes (PCEs + NCEs) was also significantly higher at dose level of 58 mg/kg body weight from control group mice. In cyclophosphamide treated group the corresponding incidence were significantly higher compared to saline treated group. The increase in the micronucleus count in treated mice as compared to control group has been considered as the parameter for the genotoxicity of the chemicals by earlier workers (Bhunya and Pati, 1990; Zang*etal*., 2000; Kocaman and

Topaktas, 2007; Tapisso *et al.,* 2009; Rasgele and Kaymak, 2010; Sekeroglu *et al.,* 2013; Jonnalagadda *et al.,* 2012). These results indicated the weak genotoxicity of spinosad. There are similar reports of several workers. Mansour *et al.* (2008) also observed that spinosad (37.38 mg a.i/kg body weight for 60 days) and malathion (13.75 and 29.00 mg a.i/kg body weight for 60 days) caused significantly increased micronucleated polychromatic erythrocyte (MN %) in rat bone marrow cells. Kocaman and Topaktas (2007) reported induction of chromosomal aberrations and micronucleus formation and also significant decrease in mitotic index and nuclear division index in human peripheral blood lymphocytes in vitro. Kocaman and Topaktas (2010) studied the combined genotoxic effects of acetamiprid and α-cypermethrin in human peripheral blood lymphocytes and reported the dose-dependent increase in CAs and SCEs at all concentrations (12.5 + 2.5, 15 + 5, 17.5 + 7.5 and 20 + 10 µg/ml of acetamiprid and α-cypermethrin) and treatment times (24 hours and 48 hours) and dose-independent induction of MN formation at all except the highest concentration in both treatment times. Significant decrease in mitotic index (MI) and proliferation index (PI) were concentration dependent at both treatment times while decrease in nuclear division index (NDI) was concentration-dependent only at 48 hours treatment period, showing the synergistic effect of acetamiprid and α-cypermethrin combination in induction of genotoxicity/cytotoxicity in human peripheral blood lymphocytes. Bansal and Chaudhry (2011) adopted dominant lethal test (DLT) for the evaluation of the genotoxic effects of acetamiprid using Culexquinquefasciatus as an experimental model and results indicated that exposure of pesticides even at small dose level proved deleterious to the genome of mosquito and its subsequent generation. Cavas *et al.* (2012) evaluated in vitro cytotoxicity and genotoxicity of technical grade acetamiprid on the human intestinal CaCo-2 cells and revealed that acetamiprid was genotoxic even at non- cytotoxic concentrations by using Micronucleus, Comet and γH2AX foci assays.

Zang *et al.* (2000) reported no significant effects on the micronuclei frequency in mice bone marrow cells until the dose reached 100 mg/kg in a single dose treatment of imidacloprid. Similarly a dose dependent in vivo induction in micronuclei frequency in PCEs of rat bone marrow cells were reported after a single dose (300 mg/kg body weight) of imidacloprid (Georgieva and Popov, 2007) and after daily exposure (50 mg/kg body weight) for 90 days (Karabay and Oguz, 2005). A similar report revealed that induction of micronuclei in human peripheral blood lymphocytes was non-significant until the imidacloprid concentration reached up to 0.1 mg/L (Feng *et al.,* 2005).

The P/N ratio determined to study the cytotoxic effects of spinosad revealed no significant change in PCEs and NCEs percent values in spinosad treated groups as compared to saline treated group. However, P/N ratio decreased significantly in cyclophosphamide treated group as compared to saline treated group. Uggini and Suresh (2013) reported that spinosad treatment did not produce any genotoxic effects at 0.15 and 0.75 mg/egg. Nevertheless, a dose of 1.5 mg/egg of spinosad induced MN in polychromatic erythrocytes and led to a decline in PCE/NCE ratio. At this high dose, fragmented nuclei were observed in the embryonic blood smears. Venitt and Parry (1984) suggested that drastic reduction of PCEs can be taken as an indication for the depression of bone marrow proliferation.

The chromosomal aberration studies (CAs) directly score the DNA damage occurring during cell division in terms of chromatid gaps, chromatid breaks, isochromatid gaps, ring chromosomes, polyploidy, endoreduplication etc. and thus far more advantageous over the micronucleus test. Chemical agents mostly lead to the appearance of chromatid aberrations; however, some of them could also induce chromosomal types of aberrations (Obe and Beck, 1982; IAEA, 1986; Carrano and Natarajan, 1988). The chromosomal types of aberrations could also arise due to misrepair of lesions in the Go stage of circulating lymphocytes as well as derived aberrations from precursor cells in bone marrow and thymus, as suggested by Carrano and Natarajan (1988).

For chromosomal aberration studies (CAs), mice were treated with colchicine that inhibits the microtubule polymerization within the mitotic spindle and arrest the cell division at metaphase stage of mitotic division. The cells were then treated stepwise to prepare the slides of metaphase chromosome spreads arrested by colchicine (Venitt and Parry, 1984). Major aberrations observed were chromatid gap (CG), isochromatid gap (ICG) while the other chromosomal anomalies (Endo Reduplication, Chromatid Break and Centromeric Break) were of lower frequency. Spinosad treatment increased the frequency of chromosomal aberrations at higher dose only, though it was non-significant as compared to saline treated group. Cyclophosphamide treatment significantly increased the frequency of chromosomal aberrations as compared to saline treated group. Mansour *et al.* (2008) also observed that spinosad (37.38 mg a.i/kg body weight for 60 days) and malathion (13.75 and 29.00 mg a.i/kg body weight for 60 days) induced structural chromosomal aberrations in rat bone marrow cells. There are conflicting reports about genotoxic effect of insecticides showing various effects varying with concentration of insecticide and duration of exposure. A

significant induction of CAs and sister chromatid exchange (SCE) by acetamiprid alone at concentration e"25 ìg/ml and of MN frequencies at concentration e"30 ìg/ml as evidenced by significant decrease in MI, NDI and PI (Kocaman and Topaktas, 2007) while report of similar effects at much lower concentration of acetamiprid (e"12.5 μg/ml) when used in combination with α-cypermethrin (e"2.5 ìg/ml) (Kocaman and Topaktas, 2010) showed the synergistic effect in induction of genotoxicity/cytotoxicity in human peripheral blood lymphocytes. Similarly, the more genotoxic and cytotoxic effects were observed in rat bone marrow cells upon combined oral exposure at a single dose of 15 mg/kg and 112.5 mg/kg for 24 hours and at dose rate of 3 mg/kg/day and 22.5 mg/kg/day for 30 days than those of individual exposure of deltamethrin and thiacloprid as evidenced by significantly increased CAs (Sekeroglu *et al.,* 2013). Karabay and Oguz (2005) have also reported the significant chromosomal aberrations in rats treated with imidacloprid (50 mg/kg body weight) for three months. Bagri *et al.* (2013) studied mutagenic effect of imidacloprid in Swiss albino mice and found that imidacloprid is a weak mutagen at a dose level of 22 mg/kg body weight administered orally for 28 days.

Thus, it is clear from the above discussion that subacute exposure of spinosad intraperitoneally for 28 days caused mild cytogenetic toxicity @ 58 mg/kg; i.p. indicated by increased MN-PCE.

The cytogenetic activity may be attributed to spinosad chemical structure and/or to certain impurities in the commercial formulation; a matter, which needs further elucidation (Mansour et al, 2008). It is apparent that, a more complete understanding of genotoxic potential of spinosad is necessary. Although, present investigation has provided first-hand information regarding genotoxic potential of spinosad in a 28 day subacute toxicity study, long term studies on low level exposure of spinosad on various parameters of genotoxicity would clearly indicate potential genotoxic effects of spinosad and this will enable to draw necessary safety measures.

The authors declare that there are no conflicts of interest.

REFERANCES

Ad-hoc committee on chromosomes methodologies in mutagen testing (1972). *J. Heredity.* **63**: 69-72.

Bagri, P., Kumar, V., Sikka, A.K. and Punia, J.S. (2013). Preliminary acute toxicity study on imidacloprid in Swiss albino mice. *V. World.* **6**(12):955-959.

Bansal,M. and Chaudhry, A. (2011). Evaluation of mutagenic potential of acetamiprid by dominant lethal test on *Culexquin quefasciatus*. *J. Appl. Nat. Sc.* **3**(2):171-175.

Bhunya,S.P. and Pati,P.C. (1990).Effect of deltamethrin, asyntheticpyrethroid, ontheinduction of chromosome aberrations, micro nuclei and sperm abnormalities in mice. *Mutagenesis* **5**: 229-232.

Boller, K. and Schmid, W. 1970. Chemical mutagenesis in mammals: The Chinese hamster bone marrow as an *in vivo* test system. Hematological findings after treatment with trenimon. *Humangenetik*. **11**: 35-54.

Carbonell, E., Valbuena, A., Xamena, N., Creus, A. and Marcos, R. (1995). Temporary variations in chromosomal aberrations in a group of agricultural workers exposed to pesticides. *Mutation Res.* **344**: 127-134.

Carrano,A.V.and Natarajan, A.T. (1988).Consideration forpopulationmonitoringusingcytogenetic techniques. *Mutation Res.* **204**: 379-406.

Cavas,T., Cinkilic, N., Vatan,O.,Yýlmaz, D. and Coskun,M. (2012). *Invitro* genotoxicity evaluation of acetamiprid in Ca Co-2 cells using the micronucleus, comet and γH2AX fociassays. *Pest. Biochem. Physiol.* **104**(3): 212-217.

Environmental Protection Agency (EPA) (1997).

Feng, S., Kong, Z., Wang, X., Peng, P. and Zeng, E.Y. (2005). Assessing the genotoxicity of imidaclopridandRH-5849 in human peripheral blood lymphocytes *in vitro* with cometassay and cytogenetic tests. *Ecotoxicol Environ. Safety.* **61**: 239-246.

Georgieva, S. and Popov,B. (2007). A study on the genotoxic effect of the pesticide imidacloprid in rabbit peripheral blood lymphocytes *in vitro* induction of SCE. *Zhivotnovdni Nauki.* **44**: 46-50.

Hagmar, L., Brogger, A., Hansteen, I.L., Heim, S., Hogstedt, B., Knudsen, L., Lambet, B., Linnainmaa, K., Mitelman, F., Reuterwall, I.C., Salomaa, C., Skerfving, S. and Sorsa, M. (1994). Cancer risk in humans predicted by increased level of chromosomal aberrations in lymphocytes: Nordic study group on the health risk of chromosome daage. *Cancer Res.* **54**: 2919-2922.

Halliwell, W.H. (1997). Cationic amphiphillic drug-induced phospholipidosis.*Toxicol. Pathol.* **25**: 53-60.

Heddle, J.A. (1973). A rapid *in vivo* test for chromosomal damage. *Mutation Res.* 18: 187-190.

Heddle,J.A.,Stuart,E. and Salamone,M.F. (1984). The bone marrow micronucleus test. *In:* Handbook ofm utagenicitytestprocedures, (Eds. KilbeY, B.J.,Legator, M.,Nichols,W. and Ramel,C.), Elsevier Science Publishers BV, Amsterdam, Netherlands. P.p.441-457.

IAEA.(1986).Biologicaldosimetry: Chromosome aberration analysis for dose assessment, technical reportsseriesno. 260. International Atomic Energy Agency, Vienna, Austria, pp.59–63.

Jonnalagadda,P.R.,Jahan,P.,Venkatasubramanian, S.,Khan,I.,Prasad,A.,Reddy,K.,Rao,M., Venkaiah, K. and Hassan, Q. (2012). Genotoxicity in agricultural farmers from Guntur district of south India- a case study. *Human and Experi.Toxicol.* **31**(7): 741-747.

Karabay,N.U.andOguz,M.G.(2005). Cytogenetic andgenotoxiceffectsoftheinsecticides, imidacloprid and methamidophos. *Gen. Mole. Res.* **4**: 653-662.

Kocaman, A.Y. and Topaktas,M. (2007). *In vitro* evaluation of the genotoxicity of acetamipridin human peripheral blood lymphocytes. *Environmental and Mole. Mutag.* **48**(6): 483-490.

Kocaman,A.Y.andTopaktas,M. (2010).Genotoxic effectsofaparticularmixtureofacetamipridand alpha-cypermethrinon chromo some aberration, sister chromatid exchange, and micronucleus formationin humanperipheral blood lymphocytes. *Environ. Toxicol.* **25**:157-168.

Mansour, S.A., Mossa, A.H. and Heikal, T.M. (2007). Haematoxicity of a New Natural Insecticide “Spinosad” on Male Albino Rats .*Inter. J. Agri. Bio.* **9**(2): 342-346.

Mansour, S.A., Mossa, A.H. and T.M. Heikal, T.M. (2008). Cytogenetic and hormonal alteration in rats exposed to recommended “safe doses” of spinosad and malathion insecticides. *Inter. J. Agri. Bio*. **10**: 9–14.

Obe, G.and Beck, B. (1982). The humanleukocyte test system.*In:* De Serres, F. J. and Hollaender, A. Rasgele,P.G. and Kaymak, F. 2010. Chromosome aberrations, micronucleus andspermhead abnormalities in mice treated with natamycin, a food preservative. *Food and Chem. Toxicol.* **48**: 789-797.

Rifkind,R.A.(1976). Fundamentalof hematology. Year Book Medical, Chicago, USA.pp. 132-148.

Salgado, V.L. (1997). The mode of action of spinosad and other insect control products. *Down to Earth*. **52**(1): 35-44.

Salgado, V.L. (1998). Studies on the mode of action of spinosad: Insect symptoms and physiology correlates. *Pest. Biochem. Physiol*. **60**(2): 91-102.

Salgado, V.L., Sheets, J.J., Watson, G.B. and Schmidt, A.L. (1998). Studies on the mode of action of spinosad: The internal effective concentration and the concentration dependence of neural excitation. *Pest. Biochem. Physiol*. **60**(2): 103-110.

Schmid, W. (1976). The micronucleus test for cytogenic analysis. In: Chemical Mutagens- principles and methods for their detection. Eds. Alexander Hollaender Plenum Press, New York. **4**: 31-53.

Sekeroglu, V., Sekeroglu, Z.A. and Kefelioglu, H. (2013). Cytogenetic effects of commercial formulations of deltamethrinand/ orthiaclopridonwist arrat bone marrow cells. *Environ. Toxicol,* **28** (9):524-531.

Sparks, T.C., Thompson, G.D., Kirst, H.A., Hertlein, M.B., Mynderse, J.S., Turner, J.R. and Worden, T.V. (1998). Fermentation Derived Control Agents TheSpinosyns. In: F.R. Hall, J.J. Menn (Eds.), Biopesticides: Use and Delivery, Humana Press, Totowa, NJ, 1996, pp. 171–188.

Tapisso, J.T., Marques, C.C., Mathias, M.D.L.and Ramalhinho, M.D.G. (2009). Induction of micronuclei and sister chromatid exchange in bone-marrow cells and abnormalities in sperm of Algerianmice (*Musspretus*)exposedto cadmium, leadandzinc. *Mutation Res.* **678**:59-64.

Uggini, G. K. and Suresh, B. (2013). Genotoxic effects of two different classes of insecticide in developing chick embryos, *Toxicol. Environ. Chem*. **95**: 992-1005.

Venitt, S. and Parry, J.M. (1984). Mutagenicitytesting: A practicalapproach.IRL Press Limited, England.

Zang,Y., Zhong,Y., Luo, Y. and Kong, Z. (2000). Genotoxicity of two novel pesticides for the earthworm, *Eiseniafetida*. *Environ. Poll.* **108**(2): 271-278.

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Research Article

EFFECT OF ALCOHOLIC EXTRACT OF *SHOREA ROBUSTA* GAERTN F. RESIN ON OXIDATIVE STRESS IN STREPTOZOTOCIN-INDUCED DIABETIC RATS

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ABSTRACT

Oxidative stress associated with diabetes causes major microvascular complications, which led to a series of detrimental effects. Plant based polyphenols, most common flavonoids and phenolics are considered beneficial antioxidants in various chronic disease conditions. Ethanolic extract of *Shorea robusta* resin was analysed and found rich in flavonoid (418.83±26 mg QE/g) and phenol content (42.65±5.48 mg GAE/g). Administration of ethanolic extract of *S. robusta* restored the activities of SOD and catalase, but reduced the MDA and NO levels significantly ($p<0.05$) in diabetic rat serum. In conclusion, findings suggest antioxidant effect of *S. robusta* resin which may counter diabetes associated oxidative stress.

Key words: Diabetes, Oxidative stress, *Shorea robusta* resin, Polyphenols

INTRODUCTION

Reactive oxygen species (ROS) play a dual role required for many important signaling reactions including cellular proliferation and differentiation. Moreover, ROS triggering a physiological or programmed event for cell death via oxidative stress. Antioxidants, basically contribute to counter the excessive number of free radicals either by enzymatically (superoxide dismutase and catalase) or non-enzymatically (vitamin C, E and selenium). Diabetes is a metabolic syndrome characterized by hyperglycemia caused impaired metabolism of proteins and lipids (Hosseini *et al.,* 2014). In fact, significant defects of antioxidant defenses which may raise susceptibility to oxidative damage and consequent diabetes complications (Ceriello, 2000). Recently, there has been an upsurge of interest in the therapeutic potential of plants as antioxidants in reducing oxidative tissue injuries. The Indian ethnomedicine *Shorea robusta* Gaertn f. (Dipterocarpaceae) is a large sub-deciduous tree, found extensively in parts of North-East and Central India known as Sal or Sala, was documented by Edward J. Waring in Pharmacopoeia of India (1866). Interestingly, monograph on *S. robusta* has been mentioned in the Ayurvedic Pharmacopoeia of India (2008) introduced by Department of AYUSH. Besides the plaster of fumigation, resin is an astringent used therapeutically, in diarrhoea, dysentery, and gonorrhea. In addition, resin has anti-inflammatory (Wani *et al.,* 2012a), wound healing (Wani *et al.,* 2012b), anti-obesity (Supriya *et al.,* 2012) and microbicidal (Bharitkar, *et al.,* 2013) activities in rat model. Since, there is no report available on the effect of ethanolic extract of *S. robusta* resin in diabetes to counter oxidative stress, the present *in vitro* and *in vivo* experiments were in diabetic rats.

MATERIALS AND METHODS

Plant material and preparation of extract

Resin of *S. robusta* was purchased from a local market of Bhubaneswar, India. It was ground into powder and then extracted with 70% ethanol under reflux for 72 h using a Soxhlet apparatus. Hydro-alcoholic extract was filtered and evaporated to dryness under reduced pressure in rotary vacuum evaporator at 40°C temperature and further kept in vacuum desiccators for complete removal of solvent and the yield of the extract was calculated. The extract (SRE) was stored at 4°C until use (Khan *et al.,* 2016).

Phytochemical study

Qualitative analysis of SRE was carried out to detect phenolic and flavonoid contents using the methods of Trease and Evans (1996).

Total phenolic content (TPC)

It was determined by Folin-Ciocalteu reagent method as described by Hossain *et al.,* 2013. Stock concentration of extract (1 mg/ml) was made up in methanol. In brief, 200µl of sample was mixed with 1.5 ml Folin-Ciocalteu reagent (10%). After 5 min, 1.5 ml of $NaCO_3$ (5%) was added and mixed thoroughly, all tubes were kept in dark for 2 h. The absorbance was measured by spectrophotometer at 750 nm. The TPC was determined from extrapolation of the calibration curve with gallic acid standard (10-100 µg/ml). Test was carried out in triplicate and total phenol was expressed as milligrams of gallic acid equivalents (GAE) per gram of

sample.

Total flavonoid content

It was determined by aluminium chloride method as described by Hossain *et al.* (2013). Reaction mixture having 500 µl sample, 2 ml water and 0.15 ml $NaNO_2$ (5%) were mixed well and after 5 min 0.15 ml $AlCl_3$ (10%) was added and kept in dark place for 5 min to complete the reaction, finally 2 ml NaOH (4%) was added and after 15 min absorbance was taken by spectrophotometer at 510 nm. Total flavonoid was determined from extrapolation of the calibration curve was made with quercetin standard (25-500 µg/ml). Test was carried out in triplicate and total flavonoid was expressed as milligrams of quercetin equivalents (QE) per gram of sample.

DPPH assay

Antioxidant activity was investigated by using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging method (Brand-Willians *et al.,* 1995). Various serially diluted concentrations of extract (50-1000 µg/ml) were mixed to equal volume (1 ml) of DPPH (0.1 mM) solution in methanol. After 30 min incubation at room temperature, absorbance was taken at 517 nm using spectrophotometer. Similarly, Ascorbic acid standard (10-100 µg/ml) and blank control readings were also taken and the per cent inhibition was calculated using formula (% Inhibition=Acontrol-Asample/Acontrol X 100). The IC50 (the concentration of a sample required for 50% scavenging of the DPPH free radical) was determined from the curve plotted against % Inhibition and concentrations. Test was carried out in triplicate and the average IC50 was calculated.

Animals

Apparently healthy adult male Wistar rats (200-220g) were procured from Laboratory Animal Resource Section, ICAR - Indian Veterinary Research Institute, Izatnagar, India. The animals were housed in polypropylene cages and kept for a week under acclimatization period with free access to standard feed and water and maintained on a 12 hour dark-to-light cycle in an air controlled room (temperature 22±2°C, humidity 55%±5%). The experimental protocols involved in this study were according to the guidelines of CPCSEA and IAEC.

Induction of diabetes

Diabetes was induced by streptozotocin (STZ) freshly prepared in citrate buffer pH 4.5 administered single injection intraperitoneal @ 60 mg/kg body weight to overnight starved rats those having fasting blood glucose level in a normal range. Similarly, control group received only equal volume of citrate buffer to nullify its effect. Initially 2% sucrose solution was given in drinking water for 48 h to alleviate the sudden hypoglycemic phase. After 72 h blood glucose was monitored by tail pricking using digital glucometer (On-Call-Plus, ACON Biotech). Rats having blood glucose on or above 300 mg/dl were selected for further study and experiment was started after 15 days of observation period.

Experimental design

The animals were randomly divided into 6 groups of 6 animals as follows: NC (non-diabetic control treated with vehicle), NE (non-diabetic rats treated with SRE 300 mg/kg), DC (diabetic control treated with vehicle), DE1 (diabetic rats treated with SRE 30 mg/kg), DE2 (diabetic rats treated with SRE 100 mg/kg) and DE3 (diabetic rats treated with SRE 300 mg/kg). SRE was dissolved in aqueous vehicle containing 0.5% carboxymethylcellulose and administered orally once in a day by using 16G gastric gavages for 8 weeks, control groups received only vehicle. At the end of experiment blood was collected from cardiac puncture and allowed to clot for 30 min and then centrifuged at 2000 rpm for 15 min, top clear supernatant was harvested and stored at -80°C until analysis.

Biochemical parameters

Malondialdehyde (MDA)

Lipid per oxidation in serum was estimated by thiobarbituric acid reactive substances TBARS method of Beuge and Aust (1978). In short, 0.1 ml of serum in 200 mM Tris-HCl buffer, pH 7.5 was treated with 2 ml of (1:1:1) TBA-TCA-HCl reagent (TBA 0.37%, 0.25N HCl and 15% TCA) and placed in water bath for 15 min and cooled then centrifuged at 2000 rpm for 10 min. The absorbance of clear supernatant was measured at 535 nm against the blank. Concentration was calculated using molar extinction coefficient of MDA which is 1.56 X $10^5 M^{-1} cm^{-1}$ and expressed as nM.

Super oxide dismutase (SOD) activity

SOD activity was measured by the method of Madesh and Balsubramanium (1998). It involves the generation of superoxide by pyrogallol auto oxidation and inhibition of superoxide dependent reduction of the tetrazolium dye (MTT) [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to its formazan. The reaction mixture contained 0.65 ml PBS (pH 7.4), 30µl MTT (1.25 mM), 10 µl serum and 75µl pyrogallol (100mM). The mixture was incubated at room temperature for 5 min and the reaction was stopped by adding 0.75 ml of DMSO which solubilizes formazan. The absorbance was read at 570 nm against the blank and the activity was expressed as Unit/ml.

Catalase (CAT) activity

CAT activity was assayed as described by Aebi (1984). Serum sample (0.1ml) was added to 1.9 ml of 50 mM phosphate buffer (1:1.5 v/v of 50mM KH_2PO_4 and 50mM Na_2HPO_4 pH 7) in a test tube then content is transferred to cuvette. The reaction was started after adding 1 ml of H_2O_2 (10mM: 0.1 ml of 30% H_2O_2 was

diluted to 100ml phosphate buffer) directly into cuvette. Optical density was recorded at every 15 sec for 1 min at 240 nm against distilled water (blank). The catalase activity was calculated using milimolar extinction coefficient of H2O2 (0.071 mmol cm^{-1}) and the activity was expressed as Unit/ml

Nitric oxide (NO)

Nitrite measurement in the serum is an indirect indicator of NO production (Bryan and Grisham, 2007). Briefly, it was measured in serum (100 µl) taking equal volume of Griess reagent (1% sulfanilamide and 0.1% naphthylenediamine in 5% phosphoric acid). After 10 min incubation at room temperature, the absorbance was measured at 550 nm in a microplate reader (SpectraMax Multi-mode). The nitrite concentration was determined from extrapolation of the calibration curve was made with standard sodium nitrite (0-100µM).

Statistical analysis

Results were expressed as mean ± S.E. The Statistical significance was analyzed by applying one-way analysis of variance (ANOVA) followed by Tukey's test using the GraphPad Prism v5.03 software program (San Diego, California USA) and the difference between the control and treatment groups were considered statistically significant at $p<0.05$.

RESULTS

Extraction of *S. robusta* resin

The hydro-alcoholic extract of *S. robusta* resin was dark brown in color with a pasty consistency and the per cent yield was 54.3% w/w dry matter.

Phytochemical analysis

Phytochemical screening of SRE revealed the presence of tannin, flavonoid and terpenes.

Total phenolic contents

TPC in the extract was calculated by extrapolation of the calibration curve with Gallic acid standard ($y=0.005x+0.010$ $R^2=0.992$) (Fig. 1a). Results revealed that the total phenolic content in SRE was 42.65±5.48 mg GAE/g dry extract.

Total flavonoid contents

Flavonoid contents in the extract was calculated by extrapolation of the calibration curve with quercetin standard ($y=0.001x-0.026$ $R^2=0.996$) (Fig. 1b). Results revealed that the flavonoid content in SRE was 418.83 ± 26 mg QE/g dry extract.

DPPH assay

The free radical scavenging activity of SRE at various concentrations (50-1000 µg/ml) showed per cent inhibition in concentration dependent manner. The scavenging activity of SRE and ascorbic acid in terms of IC50 was found 146 µg/ml and 26.08 µg/ml, respectively.

Effect of SRE on SOD and CAT activity

Results revealed that the dose dependent increase in SOD and CAT activity in diabetic rats treated with SRE in all three doses compared to control diabetic rats ($P < 0.05$). There was significant reduction in SOD and CAT activity in diabetic control as compared to normal control rat. Higher dose of extract decreased serum MDA in NE group but statistically was non-significant with NC group (Fig 2a, b; Table 1).

Effect of SRE on lipid per oxidation

The *in vivo* antioxidant effect of SRE produced a significant dose dependent decrease in the MDA level. There was significant increase in MDA level in diabetic control as compared to normal control. Highest dose exposure to NE group, exhibited reduced MDA level but statistically was non-significant with NC group (Fig 2c; Table 1).

Effect of SRE on NO

There was a significant increase in NO levels in diabetic control as compared to normal control. SRE produced a significant dose dependent decrease in the NO level. Highest dose exposure to NE, exhibited

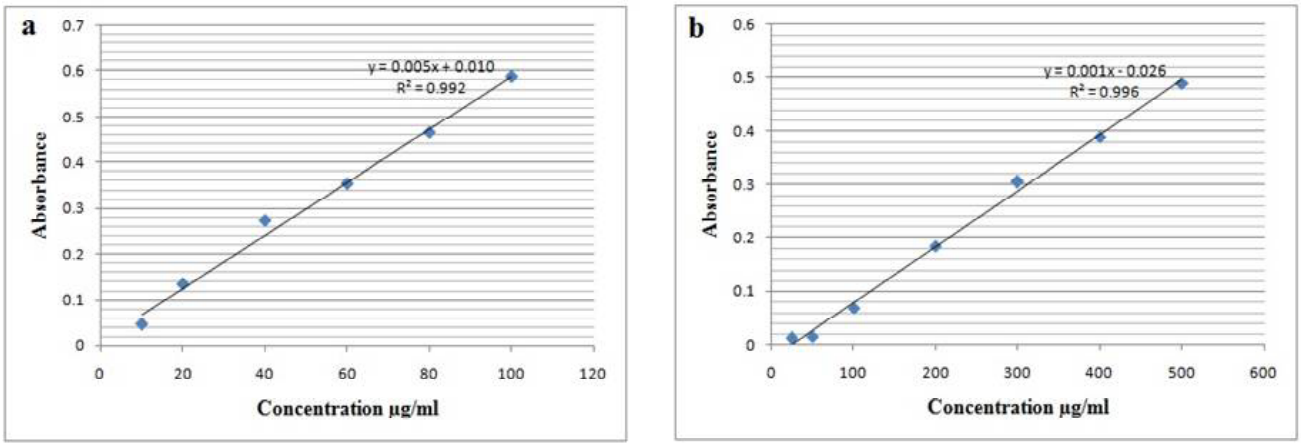

Fig. 1. Standard calibration curve of Gallic acid (a) and Quercetin (b)

Table 1.
Effects of alcoholic extract of *S. robusta* resin on superoxide dismutase (SOD), catalase, Malondialdehyde (MDA) and nitric oxide (NO) level in the serum.

Group	Treatment	SOD (U/ml)	Catalase (U/ml)	MDA (nM)	NO (µM)
1	NC	40.36±2.06	64.26±2.36	3.43±0.70	26.58±1.51
2	NE	42.32±2.60	58.68±3.59	3.08±0.47	30.77±2.04
3	DC	22.62±1.91[a]	34.50±1.72[a]	12.9±0.88[a]	158.5±18.72[a]
4	DE1	29.70±1.12	45.62±2.54	9.88±0.49[b]	94.37±9.91[b]
5	DE2	33.63±2.09[b]	55.21±2.76[b]	7.12±0.84[b]	76.42±6.41[b]
6	DE3	36.27±2.08[b]	57.12±3.03[b]	6.49±0.66[b]	59.24±4.84[b]

Values are presented as mean ± S.E. [a] p<0.05 values are significant compared with non diabetic control. [b]p<0.05 values are significant compared with diabetic control.

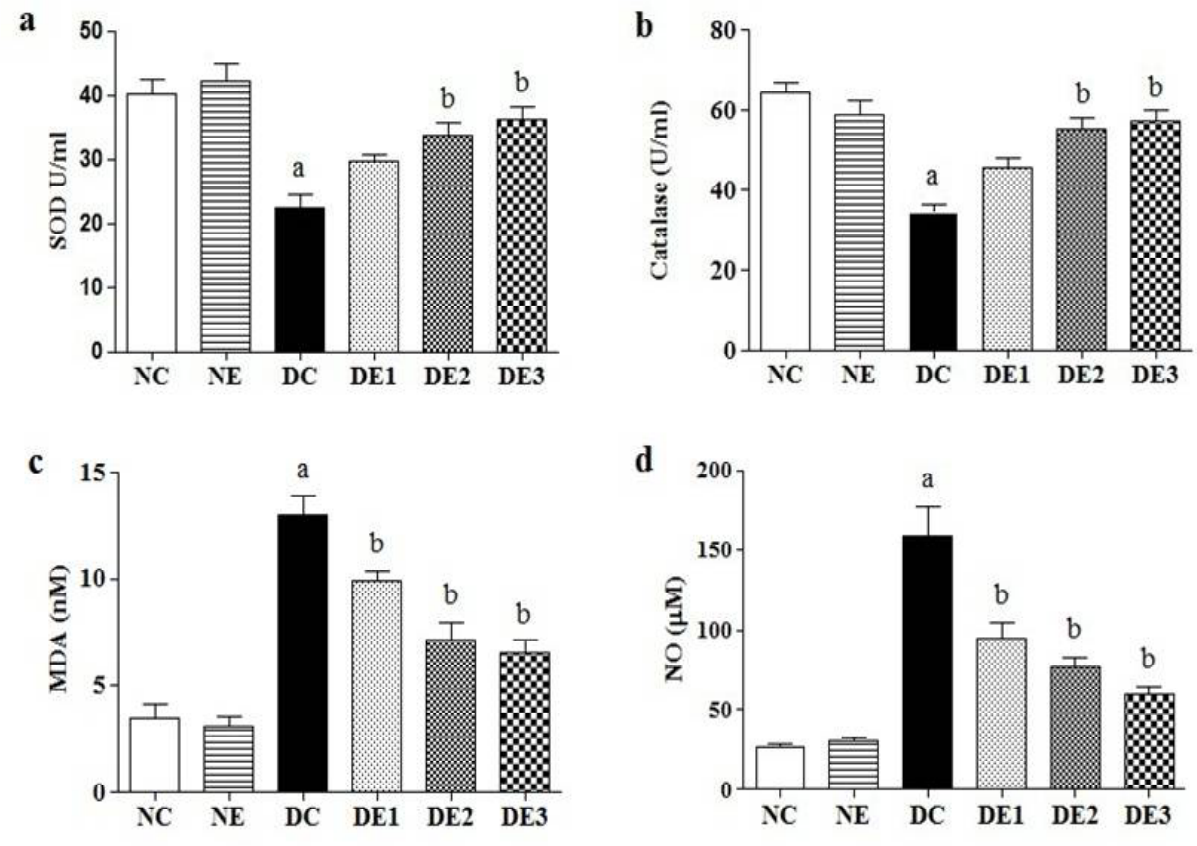

Fig. 2.
Effects of alcoholic extract of *S. robusta* resin on superoxide dismutase (SOD), catalase, Malondialdehyde (MDA) and nitric oxide (NO) level in the serum. Values are presented as mean ± S.E. [a] p<0.05 values are significant compared with non diabetic control. [b] p<0.05 values are significant compared with diabetic control.

reduced NO level but statistically was non-significant with NC group (Fig 2d; Table 1).

DISCUSSION

Hyperglycemia causes a marked deviation in antioxidant defense system. Glucose autoxidation led to increased production of ROS which enhances cellular damage and has been implicated in the pathogenesis of diabetes mellitus (Kowluru and Chan 2007). The increase in the levels of free radicals causes damage to the biological structures, such as enzymes, genetic material and cell membrane. Diabetic complications like retinopathy and neuropathy development could be prevented by use of antioxidant compounds (SOD/catalase mimetic) mediated inhibition of hyperglycemia-induced ROS production (Vincent *et al.,* 2007). In contrast to conventional antioxidants, SOD/catalase mimetic are beautiful choice to revert the hyperglycemia-induced mitochondrial superoxide overproduction which is the paramount factor to activate major five damaging pathways (AGE formation, RAGE expression, PKC activation, increased polyol flux and hexosamine flux) by inhibiting GAPDH (Giacco and Brownlee, 2010).

Polyphenols are recognized as secondary

metabolites of plants which protect them against adverse climate and pathogens. The medicinal property is highly influenced by geographical origin and time as resin was harvested in the month of April-May having higher activity (Poornima, 2009). Polyphenols include phenolic acids, flavonoid, stilbenes and lignans. Meta-analyses emphasized that long term consumption of polyphenols rich diet decrease the chronic ailments such as cancers, cardiovascular diseases, diabetes and neurodegenerative diseases (Arts and Hollman, 2005; Pandey and Rizvi, 2009). Preliminary phytochemical screening of crude resin extract revealed the presence of alkaloids, flavonoid, saponins, tannins, sterols and terpenes (Murthy *et al.,* 2011).

Here, the antioxidant activity of SRE was evaluated by in vitro (DPPH) and in vivo (SOD, CAT, MDA and NO) methods. DPPH is a reliable method most commonly used to screen general antioxidant property of a plant extract. The DPPH based antioxidant activity of various part of *S. robusta* has been studied by using different solvent for extraction and IC50 has been calculated for ethanolic extract of leaves (36.61ìg/ml) (Mathavi and Nethaji, 2014) and methanolic extract of resin (35.60 ìg/ml) (Vashisht *et al.,* 2016). Present work showing the concentration of ethanolic extract of resin is required almost four fold (146 ìg/ml) of previously claimed concentration for 50% scavenging of the DPPH free radical. In fact, IC50 for ascorbic acid was found under the standard range.

Malondialdehyde (MDA) is the most commonly used biomarker can be considered a direct index of oxidative injuries which substantially elevated in diabetes are associated with lipid per oxidation (Gallou *et al.,* 1993). In plasma NO is oxidized almost completely to nitrite, and remains stable. According to literature, hyperglycemia may enhance NO production through eNOS and iNOS. Its consequences might be beneficial by relaxation of blood vessels or detrimental by interacting with superoxide radical depending upon concentration (Adela *et al.,* 2015). In this context a marked increase in the concentration of MDA, NO and significant decreased level of SOD, CAT indicates oxidative stress in STZ exposed rats when compared to control rats. Administration of *S. robusta* significantly decreased the level of MDA, NO and restored the activity of enzymes demonstrated the reduction of oxidative stress in extract treated diabetic rats at all concentrations. In accordance with previous work exhibited that S. robusta leave extract is efficient to counter CCl_4 induced oxidative stress on isolated hepatocytes cell culture by restoring SOD and CAT activity consequently reduces MDA level (Suganya *et al.,* 2014). Similarly, extract of bark having protective effect against paracetamol induced liver damage in rats (Biswas *et al.,* 2015).

Poor glycemic control exclusively reduces the body weight in diabetes mellitus. In this context SRE-treated rats for 8 weeks improved their body weight (data not shown) compare to untreated rats is agree with earlier reports (Atangwho *et al.,* 2007). Over all findings showed that the treatment with SRE improved significant outcome probably by scavenging the ROS and strengthening antioxidants owing to resin loaded with phenolic and flavonoid contents.

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REFERENCES

Adela, R., Nethi, S.K., Bagul, P.K., Barui, A.K., Mattapally, S., Kuncha, M., Patra, C.R., Reddy, P.N. and Banerjee, S.K. (2015). Hyperglycaemia enhances nitric oxide production in diabetes: A study from south Indian patients. *PLoS ONE* **10(4)**: e0125270.

Aebi, H. (1984). Catalase in vitro. *Methods Enzymol.* **105**:121-126.

Arts, I.C.W. and Hollman, P.C.H. (2005). Polyphenols and disease risk in epidemiologic studies. *Am. J. Clin. Nutr.* **81**:317-25.

Atangwho, I.J., Ebong, P.E., Eyong, E.U., Eteng, M.U. and Uboh, F.E. (2007). Vernonia amydalina Del. A potential prophylactic antidiabetic agent in lipids complication. *Global J. Pure Appl. Sci.* **18(1)**: 103–6.

Bharitkar, Y.P., Banerjee, M., Kumar, S., Paira, R., Meda, R., Kuotsu, K. and Mondal, N.B. (2013). Search for a potent microbicidal spermicide from the isolates of *Shorea robusta* resin. *Contraception.* **88(1)**: 133-40.

Biswas, D., Gouda, S., Shankar, N.L.G. and Suresh, S.V.K. (2015). Effect of ethanol extract from *shorea robusta* (Dipterocapaceae) bark in paracetamol induced liver damage in rats. *Int. J. Phytomed.* **7(4)**: 420-426.

Brand-Williams, W., Cuvelier, M. E. and Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. *Food Sc. Technol*. **28(1)**: 25–30.

Bryan, N.S. and Grisham, M.B. (2007). Methods to detect nitric oxide and its metabolites in biological samples. *Free Radic. Biol. Med.* **43(5)**: 645–657.

Buege, J.A. and Aust, S.D. (1978). Microsomal lipid peroxidation. *Methods Enzymol.* **52**: 302-10.

Ceriello, A. (2000). Oxidative stress and glycemic regulation. *Metab. Clin. exp.* **49(2)**: 27-29.

Gallou, G., Ruelland, A., Legras, B., Maugendre, D., Allannic, H. and Cloarec, L. (1993). Plasma malondialdehyde in type 1 and type 2 diabetic patients. *Clin. Chim. Acta.* **214**: 227– 34.

Giacco, F. and Brownlee, M. (2010). Oxidative stress and diabetic complications. *Circ. Res.* **107(9)**: 1058–1070.

Hossain, M.A., Raqmi, K.A.S.,Mijizy, Z.H., Weli, A.M. and Riyami, Q. (2013). Study of total phenol, flavonoids contents and phytochemical screening of various leaves crude extracts of locally grown Thymus vulgaris. Asian Pac. *J. Trop. Biomed.* **3(9)**: 705-710.

Hosseini, S.E., Mousaei, S. and Tavakoli, F. (2014). Effect of hydro alcoholic extract of artichoke on diabetes treatment and liver enzymes in diabetic adult male rats. *Adv. Herbal Med.* **1(1)**: 17–21.

Kowluru, R.A. and Chan, P.S. (2007). Oxidative stress and diabetic retinopathy. *J. Diabetes Res.* **4**: 43603.

Madesh, M. and Balsubramanium, K.A. (1998). Microtiter plate assay for superoxide dismutase using MTT reduction by superoxide. *Indian j. Biochem. Biophys*. **35(3)**:184-188.

Mathavi, P. and Nethaji, S. (2014). *In vitro* antioxidant activity of *Shorea robusta* leaf extract. *Int. J Res. Biochem. Biophys.* **4(3)**: 22-26

Murthy, K.S.R., Lakshmi, N. and Ramulu, D.R. (2011). Biological activity and phytochemical screening of the oleoresin of *Shorea robusta* Gaertn. f. *Trop. Subtrop. Agroecosy*s. **14**: 787 – 791.

Pandey, K.B. and Rizvi, S.I. (2009). Plant polyphenols as dietary antioxidants in human health and disease. *Oxid. Med. Cell Longev.* **2(5)**: 270-278.

Poornima, B. 2009. Comparative phytochemical analysis of shorea robusta gaertn (oleoresin) WSR to its seasonal collection. Anc. Sci. life. 29(1):26-28.

Suganya, P., Nandhini, R., Jeyadoss, T. and Velavan, S. (2014). In vitro antioxidant activity of methanolic extract of shorea robusta in hepatocytes. *Int. J. Pharm. Pharma. Sc.* **6(6)**: 2014.

Supriya, K., Kotagiri, S.,rushabendra Swamy, B.M. & Archana Swamy, P & Vishwanath, K.M.. (2012). Anti-obesity activity of Shorea robusta G. Leaves extract on high fat diet induced obesity in albino rats. *Res. J. Pharmaceut., Biol. Chem. Sc.* **3**. 542-554.

Trease, G.E. and Evans, W.C. (1996). Pharmacognosy. London: Bailliere Tindall.

Vashisht, S., Singh, M.P. and Chawla, V. (2016). In-vitro antioxidant and antibacterial activity of methanolic extract of *Shorea robusta* Gaertn. F. Resin. *Int. J. Pharm. Phytopharm. Res*. **6(4)**: 68-71.

Vincent, A.M., Russell, J.W., Sullivan, K.A., Backus, C., Hayes, J.M., McLean, L.L. and Feldman, E.L. (2007). SOD2 protects neurons from injury in cell culture and animal models of diabetic neuropathy. *Exp. Neurol.* **208**: 216–27.

Wani, T.A., Chandrashekara, H.H., Kumar, D., Prasad, R., Sardar, K.K., Kumar, D. and Tandan, S.K. (2012a). Anti-inflammatory and antipyretic activities of the ethanolic extract of *Shorea robusta* Gaertn. F. resin. *Indian J. Biochem. Biophys*. **49**: 463-467

Wani, T.A., Chandrashekara, H.H., Kumar, D., Prasad, R., Gopal, A., Sardar, K.K., Tandan, S.K. and Kumar, D. (2012b). Wound healing activity of ethanolic extract of *Shorea robusta* Gaertn. f. resin. *Indian J. Exp. Biol.* **50(4)**: 277-81.

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Research Article

IMMUNOMODULATORY AND HEMATOPROTECTIVE POTENTIAL OF *WITHANIA SOMNIFERA* AQUEOUS ROOT EXTRACT (WRE) AGAINST SUBACUTE TOXICITY OF MONOCROTOPHOS IN COMMERCIAL BROILERS

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ABSTRACT

The objective of the present investigation was to access immunoprotectiveand hematoprotectiveeffect of *Withania somnifera* against subacute toxicity of monocrotophos (MCP) in chickens. The study consisted of 5 groups having 12 birds in each. Monocrotophos (2 ppm in feed daily) was administered to birds of group II to V and *Withania somnifera* aqueous root extract (100, 300 and 500 mg/kg b. wt /day) was given in drinking water to birds of group III, IV and V birds respectively for 6 weeks. Group I served as control.MCP decreased the levels of Hb, TEC, TLC, total protein, globulin, lymphocytes and AChE activity while increased heterophil, creatinine, cholesterol, AST, ALT, ALP levels in exposed birds. These hemato-biochemical changes were ameliorated by *Withania somnifera*in WRE+MCP-treated birds after 4 and 6 weeks. Activity of AChE (in brain and RBC) became almost normal in WRE-treated birds. $ZnSO_4$ turbidity test, ELISA and LST showed decrease in total Immunoglobulin (Ig) level, antibody titer and "OD of B and T cells in MCP-exposed group II birds, respectively. Increase in total Ig levels were recorded in extract-treated chickens of group IV and V while antibody titre increased in dose dependent manner in WRE-treated group III – V. WRE @ 500mg/kg significantly increased LPS stimulated cultures of B lymphocytes and @ 300 and 500 mg/kg increased PHA-stimulated cultures of T lymphocytes, respectively.MFT and DTH revealed decrease in % NBT positive macrophages and skin thickness, respectively in MCP-exposed group II birds. WRE significantly improved % NBT positive macrophages @ 300 and 500 mg/kg b. wt. Also, significant increase in skin thickness was observed following second exposure of dinitrofluorobenzene in chickens of extract treated group V at 6, 12, 24 and 72 h intervals and in group IV at 24 h interval. These observations suggested protective role of *Withania somnifera* aqueous root extract against MCP-induced immunosuppression and hematobiochemical alterations.

Keywords: Monocrotophos, immunosuppression, immunomodulation, *Withania somnifera,* chickens

INTRODUCTION

Monocrotophos is AChE inhibitingorganophosphate compound used as broad-spectrum, fast-acting insecticide, acaricide and anthelmintic agentin animal husbandry practices with both systemic and residual contact actions(Van Sitlert and Duma, 1990). It is widely used as agrochemical to control insects and pest of rice and other crops.Its indiscriminate use has caused deleterious effects in exposed birds.In India, monocrotophos and other organophosphates share 30% of the total production of the pesticides (Gupta, 2004).Medicinal herb like *W. somnifera* is a known antistress, adaptogenic and hemopoietic substance since ayurvedic times (Mishra *et al.,*2000**).**In Ayurvedic system of medicine*Withania somnifera*has been described as immunomodulators (Agarwal and Singh, 1999). Withanolides, highly oxygenated C-28 steroid are the principal bioactive compounds present in *Withania somnifera* root. Their pharmacological activities have been attributed to withaferin A and withanolide D (Kaur *et al.,* 2003).

The present investigation was undertaken to evaluate ameliorating potential of *Withania somnifera* aqueous root extract (WRE) on hematobiochemical and immunological profile in subacutemonocrotophos (MCP) toxicity (2 ppm in feed) daily in broiler chickens.

MATERIALS AND METHODS

Preparation of plant extract

Withania somnifera aqueous roots were grinded, soaked in distilled water for 24 h and filtrate was dried in incubator with fan and finally lyophilized.

Experimental animals and design

In this experiment, 60 day-old commercial broilers, divided into five groups (12 in each), were used. The broilers of group II to V were treated with 2 ppm of MCP in feed daily with effect from day 7 till the end of experiment. *Withania somnifera* root extract was given @ 100, 300 and 500 mg kg^{-1} b. wt in morning drinking water daily to the birds of group III to V and thereafter till end of the experiment. Birds of group I did not receive any treatment except routine feed and water served as

untreated control.

Hematological parameters

On 29th day of treatment, blood samples were collected from six birds from each group to evaluate haemato-biochemical parameters. Heparinized blood samples (1ml) were used. Packed cell volume (PCV) and hemoglobin was estimated by the method of Jain (1986). Total erythrocytes count (TEC) and total leucocyte count (TLC) was determined by the method of Natt and Herric (1952) using poultry diluting fluid. Differential leucocyte count (DLC) was done by preparing blood smear from drop of uncoagulated blood and leucocytes were counted by method as described by Lucas and Jamroz (1961). Erythrocytic indices were calculated by the method as mentioned by Chauhan and Agarwal (2006).

Immunological parameters

After 35 days of treatment, blood samples were collected from rest of birds from each group for evaluation of immunological parameters. Lymphocyte stimulation test (LST) was estimated using method of Rai-el-Balha (1985) and Chauhan (1995). Macrophage function test (MFT) was determined using method of Talwar (1983). Delayed type hypersensitivity test (DTH) was calculated using method of Phanuphak (1974). Enzyme linked immunosorbent essay was estimated using method of Miers (1983) using antibody titre against RD antigen. Total immunoglobulin (Ig) was estimated using method of McEvan (1969). On 43rd day, all the experimental birds were sacrificed, blood samples and tissues (liver, brain and kidney) were collected to evaluate haematological and biochemicalparameters. After completion of experiments all the birds were sacrificed buried scientifically. Date was statically analysed by ANOVA for signifiant difference at 5% Level of significance.

RESULTS

In the present investigation immunoprotective and hematoprotective effect of *Withania somnifera* against subacute toxicity of monocrotophos (MCP) in chickens was accessed. The study consisted of 5 groups having 12 birds in each. Birds of group II to V were administered MCP (2 ppm in feed daily).WRE, *Withania somnifera* aqueous root extract was given in drinking water to birds of group III, IV and V @ 100, 300 and 500 mg/kg b.wt/day respectively for 6 weeks. Group I served as control. MCP exposed birds of group II remained dull and depressed throughout the study period. The levels of Hb, TEC, TLC, total serum protein, globulin, lymphocytes and AChE activity were decreased while heterophil, creatinine, cholesterol, AST, ALT, ALP levels increased in exposed birds. The levels of Hb, TEC, TLC, PCV and lymphocyte count were significantly increased and heterophil counts were decreased after 4 and 6 weeks in WRE+MCP-treated birds (Table 1 and 2).There was increase in level of serum total protein, albumin and globulin while decrease in A: G ratio, creatinine, cholesterol, AST, ALP and ALT in birds of group III-V as compared to MCP group (Table 3 and 4). Activity of AChE (in brain and RBC) became almost normal in WRE-treated birds. $ZnSO_4$ turbidity test, ELISA and LST showed decrease in total Immunoglobulin (Ig) level, antibody titer and "OD of B and T cells in MCP-exposed group II birds, respectively (Table 5). Increase in total Ig level were recorded in extract-treated chickens of group IV and V. Birds of WRE-treated group III – V exhibited dose dependent increase in antibody titer. WRE @ 500mg/kg significantly increased LPS stimulated cultures of B lymphocytes and @ 300 and 500 mg/kg increased PHA-stimulated cultures of T lymphocytes, respectively.MFT and DTH revealed

Table 1:
Effect of aqueous root extract of *W. somnifera* (WRE) on haematological profile in broiler chickens after 4 weeks of monocrotophos (MCP, 2 ppm in feed) treatment (mean±S.E., n=6).

Group	I(Control)	II	III	IV	V	One way ANOVA		
Extract / Drug	-	MCP	MCP+WRE	MCP+WRE	MCP+WRE			
WRE(mg/kg/day)	-	-	100	300	500	C.D	d.f	F
Hb(g/dl)	8.70±0.27	6.30±0.97[a](27)	7.73±0.29(11)	7.13±0.30(18)	8.23±0.15[b] (5)	1.43	4,25	3.68
PCV (%)	27.5±1.77	22.5± 0.89(18)	25.5±2.47(7)	23.8±0.70(13)	25.2±2.12(8)	5.02	4,25	1.17
TEC ($x10^6$/µl)	3.75±0.43	2.48± 0.35[a](34)	2.65± 0.16(29)	2.95±0.13(21)	3.43±0.35[b](8)	0.90	4,25	2.99
TLC ($x10^3$/ µl)	21.7±1.56	16.8±1.51[a](22)	18.5±1.86(14)	20.7±1.67(4)	21.8±1.51[b](1)	4.72	4,25	1.77
Lym(%)	58.3±2.01	40.7±1.69[a](30)	48.8±1.14[b](16)	51.8±1.25[b](11)	57.2±1.42[b](2)	4.45	4,25	21.6
Het(%)	34.2±2.21	52.7±2.03[a](54)	43.3±1.28[b](27)	41.2±1.06[b](20)	35.5±1.65[b](4)	5.17	4,25	17.1
Mon(%)	4.17±0.60	4.17± 0.91(0)	4.50±0.43(8)	4.50±0.85(8)	4.83± 0.87(16)	2.19	4,25	0.14
Bas(%)	0.67±0.21	0.50± 0.34(25)	0.83±0.31(24)	0.33±0.33(51)	0.67± 0.33(0)	0.90	4,25	0.38
Eos(%)	2.17±0.65	2.00± 0.73(7)	2.50±0.72(15)	2.17±0.75(0)	1.83± 0.65(15)	2.04	4,25	0.12
MCV (μm^3)	81.2±13.9	99.5±13.6(22)	99.1±12.6(22)	81.4±3.24(0)	74.9±4.66(7)	30.9	4,25	1.13
MCH (pg)	25.3±3.82	26.8±4.39(6)	29.9±2.54(18)	24.3±0.97(4)	25.2±2.29(0.5)	8.84	4,25	0.51
MCHC (%)	32.6±3.26	28.3±4.73(13)	32.0±3.69(2)	30.1±1.80(7)	33.8±2.73(4)	9.82	4,25	0.41

[a] = p< 0.05 as compared to untreated control in the same row. [b] = p< 0.05 as compared to MCP group in the same row.
Lym: Lymphocytes, Het : Heterophils, Mon : Monocytes, Bas : Basophils, Eos : Eosinophils
Values in parenthesis are % change as compared to control group

Table 2:
Effect of aqueous root extract of *W. somnifera* (WRE) on haematological profile in broiler chickens after 6 weeks of monocrotophos (MCP, 2 ppm in feed) treatment (mean ± S.E., n=6).

Group	I(Control)	II	III	IV	V	One way ANOVA		
Extract / Drug	-	MCP	MCP+WRE	MCP+WRE	MCP+WRE			
WRE(mg/kg/day)	-	-	100	300	500	C.D	d.f	F
Hb(g/dl)	8.07±0.30	6.67±0.28(17)	7.63±0.86(5)	7.17±0.38(11)	7.67±0.34(5)	1.42	4,25	1.23
PCV(%)	28.7±1.82	20.5±1.02[a](28)	19.8±1.38(31)	25.0±1.37[b](13)	27.0±1.65[b](6)	4.26	4,25	7.05
TEC (x10^6/µl)	3.39±0.42	2.13±0.37[a](37)	2.74±0.14(19)	2.98±0.23(12)	3.36±0.35[b](1)	0.93	4,25	2.64
TLC (x10^3/µl)	22.0±1.88	15.2±0.91[a](31)	20.2±1.05[b](8)	16.8±2.06(23)	21.5±1.73[b](2)	4.61	4,25	3.54
Lym(%)	60.2±1.56	43.7±1.69[a](27)	49.5±1.61[b](17)	56.2±1.49[b](6)	58.3±2.33[b](3)	5.60	4,25	12.6
Het(%)	33.8±0.91	48.8±1.62[a](44)	43.3±1.15[b](28)	36.2±1.62[b](7)	33.2±1.25[b](2)	3.87	4,25	25.7
Mon(%)	3.67±0.88	4.67±1.02(27)	4.00±1.06(9)	4.17±1.08(13)	4.67±1.41(27)	3.19	4,25	0.15
Bas(%)	0.67±0.33	0.83±0.48(24)	1.17 ±0.40(74)	0.83±0.31(24)	1.17±0.48(74)	1.17	4,25	0.30
Eos(%)	1.50±0.67	2.00±0.58(33)	2.17±0.87(44)	2.67±0.80(78)	2.67±1.17(78)	2.45	4,25	0.34
MCV (µm^3)	99.6±25.1	106.2±12.9(6)	73.3±5.57(26)	92.9±8.00(7)	85.1±9.83(14)	40.7	4,25	0.83
MCH (pg)	26.9±5.35	34.7±4.46(29)	28.7±4.33(7)	24.9±2.79(7)	24.4±3.08(9)	11.9	4,25	1.02
MCHC (%)	29.1±2.99	33.1±2.47(14)	38.7±4.41(33)	29.2±2.60 (0.5)	28.6±1.15 (2)	8.45	4,25	2.17

[a] = p< 0.05 as compared to untreated control in the same row. [b] = p< 0.05 as compared to MCP group in the same row.
Lym: Lymphocytes, Het : Heterophils, Mon : Monocytes, Bas : Basophils, Eos : Eosinophils
Values in parenthesis are % change as compared to control group.
MCH: Mean corpuscular hemoglobin, MCV: Mean corpuscular volume, MCHC: Mean corpuscular hemoglobin concentration

Table:3.
Effect of aqueous root extract of *W. somnifera* (WRE) on biochemical profile in broiler chickens after 4 weeks of monocrotophos (MCP) treatment (mean ± S.E., n=5).

Group	I(Control)	II	III	IV	V	One way ANOVA		
Extract / Drug	-	MCP	MCP+WRE	MCP+WRE	MCP+WRE			
Dose of WRE(mg/kg/day)	-	-	100	300	500	C.D	d.f	F
Total protein (g/dl)	4.26±0.44	2.04±0.41[a](52)	2.66±0.10(37)	2.36±0.29(44)	3.55±0.47[b](16)	1.24	4,20	4.62
Alb (g/dl)	1.56±0.24	1.08±0.32(31)	1.83±0.19[b](17)	1.20±0.14(23)	1.35±0.22(13)	0.69	4,20	1.70
Glob (g/dl)	2.70±0.65	0.95±0.37[a](65)	0.83±0.11(69)	1.16±0.26(57)	2.20±0.30[b](14)	1.12	4,20	4.74
A:G Ratio	0.90±0.35	2.13±0.86(136)	2.45±0.46(172)	1.30±0.36(44)	0.62±0.08(32)	1.45	4,20	2.56
Creat(mg/dl)	0.42±0.04	0.62±0.09(47)	0.58±0.09(38)	0.46±0.09(9)	0.54±0.07(28)	0.21	4,20	1.14
Chol(mg/dl)	120.8±19.4	216.9±41.6(79)	178.8±23.5(48)	177.3±17 (46)	168.2±52(38)	99.7	4,20	1.03
AST (IU/L)	46.4±6.05	74.8±4.08[a](43)	73.6±1.83(58)	68.4±3.66(47)	51.6±5.11[b](11)	12.8	4,20	8.93
ALT (IU/L)	17.2±0.80	30.0±1.90[a](74)	26.4±1.47(53)	27.6±0.75(60)	20.0±0.75[b](16)	3.77	4,20	17.8
ALP (KAU)	54.8±17.05	172.5±37.7(214)	124.5±11.4(28)	127.4±34(26)	75.3±16.4(56)	75.6	4,20	3.29
AChE* (RBC)	10.5±0.07	9.77±0.42(13)	10.5±0.14(6)	10.7±0.2[b] (4)	10.9±0.34[b](3)	0.74	4,25	3.05

[a] = p< 0.05 as compared to untreated control in the same row. [b] = p< 0.05 as compared to MCP group in the same row.
Alb: albumin, A:G ratio : albumin:globulin ratio, Creat : creatinine, Chol : cholesterol.
AChE: Acetylcholinesterase, * nmoles/ min/ ml
Values in parenthesis are % change as compared to control group.

Table 4:
Effect of aqueous root extract of *W. somnifera* (WRE) on biochemical profile in broiler chickens after 6 weeks of monocrotophos (MCP) treatment (mean±S.E., n=5).

Group	I(Control)	II	III	IV	V	One way ANOVA		
Extract / Drug	-	MCP	MCP+WRE	MCP+WRE	MCP+WRE			
WRE(mg/kg/day)	-	-	100	300	500	C.D	d.f	F
Total protein (g/dl)	3.28±0.34	1.43±0.12[a](56)	1.62±0.09(50)	1.82±0.14(44)	2.52±0.33[b] (23)	0.69	4,20	10.8
Alb (g/dl)	1.02±0.06	0.74±0.05[a](27)	0.79±0.10(22)	0.96±0.1[b] (6)	0.86±0.04(15)	0.21	4,20	3.42
Glob (g/dl)	2.26±0.31	0.70±0.13[a](69)	0.83±0.06(63)	0.86±0.12(62)	1.66±0.4[b] (26)	0.69	4,20	8.62
A:G Ratio	0.49±0.07	1.54±0.67[a](214)	0.99±0.19(102)	1.19±0.15(142)	0.64±0.15(30)	0.98	4,20	1.64
Creat(mg/dl)	0.84±0.31	2.56±0.74[a](204)	2.12±0.56(152)	1.36±0.29(62)	0.92±0.3[b] (9)	1.40	4,20	2.54
Chol (mg/dl)	143.1±21	169.2±18.2(18)	168.4±9.72(17)	203.2±16.5 (42)	153.1±34.9 (7)	64.3	4,20	1.09
AST (IU/L)	47.6±6.05	68.8±4.32[a](44)	68.4±4.96(43)	50.8±7.9(6)	48.0±7.40[b](1)	18.6	4,20	3.01
ALT (IU/L)	20.4±2.32	40.0±5.83[a](96)	30.4±3.43(49)	36.0±5.37(76)	21.2±2.73[b](4)	12.3	4,20	4.36
ALP (KAU)	95.4±31.8	153±30.3(61)	120.7±24.2(21)	126.7±15.5 (17)	100.9±21.2 (34)	74.6	4,20	0.83
AChE* (RBC)	9.67±0.16	8.48±0.40[a](9)	8.96±0.28(8)	9.33±0.2[b] (3)	9.55±0.12(3)	0.75	4,25	3.48
AChE+ (Brain)	4.41±0.36	2.21±0.22[a] (41)	3.44±0.49[b](17)	2.40±0.28(42)	2.78±0.44(37)	1.09	4,25	5.77

[a] = p< 0.05 as compared to untreated control in the same row. [b] = p< 0.05 as compared to MCP group in the same row.
Alb: albumin, A:G ratio : albumin:globulin ratio, Creat : creatinine, Chol : cholesterol. Values in parenthesis are % change as compared to control group. AChE: Acetylcholinesterase, * nmoles/ min/ ml; + nmoles/ min/ g

Table 5:
Effect of aqueous root extract of *W. somnifera* (WRE) on immunological profiles in broiler chickens after 5 weeks of monocrotophos (MCP) treatment (mean±S.E., n=6).

Group I(Control)		Ⅱ	Ⅲ	IV	V		One way ANOVA		
Extract / Drug		-	MCP	MCP+WRE	MCP+WRE	MCP+WRE			
WRE(mg/kg/day)		-	-	100	300	500	C.D	d.f	F
LST	B cells	0.36±0.03	0.16±0.03[a](55)	0.25± 0.03(30)	0.28±0.06(22)	0.31±0.08[b](13)	0.15	4,25	3.18
(Ä OD)	Tcells	0.34±0.09	0.15±0.05[a](55)	0.20±0.03(41)	0.29±0.03[b](14)	0.32±0.12[b](5)	0.02	4,25	3.26
MFT*		61.4±1.31	40.1±1.65[a](34)	46.7±1.76(23)	52.5±2.21[b](14)	54.0±4.68[b](12)	7.72	4,20	9.31
ELISA**(Ä OD)		5.00±0.11	2.28±0.17[a](54)	3.66±0.09[b](27)	3.70±0.27[b](26)	4.31±0.17[b](13)	0.50	4,25	33.9
Total Ig	(g/l)	1.50±0.20	0.67±0.12[a](55)	0.90±0.17 (34)	1.65±0.38[b](100)	1.26±0.46(88)	0.82	4,25	2.89
DTH† (in cm)	0h	0.48±0.01	0.43±0.02(10)	0.44 ±0.01(8)	0.44±0.02(8)	0.47±0.02(2)	0.04	4,25	2.60
	6h	0.54±0.02	0.46±0.01[a](14)	0.46±0.01(14)	0.50±0.02(7)	0.51±0.03[b](5)	0.04	4,25	2.60
	12h	0.67±0.03	0.51±0.02[a](23)	0.51±0.01(23)	0.53±0.02(21)	0.64±0.04[b](4)	0.06	4,25	10.8
	24h	0.72±0.02	0.60±0.02[a](16)	0.63 ± 0.02(12)	0.66±0.01[b](8)	0.70±0.01[b](3)	0.05	4,25	8.84
	48h	1.10±0.04	0.85±0.03[a](22)	0.87 ± 0.03(21)	0.93±0.08(15)	1.01±0.13(8)	0.16	4,25	3.25
	72h	0.90±0.03	0.70±0.02[a](22)	0.73 ± 0.03(19)	0.74±0.05(18)	0.84±0.08[b](6)	0.10	4,25	5.31
	Avg	0.79±0.10	0.61±0.08(22)	0.64±0.08(19)	0.67±0.08(15)	0.74±0.09(6)	0.22	4,20	0.76

[a] = p< 0.05 as compared to untreated control in the same row. [b] = p< 0.05 as compared to MCP group in the same row.
* Percentage of NBT positive macrophages. ** Antibody titer, † Skin thickness. Values in parenthesis are % change as compared to control group.

decrease in % NBT positive macrophages and skin thickness, respectively in MCP-exposed group II birds (Table 5). WRE significantly improved % NBT positive macrophages @ 300 and 500 mg/kg b. wt. Also, significant increase in skin thickness was observed following second exposure of dinitrofluorobenzene in chickens of extract treated group V at 6, 12, 24 and 72 h intervals and in group IV at 24 h interval.

DISCUSSION

Haematological profile

Monocrotophos is an organophosphate which irreversibly at esteratic site of acetylcholine esterase and inhibits hydrolysis of acetylcholine (Ach). This inhibition causes accumulation of Ach at neuroeffector sites causing persistent stimulation of cholinergic receptors which could be attributed to toxicity of cockerels (Echobichon, 2001). In present study MCP (2ppm in feed) after 4 and 6 weeks of subacute exposure decreased values of Hb, TEC, TLC and lymphocytes. Progressive development of anemia due to increased RBC fragility, decreased Hb synthesis and hemolysis accounts for the hematotoxic effect of MCP in broilers (Ahmad *et al.,* 1995) and suppression in differentiation and proliferation of stem cell and bone marrow by MCP (Skripsky and Loosli., 1995).Lymphocytopenia and heterophiliawas observed in cockerels of MCP group II after 4 and 6 week of feeding trial due to degenerative effect of MCP on lymphoid system (Venkatarghavan*et al.,* 1980; Cho *et al*., 1989, Girija *et al.,* 1996; Aphale *et al.,* 1998). Multiple intraperitoneal dose of Nuvacron (monocrotophos, 0.8 mg/kg) in mice revealed similar findings of neutrophilic and lymphocytopenia (Gupta *et al.,* 1982). These changes have been reported by other workers in MCP treated chickens and rats (Cho *et al.,* 1989).

Less severe toxic signs were noticed in birds of WRE treated groups indicating *W.somnifera* might have protected enzyme AchE from action of MCP. WRE exhibited significant and dose dependent increase in values of Hb, TEC, PCV and lymphocytes and decrease in heterophil count in extract treated group birds. These findings were in agreement with earlier reports in which *W. somnifera* significantly increased the concentration of Hb and PCV in broiler chicks and increased TLC and lymphocyte count in urethane-intoxicated animals (Samarth *et al.,* 2003 and Singh *et al.,* 1986). There was increase in bone marrow cellularity by methanolic extract of ashwagandha and the ratio of normochromic and polychromic erythrocytes in γ-irradiated mice was normalized (Girija *et al.,* 1996). Ashwagandha extract reduced cyclophosphamide-included leucopenia in mice (Davis and Kuttan, 1998; Gupta *et al.,* 2001). *W. somnifera* treatment in children for 60 days significantly increased the level of Hb, PCV, MCV and serum iron (Venkataragavan *et al.,* 1980). It is thus inferred that WRE has hematoprotective effect on MCP exposed chickens.

Biochemical profile

Significant decrease in total protein, globulin levels and increase in creatinine level after 4 and 6 weeks of MCP exposure in group II birds were similar tostudies of Garg and co-workers (2004a, b).MCP-induced hypoproteinemia may be caused by diminution in the protein biosynthesis due to hepatic dysfunction and excess elimination of proteins from damaged kidneys. This was evidenced by hepatorenaland biochemical changes in broilers. Decrease in level of globulin synthesis could be caused by dysfunctioning of rough endoplasmic reticulum due to pesticide exposure in broiler chicken (Reyes and Moore, 1979). Decreased elimination of creatinine due to renal toxicity (Rahman *et al.,* 2000) and ACh-mediated muscle hyperactivity

leading to free radical- induced lipid peroxidation and muscle injury (Yang and Dettbarn, 1996)might have increased creatinine level in MCP-treated birds.

Hepatoprotective potential of *W. somnifera*(Mishra *et al.,* 2000) has significantly increased the total protein and globulin levels in present study. Similar effect of *W. somnifera* on serum protein and globulin was also found in normal and lead-treated broilers (Samarth *et al.,* 2003).Increased in the activities of AST, ALT and ALP in serum of MCP exposed group II birds were in agreement with earlier reports of Garg and coworkers*,* 2004a (in chicken); Rao, 2006 (in plasma and liver of euryhaline fish) and Kaur and Dhanju, 2004; Rahman and Siddiqui, 2003; Rahman *et al.,* 2000a (in plasma and liver of female rats). Increase in transaminase activities and in level of ALP in MCP-treated birds might be due to necrotic hepatitis and intrahepatic and extrahepatic bile duct obstruction (Cornelius. 1989). These findings suggest hepatotoxic potential of MCP in commercial broilers.

Effect on humoral system

To access humoral immune response in birds, Zinc sulphate turbidity test for estimating Ig level and ELISA (against Ranikhet Disease vaccine) were employed.Significant reduction in serum total immunoglobulin (Ig), B lymphocytes and RD vaccine – induced antibody titre (expressed as ELISA) were significantly decreased in the MCP group birds when compared to control. Reduction in antibody titre could be attributed to hampered proliferation and activation of B-lymphocytes responsible for biosynthesis of immunoglobulins. Marked reduction in TLC, DLC and serum globulin levels in this study supported immunosuppressive effect of MCP exposed chickens.Prolonged feeding of MCP (2 ppm in feed) for 20 weeks has been reported to cause immunosuppression in broiler chickens**.** Decrease in delayed hypersensitivity reaction and haemaglutinationtitre in MCP exposed rats indicated suppression of both cellular and humoral responses. As per reports of Garg and coworkers, 2004a, MCP (2 ppm in feed) for 8 weeks caused leucocytopenia and lowered level of T-lymphocyte. MCP has also been found to cause apoptosis and necrosis in cultured human lymphocytes (Das *et al.,* 2006). In the present study similar observations have been recorded.

WRE significantly improved humoral status in birds of III to V group. Immunostimulatory properties of ashwangandha root extract were detected in cyclophosphamide-induced immunocompromised mice (Agarwal *et al.,* 1999).Immunomodulatory property of *W. somnifera* has also been reported earlier by other workers (Ghosal *et al.,* 1989; Ziauddin *et al.,* 1996; Davis and Kuttan, 2000; Gautam*et al.,* 2004). Different polyherbal preparations like Zeetress and Immo-21 contain *W. somnifera*as an ingredient (Babu *et al.,* 2002; Nemmani *et al.,* 2002; Sujata *et al.,* 2002).

Effect on cell mediated immunity

In this study, B-lymphocyte transformation assay (LST) was carried out by using lipopolysaccharide (LPS) as mitogen. Lower capacity of B lymphocyte to form clones and to convert into plasmocyteswas evident by significant decrease in LPS-stimulated cultures of lymphocytes from MCP treated birds in comparison to control. Thus, MCP has direct inhibitory effect on B lymphocytes leading to reduced blastogenesis and antibody synthesis, which in turn might be responsible for immnosuppression in MCP-treated birds. Delayed type of hypersensitivity reaction, characterized by decrease in skin thickness was significantly reduced in MCP group II birds. It may party be due to increased synthesis of corticosteroids and/or quantitative change in T-cell population**.** Corticosteroids are well known immunosuppressive agents. Macrophage function test (MFT) showed MCP significantly reduced the percentage of NBT positive macrophages in group II birds. Thus, monocrotophos was observed to have the toxic potential on cell-mediated immunity. Its toxicity was detected in lymphocytes from peripheral blood of healthy donors (Jamil *et al.,* 2004). It decreased size of lymphoid follicles and depleted level of lymphocytes (Garg *et al.,* 2004). Casale and co-workers (1993) also witnessed adverse effect of MCP on CMI in mice.

In present investigation, WRE stimulated the cell-mediated immunity as evidenced by increased level of NBT positive macrophages (MFT), T and B lymphocytes (LST), serum total immunoglobulin (Ig) and skin thickness (DTH) in MCP-treated broilers after 5 weeks of experimental trial. The significant increase in TLC, lymphocytes count and the level of total protein and globulin by WRE further supported immunostimulatory potential of ashwagandha in MCP-treated birds. Cyclophosphamide-induced immunosuppression, as evidenced by reduced lymphocyte proliferation, IL-2, γ-interferon and GM-CSF production was significantly improved by the ashwagandha plant in mice (Davis and Kuttan, 1999).Iuvone and co-workers (2003) reported immunostimulatorypotenital of *W. somnifera* through increase in nitric oxide potential in macrophage. The macrophage leucocyte production, bone marrow cellularity, antibody against sheep RBC, number of plaque forming cells in spleen and phagocytic activity of peritoneal macrophages were also supposed to be the indicators of immunodulaotry effect of ashwagandha (Davis and Kuttan, 2000a). The significant reduction in inflammation and edema in adjuvant-induced arthritic (AIA) rats were also evidenced to prove

immunomodulatory property of ashwagandha.

It is concluded front this study that WRE produced protetive effect on MCP-induced altered immunity and hematobiochemical profile.

REFERENCES

Agarwal, S.S. and Singh, V.K. (1999). Immunomodulators: A review of studies on Indian Medicinal Plants. *Proc Ind Natl Sci Acad.* **65**:179-204.

Aphale; A.A.; Chhiba, A.D.; Kumbhkaran, N. R.; Mateenuddin, M. and Dahat, S.H. (1998). Subacute toxicity study of the combination of ginseng (*Panax ginseng*) and ashwagandha (*Withania somnifera*) in rats: a safety assessment. *Indian J. Physiol. Pharmacol.* **42:** 299-302.

Ahmad, F.; Ali, S.S. and Shakoori, A.R. 1995. Sublethal effects of Danitol(Fenpropathrin), a synthetic pyrethroid, on freshwater Chinese grass carp,Ctenopharyngodonidella. Folia. Biol. (Krakow). 43:151-159.

Babu, B.J.; Kalakumar, B.; Reddy, K.S. and Rao, A.S. (2002). Effect of Zeetress on cypermethrin induced suppression of growth and immunity in broiler chicks. *Indian J. Toxicol.* **9**: 135-138.

Chauhan, R.S. and Agarwal, D.K. (2006)**.** Textbook of Veterinary Clinical & Laboratory diagnosis, 2nd Edn. Jaypeebrothers medical publishers (p) Ltd. New Delhi. pp.149.

Chauhan, R.S. (1995)**.** Veterinary Clinical and Laboratory Diagnosis. Jaypeebrothers medical publishers (p) Ltd. New Delhi. pp. 326.

Cho, J.H.; Park, J.M. and Jean, Y.H. (1989).Studies on the diagnosis of organophosphate insecticides poisoning: Clinical and hematological diagnosis. *Resp. Rep. Rural Dev. Adm. Vet.* **31**: 8-13.

Cornelius, A.S.; Womer, R.B. and Jakacki, R. (1989). Multiple hemangioendotheli-omas of the liver. *Med. Pediat. Oncol.***17:**501-507.

Das, G.P.; Shaik, A.P. and Jamil, K. (2006). Estimation of apoptosis and necrosis caused by pesticides in vitro on human lymphocytes using DNA diffusion assay. *Drug. Chem. Toxicol.* **29**:147-56.

Davis, L. and Kuttan, G. (1998). Suppressive effect of cyclophosphamide-induced toxicity by *Withania somnifera* extract in mice. *J. Ethnopharmacol*. **62:**209-214.

Davis, L. and Kuttan, G. (1999). Effect of *Withania somnifera* on cytokine production in normal and cyclophosphamide-treated mice. *Immunopharmacol. Immunotoxicol.* **21:**695-703.

Davis, L. and Kuttan, G. (2000).Immunomodulatory activity of *Withania somnifera*.*J Ethnopharmacol.***71**:193-200.

Davis, L. and Kuttan, G. (2002a). Effect of *Withania somnifera* on cell mediated immune responses in mice. *J. Exp. Clin. Cancer Res*. **21:**585-590.

Davis, L.andKuttan,G.(2002b).Effect of *Withania somnifera* on CTL activity. *J. Ethnopharmacol.,***22:**115-118.

Echobichon, D.J. (2001). Toxic effects of pesticides-anticholinesterase agents. In: Casarette and Doull's Toxicology: The basic science of poisons. Ed. Klaassen, C.D. 6th Edition, Mc Graw Hills, New Delhi. P.774-784.

Garg, U. K.; Pal, A. K.; Jha, G. J. and Jadhao, S.B. (2004b)**.** Pathophysiological effects of chronic toxicity with synthetic pyrethroid, organophosphate and chlorinated pesticides on bone health of broiler chicks. *Toxicol. Pathol.* **32**:364-409.

Garg, U. K.; Pal, A.K.; Jha, G. J. and Jadhao, S. B. (2004a).Haemato-biochemical and immuno-pathophysiological effects of chronic toxicity with synthetic pyrethroid, organophosphate and chlorinated pesticides in broiler chicks. *Int. Immunopharmacol.***4**:1709-1722.

Gautam, M.; Diwanay, S. S.; Gairola, S.; Shinde, Y. S.; Jadhav, S. S. and Patwardhan, B. K. (2004)**.** Immune response modulation to DPT vaccine by aqueous extract of *Withania somnifera* in experimental system. *Int. Immunopharmacol*. **4:**841-849.

Ghosal. S.; Lal, J. and Srivastava, R. (1989). Immunomodulatory and CNS effects of sitoindosides IX and X, two new glycowithanolides from *Withania somnifera*. *Phytother. Res*. **3:**201-206.

Girija, K.; Kuttan, G.; Uma, D.P. and Bisht, K.S. (1996).Use Of*Withania somnifera* as an adjuvant during radiation therapy. *Indian J. Exp. Biol.* **34:**854-856.

Gupta, M.; Bagchi, G.; Bandopadhyay, S.; Sasmal, D.; Chatterjee, T. and Dey, S.N. (1982)**.** Haematological changes produced in mice by Nuvacron or Furadon. *Toxicol.* **25**:255-260.

Gupta, Y. K.; Sharma, S. S.; Rai, K. and Katiyar, C. K. (2001).Reversal of placitaxel- induced neutropenia by *Withania somnifera* in mice. *Indian J. Physiol. Pharmacol.,* **45**:253-257.

Iuvone, T.; Esposito, G. and Izzo, A.A. (2003).Induction of nitric oxide synthase expression by *Withania somnifera* in macrophages. *Life Sci*. **72:**1617-1625.

Jamil, K.; Shaik, A.P.; Mahaboob, M. and Krishna, D. (2004). Effect of organophosphorus and organochlorine pesticides (monocrotophos, chlorpyriphos, dimethoate and endosulfan) on

human lymphocytes *in vitro.Drug Chem. Toxicol.* **27**:133-44.

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

Kaur, P.; Sharma, M.; Mathur, S.; Tiwari, M.; Divekar, H. M.; Kumar, R. and Srivastava, K.K. (2003). Effect of 1-oxo-5beta, 6beta-epoxy-witha-2-ene-27-ethoxy-olide isolated from the roots of *Withania somnifera*on stress indices in Wistar rats. *J. Altern. Complement Med.* **9**:897-907.

Kaur, S. and Dhanju, C. K. 2004. Enzymatic changes induced by some organophosphorus pesticides in female rats. *Indian J. Exp. Biol.* **42**:1017-9.

Lucas, A. M. and Jamroz, C. (1961).Atlas of Avian Haematology. Govt. Priner, Washington, D.C.35p.

McEvan, A. D.; Fisher, W.; Selman, I. F. and Perihale, W. J. (1969). A turbidity test for estimation of immunoglobulin levels in neonatal calf serum. *Clinical Chem. Acta.* **27**:155-163.

Mishra, L.C.; Singh, B. B. and Dagenias, S. (2000).Scientific basis for the therapeutic use of *Withania somnifera*(Ashwagandha): A review. *Alt. Med. Rev.* **5:**334-346.

Miers, L.A.; Bankowski, R.A. and Zee, Y. C. (1983).Optimizing the Enzyme Linked Immunosorbent Assay for evaluating immunity of chickens to New Castle disease. *Avian Diseases.* **27**:1112-1125.

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

Nemmani, K. V.; Jena, G. B.; Dey, C. S.; Kaul, C.L. and Ramarao, P. (2002). Cell proliferation and natural killer cell activity by polyherbal formulation. *Indian J. Exp. Biol.* **40**:282-287.

Phanuphak, P.; Moorhead, J. W. and Clamam, H. N. (1974).Tolerance and Contact Sensitivity to DNFB in Mice. *J. Immunol.***112:**115-123.

Rahman, M. F. and Siddiqui, M. K. and Jamil, K. (2000).Acid and alkaline phosphatase activities in a novelphosphorothionate (RPR-V) treated male and female rats. Evidence of dose and time-dependent response. *Drug.ChemToxicol.* **23**:497-509.

Rai-el-Balhaa, G.; Pellering, J. L.; Boddin, G. and Abdullah, A. (1985). Lymphoblastic transformation assay of sheep peripheral blood lymphocyte: a new rapid and easy to read technique. *Comp. Immunol. Microbil. Infect. Dis.***8**:311-318.

Rao, N.R.; Pande, C.B. and Kiran, A.R. (1999).Effect of zeetress on broiler performance. *Pashudhan.* **14:**4.

Rao, J.V. (2006).Biochemical alterations in euryhaline fish, *Oreochromismossambicus*exposed to sub-lethal concentrations of an organophosphorus insecticide, monocrotophos. *Chemosphere.* [Epub ahead of print].

Rehman, M.F. and Siddiqui, M. K. (2003).Biochemical enzyme activity in different tissues of rats exposed to novel phosphorothionate (RPR-V). *J. Environ. Sci. Health B.* **38**:59-71.

Rehman, M. F.; Siddiqui, M. K. and Jamil, K. (2000a).Acid and alkaline phosphatase activities in a novel phosphorothionate (RPR-II) treated male and female rats.

Reyes, M. D. and Moore, E .C. (1979).Tissue residues and ultrastructural changes induced by DDT in chickens. *Poult. Sci.* **58**:1183-1191.

Samarth, V. R.; Dakshinkar, N. P.; Jagtap, D. G. and Bhojne, G. R. (2003).Effect ofAshwagandha root powder (*Withania somnifera)* onhemato-biochemical profile of broilers. *Ind. J. Ani. Sci.* **73:**648-649.

Singh, N.; Singh, S. P. and Nath, R. (1986). Prevention of urethane-induced lung adenomas by *Withania somnifera* (L.) Dunal in albino mice. *Int. J. Crude Drug Res*. **24:**90-100.

Sujata, K.; Dhanlakshmi, K. and Rao, A. S. (2002). Immunomodulatory effect of *stressroak* and zinc sulfate in birds vaccinated with *Salmonella gallinarum* vaccine. *Indian Vet. J.***79**:883-886.

Skripsky, T. and Loosli, R. (1994). Toxicology of monocrotophos. *Rev. Environ. Contam. Toxicol.* **139**:13-39.

Talwar, G.P. (1983). A handbook of practical Immunology. New Delhi, Vikas publishing house Pvt. Ltd.

Van Sitlert, N. J. and Duma, E. P. (1990). Field study on explosive and health effect of an organophosphorus pesticides for maintaining in registration in the Philippines. Med. Lav. **81**:463-473

Venkataraghavan, S.; Seshadri, C. and Sundaresan, T.P. (1980).The comparative effect of milk fortified with Aswagandha, Aswagandha and Punarnava in children -double-blind study. *J. Res. Ayur Sid*. **1:**370-385.

Yang, Z. P. and Dettbarn, W. D. (1996). Diisopropylphosphoro-fluoridate-induced cholinergic hyperactivity and lipid peroxidation. *Toxicol. Appl. Pharmacol.***138**:48-53.

Ziauddin, M.; Phansalkar, N. and Patki P. (1996). Studies on the immunomodulatory effects of ashwagandha. *J. Ethnopharmacol*. **50:**69-76.

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Research Article

EFFECT OF ORGANIC MINERAL MIXTURE, PROBIOTICS, ENZYMES, EMULSIFIER AND LIVER SUPPLEMENTS ON HAEMATOLOGICAL PARAMETERS OF BROILERS

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ABSTRACT

A feeding trial was conducted to evaluate the effects of organic mineral mixture, probiotics, enzymes, emulsifier and liver supplements on the haematological parameters of broilers. For this purpose, a total of 396 day-old commercial broiler chicks (Cobb) were used and randomly allocated into 11 groups with three replicates of 12 chicks each. Broilers of T_2, T_3 and T_4 groups showed significantly ($P<0.05$) higher values of TEC, TLC, PCV and Hb as compared to T_0 group. Significant ($P<0.05$) decrease in MCH and MCV were observed in supplemented groups. All the supplemented groups showed significant ($P<0.05$) higher values of TEC, TLC, PCV and Hb compared to T_0 group at 21st and 42nd day. Significant ($P<0.05$) decrease in MCV and MCH compared to T_0 group at 21st and 42nd day. Significant ($P<0.05$) increase in MCHC at 21st day while significantly ($P<0.05$) decrease in MCHC at 42nd day compared to T_0 group.

Key words: Broilers, BUN, enzymes, Hb, PCV, probiotics, TEC, TLC

INTRODUCTION

Poultry is one of the fastest growing segments of the agricultural sector in India. India is the third largest egg producer after China and USA and the fourth largest poultry producer after China, Brazil and USA. The annual egg and broiler production of India is 70 billion eggs and 3.8 million tons respectively, with per capita consumption of 68 eggs and 2.5 kg chicken meat against the ICMR recommendations of 180 eggs and 11 kg poultry meat (CARI VISION 2050). Poultry meat has significant role in Indian diet valued at US $ 6.6 billion. Favoured by socio- economic conditions like rising purchasing power and changing food habits of the people this sector is driven by ever increasing domestic demand. Poultry meat is an excellent source of high quality protein, vitamins, and minerals and is not subjected to cultural and religious restrictions. Rising input cost in poultry production has necessitated the need to look for feed supplements which can enhance the nutrient utilization efficiency of feeds thereby improving performance of poultry and resultant increase in profitability. In this context use of organic minerals, probiotics, enzymes, emulsifiers and liver supplements seems promising. Use of organic minerals in poultry diets has been shown to have multiple beneficial effects including higher absorption and increased antibody levels as they may provide alternative pathways for absorption, by decreasing mineral excretion. Similarly, use of probiotics and feed enzymes have been reported to regulate gut integrity, reduce digestive disorders, improve nutrient absorption/ feed efficiency, increases production, check the mortality and lowering of feed cost. Poultry produces emulsifiers in the form of bile, however, at times it is insufficient in view of added fats and oils. Also, as the digestive tract in young birds is not completely developed, fat absorption from the feed matrix is hampered. Hence, addition of emulsifier into the diet can overcome this problem by reducing the size of the fat globules forming small micelles and increasing the total surface available for enzymatic digestion.The addition of synthetic emulsifier to broiler diets is a recent practice as compared to other dietary supplements. Polyherbal liver stimulants possess hepato – protective, hepatogenic, immunomodulatory and antioxidant properties, which tone up liver resulting in increased utilization of feed and better performance.Keeping the above facts in view, an experiment was conducted to determine the effect of supplementation of organic mineral mixture, probiotics, enzymes, emulsifier and liver stimulants on the haematological parameters of broilers.

MATERIALS AND METHODS

A total number of 396 day old commercial broiler chicks (Cobb) were procured for undertaking the experiment. All the chicks were individually weighed and randomly allotted to eleven different groups each with three replicates of 12 chicks. The groups were designated as T_0; basal diet, T_1; chicks fed basal diet along with organic mineral mixture 1 (Organomin forte) @ 0.5 g per kg feed, T_2; basal diet along with organic mineral mixture 2 (Vannamin) @ 0.5 g per kg feed,T_3; basal diet along with probiotics (Microguard) @ 0.1g per kg feed,T_4; basal diet along with enzymes + probiotics (Brozyme - XPR) @ 0.5 g per kg feed, T_5; basal diet along with emulsifier (Lipigon) @ 0.5 g per kg feed, T_6; basal diet with 3% less energy, T_7; basal diet

Table 1.
Effect on haematological parameters related to erythrocytes in different groups at the end of 4th week of experiment (Mean±S.E., n=6)

Groups	Hb(g/dL)	PCV(%)	TEC(10^6/cumm)	MCV(fL)	MCH(pg)	MCHC(g/dL)
T1	11.18^{ab}±0.138	38.25^{ab}±0.738	5.53^{ab}±0.19	69.47±2.102	20.37±0.856	29.30±0.696
T2	10.47^{c}±0.150	35.33^{c}±1.327	5.18^{b}±0.162	68.23±2.257	20.29±0.706	29.82±0.983
T3	11.73^{a}±0.201	40.00^{a}±0.577	5.80^{a}±0.071	69.05±1.702	20.25±0.454	29.34±0.308
T4	10.84^{bc}±0.236	36.07^{bc}±0.503	5.26^{b}±0.151	68.80±2.117	20.65±0.563	30.05±0.519
T5	11.01^{bc}±0.295	37.95^{ab}±0.402	5.78^{a}±0.093	65.70±0.585	19.10±0.785	29.05±1.055

Mean values with common alphabet as superscript do not differ significantly.

Table 2.
Effect on haematological parameters related to leucocytes in different groups at the end of 4th week of experiment (Mean±S.E., n=6)

Groups	TLC (10^3/cu mm)	Absolutelymphocyte	Absoluteheterophil	Absolute monocyte	Absoluteeosinophil	AbsoluteBasophil
T1	11.25^{ab}±0.383	6727.00^{ab}±324.437	4143.5^{b}±101.57	225.83±34.141	153.66±40.829	0±0
T2	10.32^{c}±0.182	4416.46^{d}±136.029	5545.28^{a}±209.557	171.43±21.253	172.31±35.379	17.83±17.833
T3	11.89^{a}±0.109	7277.90^{a}±223.566	4264.40^{b}±214.177	177.73±39.954	138.65±19.499	39.65±25.076
T4	10.82^{bc}±0.273	5163.81^{c}±240.822	5392.41^{a}±193.338	108.45±30.344	158.65±55.621	0±0
T5	11.02^{bc}±0.253	6213.01^{b}±193.422	4517.63^{b}±140.344	146.28±45.387	144.73±36.079	0±0

Mean values with common alphabet as superscript do not differ significantly

with 3% less energy along with liver supplement 1(Superliv premix) @ 0.5 g/kg feed, T_8; basal diet with 3%2 less energy along with liver supplement 2 (X- liv Pro) @ 0.5 g/kg feed, T_9; basal diet along with enzymes with probiotics (Brozyme - XPR) and liver supplement 1(Superliv premix) @ 0.5 g/kg feed, and T_{10}; basal diet along with enzyme with probiotics (Brozyme - XPR), liver supplement 1(Superliv premix) and emulsifier (Lipigon) @ 0.5 g/kg feed.Average body weight of chicks was similar for all the treatment groups. The broiler chicks were housed in deep litter system under standard management practices. Blood samples were collected from six experimental birds of each group i.e. two broiler chicks from each replicate on 21st and 42nd day of experimental feeding. Blood samples (about 4.0 ml) were collected aseptically from their wing vein, using sterilized syringes and needles (24 gauge needle). Collected blood samples was transferred to the vials containing anticoagulant ethylene diamene tetra acetate (EDTA) and used for estimation of haemoglobin (Hb), packed cell volume (PCV), red blood cells (RBC), white blood cells (WBC), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), Mean corpuscular haemoglobin concentration (MCHC) and estimation. Total erythrocytes count (TEC) was done as described by Natt and Herrick (1952). Total leukocyte count (TLC) was performed with Neubauer's counting chamber (Jain, 1986). Packed cell volume (PCV) was estimated using micro haematocrit method as described by Sharma and Singh (2000). Haemoglobin (Hb) concentration was estimated spectrophotometrically at 540 nm by cyanomethemoglobin method, using Drabkin's solution (Fudge, 2000). MCV, MCH and MCHC were done by fully automatic blood cell counter model – PCE – 210. The experimental data obtained were analyzed statistically using completely randomized design (CRD) as per the methods given by (Snedecor and Cochran, 1994). The significant mean differences between the treatments were determined by using Duncan's Multiple Range Test (DMRT) as given by Kramer (1957).

RESULTS AND DISCUSSION

Haematological parameters (21st day)

The data representing the haematological parameters in broilers fed diet supplemented with organic mineral mixture, probiotics, enzymes, emulsifier and liver supplements on 21st day of feeding trial are summarized in Table1.

Total erythrocyte counts (TEC)

Total erythrocyte count in the present investigation revealed a significant ($P< 0.05$) positive impact of these supplementation in broilers. Total erythrocyte count values in $T_1, T_2, T_3, T_4, T_5, T_6, T_7, T_8, T_9$ and T_{10} groups were significantly ($P<0.05$) higher than T_0 (control) group, however, there were no significant differences in the total erythrocyte count values between T_1 and T_2, T_3 and T_5, T_9 and T_{10} groups of broilers.

Total erythrocyte count value was maximum ($2.97 \pm 0.01 \times 10^6$/ µl) in T_{10} and minimum ($2.22 \pm 0.04 \times 10^6$ / µl) in T_0 group. Similar results of significant increase in total erythrocyte count (TEC) were also reported by Rahman *et al.*(2013) and Cowieson *et al.*(2006)

Total leukocyte counts (TLC)

Total leukocyte count values in $T_1, T_2, T_3, T_4, T_5, T_7, T_8, T_9$ and T_{10} groups were significantly ($P<0.05$) higher than T_0 (control) group except T_6, however, there were no significant differences in the total leukocyte count values between T_1 and T_2, T_3, T_4, T_5, T_9 and T_{10} groups of broilers. Total leukocyte count value was maximum ($26.89 \pm 0.05 \times 10^3$/ µl) in T_{10} and minimum ($24.29^a \pm$

0.01x 10^3 / µl) in T_0 group. Similar results of significant increase in total leukocyte count (TLC) were also reported by Rahman *et al.*(2013) and Cowieson *et al.*(2006).

Packed cell volume (PCV)

Packed cell volume values in in $T_1, T_2, T_3, T_4, T_5, T_7, T_8, T_9$ and T_{10} groups were significantly ($P<0.05$) higher than T_0 (control) group except T_6, however, there were no significant differences in the packed cell volume values between T_1 and T2, T_3, T_4 T_5 and T_9 groups of broilers. Packed cell volume value was maximum ($28.86^e \pm 0.05$ per cent) in T_{10} and minimum ($25.81^a \pm 0.09$ per cent) in T_0 group. Similar results of significant increase in packed cell volume (PCV) were also reported by Rahman *et al.*(2013) and Cowieson *et al.*(2006).

Haemoglobin (Hb)

Haemoglobin values in $T_1, T_2, T_3, T_4, T_5, T_7, T_8, T_9$ and T_{10} groups were significantly ($P<0.05$) higher than T_0 (control) group except T_6, however, there were no significant differences in the haemoglobin values between T_1 and T_2, T_3 and T_5 groups of broilers. Haemoglobin was maximum (9.92 ± 0.02 g/ dl) in T_{10} and minimum (8.45 ± 0.05 g /dl) in T_0 group. Similar results of significant increase in haemoglobin (Hb) were also reported by Shareef and Al- Dabbagh(2009), Rahman *et al.*(2013) and Cowieson *et al.*(2006).

Mean corpuscular volume (MCV)

Mean corpuscular volume values in $T_1, T_2, T_3, T_4, T_5, T_6, T_7, T_8, T_9$ and T_{10} groups were significantly ($P<0.05$) lower than T_0 group, however, there were no significant differences in the mean corpuscular volume values between T_7 and T_8, T_9 and T_{10} groups of broilers. Mean corpuscular volume value was maximum (116.26 ± 0.03 fl) in T_0 and minimum (97.17 ± 0.03 fl) in T_{10} group. Similar results of significant decrease in mean corpuscular volume were also reported by Hosseini (2011c).

Mean corpuscular haemoglobin (MCH)

Mean corpuscular haemoglobin values in $T_1, T_2, T_3, T_4, T_5, T_6, T_7, T_8, T_9$ and T_{10} groups were significantly ($P<0.05$) lower than T_0 group, however, there were no significant differences in the mean corpuscular haemoglobin values between T_6 and T_7, $T_1, T_2, T_3, T_4, T_5, T_8, T_9$ and T_{10} groups of broilers. Mean corpuscular haemoglobin was maximum (38.06 ± 0.01pg) in T_0 and minimum (33.40 ± 0.03 pg) in T_{10} group. Similar results of significant decrease in mean corpuscular volume were also reported by Hosseini (2011c).

Mean corpuscular haemoglobin concentration (MCHC)

Mean corpuscular haemoglobin concentration values in $T_1, T_2, T_3, T_4, T_5, T_7, T_8, T_9$ and T_{10} groups were significantly ($P<0.05$) higher than T_0 group, however, there were no significant differences in the mean corpuscular haemoglobin concentration values between $T_1, T_2, T_3, T_4, T_5, T_7, T_8, T_9$ and T_{10} groups of broilers. Mean corpuscular haemoglobin concentration was maximum (34.37 ± 0.02) in T_{10} and minimum (32.73 ± 0.01) in T_0 group. The present experiment indicates that the inclusion of probiotic significantly increase the value of MCHC concentration in broilers. The observations of Shareef and Al-Dabbagh (2009) who found no significant difference in mean corpuscular haemoglobin concentration of broilers supplemented with probiotics.

Haematological parameters (42nd day)

The data representing the haematological parameters in broilers fed diet supplemented with organic mineral mixture, probiotics, enzymes, emulsifier and liver supplements on 42nd day of feeding trail are summarized in Table 2.

Total erythrocyte counts (TEC)

Total erythrocyte count in the present investigation revealed a significant ($P< 0.05$) positive impact of these supplementations in broilers. Total erythrocyte count values in $T_1, T_2, T_3, T_4, T_5, T_6, T_7, T_8, T_9$ and T_{10} groups were significantly ($P<0.05$) higher than T_0 group, however, there were no significant differences in the total erythrocyte count values between T_1 and T_2, T_9 and T_{10} groups of broilers. Total erythrocyte count value was maximum (3.14 ± 0.00

Table1:

Effect of feed supplementation on haematological profile of broilers (21stday)

Treatments	TEC(10^6/µl)	TLC(10^3/µl)	PCV(%)	Hb(g /dl)	MCV*(fl)	MCH*(pg)	MCHC*(%)
T_0	2.22^a±0.04	24.29^a±0.01	25.81^a±0.09	8.45^a±0.05	116.26^a±0.03	38.06^a±0.01	32.73^a±0.01
T_1	2.70^b±0.02	25.70^b±0.13	27.31^b±0.14	9.23^b±0.04	101.14^b±0.19	34.18^b±0.04	33.79^b±0.02
T_2	2.75^b±0.00	25.98^b±0.02	27.63^b ±0.04	9.36^{bc}±0.02	100.47^{be}±0.08	34.03^b±0.00	33.87^b±0.02
T_3	2.83^c±0.00	26.51^c±0.01	28.25^c±0.02	9.60^c±0.02	99.82^{be}±0.29	33.92^b±0.03	33.98^b±0.05
T_4	2.88^{cg} ±0.02	26.63^c±0.04	28.46^{ce}±0.09	9.73^{ce}±0.04	98.81^{be}±0.24	33.78^b±0.05	34.18^b±0.02
T_5	2.79^c±0.01	26.23^c±0.13	27.95^c±0.13	9.49^c±0.04	100.17^{be}±0.09	34.01^b±0.01	33.95^b±0.00
T_6	2.38^d±0.04	24.53^{ad}±0.12	26.05^{ad}±0.02	8.64^{ad}±0.04	109.45^c±0.03	36.30^c±0.01	33.16^{ba}±0.01
T_7	2.51^e±0.03	24.89^d±0.07	26.46^d±0.18	8.87^d±0.09	105.41^d±0.02	35.33^c±0.01	33.52^b±0.08
T_8	2.62^f±0.02	25.28^{db}±0.12	26.91^{db}±0.08	9.10^{db}±0.02	102.70^d±0.01	34.73^b±0.01	33.81^b±0.00
T_9	2.93^g±0.00	26.75^c±0.01	28.70^{ce}±0.02	9.84^{ce}±0.01	97.95^{be}±0.23	33.58^b±0.04	34.28^b±0.45
T_{10}	2.97^g±0.01	26.89^c±0.05	28.86^e±0.05	9.92^e±0.02	97.17^e±0.03	33.40^b±0.03	34.37^b±0.02

Table 2:
Effect of feed supplementation on haematological profile of broilers (42nd day)

Treatments	TEC(10^6/µl)	TLC(10^3/µl)	PCV(%)	Haemoglobin(g/dl)	MCV(fl)	MCH(pg)	MCHC(%)
T_0	2.14^{a}±0.06	26.63^{a}±0.10	26.85^{a}±0.01	9.45^{a}±0.08	125.46^{a}± 0.12	44.15^{a}±0.09	35.52^{a}±0.02
T_1	2.72^{b}±0.01	27.50^{b}±0.05	28.84^{b}±0.15	9.93^{b}±0.04	106.02^{b}±0.13	36.50^{b}± 0.02	34.43^{b}± 0.08
T_2	2.78^{be}±0.01	27.64^{bc}±0.02	29.15^{b}±0.03	10.05^{bc}±0.02	104.85^{bc}±0.03	36.15^{bc}± 0.14	34.47^{b}±0.02
T_3	2.93^{c}±0.03	27.84^{bc} ±0.01	29.64^{c}±0.02	10.23^{c}±0.04	101.16^{c} ±0.21	34.91^{c}± 0.03	34.51^{b}± 0.02
T_4	3.03^{d}±0.01	27.90^{bc} ±0.02	29.85^{c}±0.09	10.32^{ce}±0.01	98.51^{ce}± 0.10	34.05^{cf}± 0.14	34.57^{b}± 0.12
T_5	2.84^{e}±0.01	27.74^{bc}±0.03	29.42^{c}±0.11	10.12^{c}±0.00	103.59^{bc} ±0.10	35.63^{cb}± 0.03	34.39^{b}±0.03
T_6	2.30^{f} ± 0.02	26.84^{a}±0.01	27.05^{a}±0.02	9.61^{d}±0.01	117.60^{d} ±0.16	41.78^{d}±0.15	35.19^{a}±0.09
T_7	2.44^{g}±0.04	27.02^{b} ±0.09	27.81^{d} ±0.14	9.73^{d}± 0.04	113.97^{d}± 0.04	39.87^{e}±0.01	34.98^{ba}±0.02
T_8	2.62^{h}±0.04	27.30^{b} ±0.05	28.30^{d}±0.15	9.82^{b}±0.00	108.01^{b}± 0.03	37.48^{b}±0.10	34.69^{b}±0.08
T_9	3.09^{di}±0.01	27.68^{bc}±0.33	30.14^{c}± 0.06	10.38^{ce}±0.01	97.54^{ce} ± 0.03	33.59^{cf}±0.03	34.43^{b}±0.02
T_{10}	3.14^{i}±0.00	28.14^{c}±0.02	30.36^{e}±0.05	10.44^{e}±0.02	96.68^{e}± 0.13	33.24^{f}±0.06	34.38^{b} ±0.06

x 10^6/µl) in T_{10} and minimum (2.14 ± 0.06 x 10^6 / µl) in T_0 group. Similar results of significant increase in total erythrocyte count (TEC) were also reported by Rahman *et al.*(2013), Hassan *et al.*(2012) and Hosseini (2011c).

Total leukocyte counts (TLC)

Total leukocyte count values in T_1, T_2, T_3, T_4, T_5, T_7, T_8, T_9 and T_{10} groups were significantly (P<0.05) higher than T_0 (control) group, however, there were no significant differences in the total leukocyte count values between T_7 and T_8, T_2, T_3, T_4, T_5 and T_9 groups of broilers. Total leukocyte count value was maximum (28.14 ± 0.02x 10^3 / µl) in T_{10} and minimum (26.63 ± 0.10x 10^3 / µl) in T_0 group. Similar results of significant increase in total leukocyte count (TLC) were also reported by Rahman *et al.*(2013), Cowieson *et al.*(2006), Cetin *et al.*(2005) and Hassan *et al.*(2012).

Packed cell volume (PCV)

Packed cell volume values in in T_1, T_2, T_3, T_4, T_5, T_7, T_8, T_9 and T_{10} groups were significantly (P<0.05) higher than T_0 (control) group, however, there were no significant differences in the packed cell volume values between T_1 and T2, T_3, T_4 and T_5, T_7 and T_8 groups of broilers. Packed cell volume value was maximum (30.36^{e} ± 0.05 per cent) in T_{10} and minimum (26.85 ± 0.01 per cent) in T_0 group. Similar results of significant increase in packed cell volume (PCV) were also reported by Rahman *et al.*(2013), Hosseini (2011c), Cowieson *et al.*(2006) and Cetin *et al.* (2005).

Haemoglobin (Hb)

Haemoglobin values in T_1, T_2, T_3, T_4, T_5, T_6, T_7, T_8, T_9 and T_{10} groups were significantly (P<0.05) higher than T_0 (control) group, however, there were no significant differences in the haemoglobin values between T_3 and T_5, T_6 and T_7, T_1 and T_8 groups of broilers. Haemoglobin was maximum (10.44± 0.02g/dl) in T_{10} and minimum (9.45±0.08g/dl) g in T_0 group. Similar results of significant increase in haemoglobin (Hb) were also reported by Shareef and Al- Dabbagh(2009), Rahman *et al.*(2013) and Cowieson *et al.*(2006).

Mean corpuscular volume (MCV)

Mean corpuscular volume values in T_1, T_2, T_3, T_4, T_5, T_6, T_7, T_8, T_9 and T_{10} groups were significantly (P<0.05) lower than T_0 group, however, there were no significant differences in the mean corpuscular volume values between T_6 and T_7, T_1 and T_8 groups of broilers.

Mean corpuscular volume value was maximum (125.46 ± 0.12fl) in T_0 and minimum (96.68 ± 0.13 fl) in T_{10} group. Similar results of significant decrease in mean corpuscular volume were also reported by Hosseini (2011c).

Mean corpuscular haemoglobin (MCH)

Mean corpuscular haemoglobin values in T_1, T_2, T_3, T_4, T_5, T_6, T_7, T_8, T_9 and T_{10} groups were significantly (P<0.05) lower than T_0 group, however, there were no significant differences in the mean corpuscular haemoglobin values between T_1 and T_8 groups of broilers. Mean corpuscular haemoglobin was maximum (44.15 ± 0.09 pg) in T_0 and minimum (33.24 ± 0.06 pg) in T_{10} group. Similar results of significant decrease in mean corpuscular volume were also reported by Hosseini (2011c).

Mean corpuscular haemoglobin concentration (MCHC)

Mean corpuscular haemoglobin concentration values in T_1, T_2, T_3, T_4, T_5, T_7, T_8, T_9 and T_{10} groups were significantly (P<0.05) higher than T_0 group, however, there were no significant differences in the mean corpuscular haemoglobin concentration values between T_1, T_2, T_3, T_4, T_5, T_7, T_8, T_9 and T_{10} groups of broilers. Mean corpuscular haemoglobin concentration was maximum (35.52 ± 0.02) in T_0 and minimum (34.38 ± 0.06) in T_{10} group. Similar results of significant decrease in mean corpuscular volume concentration were also reported by Hosseini (2011c).

REFERENCES

CARI VISION. (2050). Central Avian Research Institute, Izatnagar, Bareilly, India.

Cetin, N., Guclu B. K. and Cetin, E. (2005). The effects of probiotic and mannanoligosaccharide on some haematological and immunological parameters in Turkeys. *J. of Vet. Med.* 52: 263-

267.

Cowieson, A. J., Acamovic, T. and Bedford, M. R. (2006). Supplementation of corn-soy based diets with an *Eschericia coli*-derived phytase: effects on broiler chick performance and the digestibility of amino acids and the metabolisability of minerals and energy and haematology. *Poult. Sci.* **85**: 1389-1397.

Fudge, A. M. (2000). Avian Complete Blood Count. *In:* Laboratory Medicine: Avian and Exotic Pets, 1st ed. Saunders Company, U.S.A. 9-18.

Hassan, A. M., Mahmoud, M. M. A., Hamed, D. H. and Kilany, O. E. (2012). Effect of dietary yeast supplementation on growth performance and colonization of *salmonella enteritidis* in japanese quails. *YYU Veteriner Fakultesi Dergisi.* **23 (1)**: 45-50.

Hosseini, S. M. (2011c). The effect of *Saccharomysec cerevisiae* on blood parameters of broiler chickens. *Global Veterinaria* **7 (4)**: 411-414.

Hosseini, S. M. (2011a). The effect of utilization of different levels of *Saccharomysec cerevisiae* on broiler chickens performance. *Global Veterinaria* **6 (3)**: 233-236.

Jain, N. C. (1986). Schalm's Veterinary Haematology. Lea and Febiger, Philadelphia, USA. 2: 56-61.

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

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

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

Shareef, A. M. and Al-Dabbagh, A. S. A. (2009). Effect of probiotic (*Saccharomyces cerevisiae*) on performance of broiler chicks. *Iraqi J. Vet. Sci.* **23:** 23-29.

Sharma, I. J. and Singh, H. S. (2000). Student's Laboratory Manual of Veterinary Physiology. Kalyani Publishers, New Delhi. 5: 26-38.

Snedecor, G. W. and Cochran, W. B. (1994). *Statistical Methods.* 8th ed. The Iowa State University Press, Ames, IOWA, USA.

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Research Article

INVESTIGATION OF THE INFLUENCE OF ALPHA-TOCOPHEROL ON COPPER, IRON, ZINC AND MANGANESE LEVELS IN LIVER FOLLOWING CHRONIC EXPOSURE OF FLUBENDIAMIDE AND COPPER TO WISTAR RATS

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ABSTRACT

Present study was designed to examine the effects of alpha-tocopherol on the levels of Copper (Cu), Iron (Fe), Zinc (Zn) and manganese (Mn) in liver of copper and/or flubendiamide exposed (90 days oral) Wistar rats. Fifty four male Wistar albino rats were randomly divided into nine groups containing six animals in each: groups I (deionized water), II (corn oil) and III (α-tocopherol @ 100 mg/kg) served as negative controls while the remaining six groups, namely-IV (copper sulphate-@ 33 mg/kg), V (flubendiamide-@ 200 mg/kg), VI (flubendiamide-@ 200 mg/kg + copper sulphate- @ 33 mg/kg), VII (copper sulphate-@ 33 mg/kg + α-tocopherol-@ 100 mg/kg), VIII (flubendiamide-@ 200 mg/kg + α-tocopherol-@ 100 mg/kg) and IX (flubendiamide-@ 200 mg/kg + copper sulphate-@ 33 mg/kg + α-tocopherol-@ 100 mg/kg) were treatment groups. Animals were humanly sacrified on 91st day. Cu, Fe Zn, and Mn were estimated in liver by Atomic Absorption Spectrophotometer. Copper and iron levels were markedly higher in copper, flubendiamide alone and combined exposed groups while manganese level was low in the xenobiotics exposed groups. No significant alteration was observed in level of zinc in any of the xenobiotics exposed as well as xenobiotics + α-tocopherol treatment groups. α-tocopherol-treatment improve the values of copper, iron , zinc and manganese towards the levels of control groups indicates antioxidant potential of α-tocopherol.

Key words: Copper, flubendiamide, Cu, Fe, Zn, Mn, wistar rats.

INTRODUCTION

Everyday human and animals are exposed simultaneously number of xenobiotics mainly pesticides and heavy-metal due to intensive agriculture practices and rapid industrial expansion (Swarup and Dwivedi, 2002; INSA, 2011). However, current trends in the risk assessment of insecticides residue in food and biological system is generally based upon studies of a single compound. Knowledge of adverse health effect on following combined exposure to insecticide and metal is very limited. Flubendiamide is a phthalic acid diamide insecticide. It is recently introduce in India by Bayer Crop Science and attracted good attention for use on vegetable crops, namely-rice, cotton, corn, grapes, other fruits and vegetables (Bayer, 2003; Tohnishi *et al.,* 2005) for control of lepidopteron insects. Flubendiamide activates ryanodine-sensitive intracellular calcium release channels in neuromuscular junctions of insect, leading to overstimulation of insect skeletal muscles. Flubendiamide does not bind to mammalian type 1, 2 and 3 ryanodine receptors (JMPR, 2010). It is available both as a liquid formulation and also as granules, and it's display many favourable ecological, ecotoxicological and environmental profile, such as high and selective activity against a broad range of harmful lepidopteron species, selective insect rynodine receptor action, low toxicity against mammals and safe during use on crops (Nauen, 2006; Hirooka *et al.,* 2007). Due to lipophilic in nature, it is moderately well absorbed following oral exposure and is mostly excreted in faeces due to biliary excretion (Motoba, 2004). Short term, sub-chronic and chronic dosing regimens of flubendiamide via oral and dermal routes revealed that liver was the most sensitive organ in all species of animals (JMPR, 2010)

Copper exposure in the environment is inevitable. Copper is an essential micronutrient and is normally subjected to effective homeostatic control. Food and drinking water and athropogenic are important sources of copper intake. Populations living near sources of copper emissions, such as copper smelters and refineries and workers in these and other industries may also be exposed to high levels of copper in dust by inhalation. It is estimated that in excess of 75,000 tons of copper is released into the atmosphere annually of

which a quarter is thought to come from natural sources, whilst the rest is of anthropogenic origin. It is uses as anti-fouling, fungicide, pesticide, plumbing, antioxidant and specifically for arthritis and osteoporosis. Copper is actively absorbed, primarily from stomach and duodenum and transferred from the intestinal mucosa to hepatocytes (Luza and Speisky, 1996). Indian childhood cirrhosis (ICC) is a fatal disorder associated with accumulation of massive levels of copper in liver. Ingestion of 100 g copper-sulphate may produce intravascular haemolysis, acute hepatic failure, acute tubular renal failure, shock, coma or death (Baker *et al.* 2012). Various xenobiotics exposure produced interaction between xenobiotics and xenobiotics and biological system at organ/cell level.

Proper absorption and metabolism of copper requires an appropriate balance with the minerals zinc and manganese. α-tocopherol (α-TOH) exhibits highest vitamin E bioactivity among the eight natural forms of vitamin E. α-tocopherol is the major lipid soluble antioxidant and is known to protect cellular membranes and lipoproteins from peroxidation (Yavuz *et al.,* 2004). To date, several studies have been conducted to examine the protective effects of α-tocopherol on pesticide induced oxidative stress (Aldana *et al.,* 2001; Kalender *et al.,* 2005; Durak *et al.,* 2008; Sodhi *et al.,* 2008; Mandil *et al.,* 2016; Mandil *et al.,* 2017) but no information is available on the effect of α-tocopherol on minerals profile of liver following concurrent exposure of flubendiamide and/copper.

Therefore, present study was planned with the objectives to estimate influence of α-tocopherol on copper, iron, zinc and manganese levels in liver tissue following 90 days exposure of flubendiamide and/copper.

MATERIALS AND METHODS

Experimental animals

Fifty four male Wistar rats were obtained from Laboratory Animal Resource Section, Indian Veterinary Research Institute, Izatnagar, India and maintained in the Departmental Experimental Laboratory Animal House. After a fifteen-day's of acclimatization period, nine groups of six animals each were formed by randomization. Pelleted feed and deionized drinking water were fed ad lib. A 12h dark/light cycle was kept. Study was approved by the Institutional Animal Ethics Committee, DUVASU, Mathura (No. 79 IAEC/13).

Experimental design

Animals of groups I (deionized water), II (corn oil) and III (α-tocopherol @ 100 mg/kg) served as negative controls while the remaining six groups, namely-IV (copper sulphate-@ 33 mg/kg), V (flubendiamide-@ 200 mg/kg), VI (flubendiamide-@ 200 mg/kg + copper sulphate- @ 33 mg/kg), VII (copper sulphate-@ 33 mg/kg + α-tocopherol-@ 100 mg/kg), VIII (flubendiamide-@ 200 mg/kg + α-tocopherol-@ 100 mg/kg) and IX (flubendiamide-@ 200 mg/kg + copper sulphate-@ 33 mg/kg + α-tocopherol-@ 100 mg/kg) were treatment groups. Solutions of copper sulphate (Copper sulphate pentahydrate; CuSO4. $5H_2O$; SDFCL) and flubendiamide (commercial grade; FAME®, BAYER) were prepared by dissolving in deionized water. α-tocopherol (Simga-Aldrich, USA) dissolved in corn oil and was administered daily by oral gavage for 90 days. The dose of flubendiamide and copper sulphate were $1/10^{th}$ of the values of LD_{50} values of flubendiamide (> 2000 mg/kg) and copper-sulphate (> 333 mg/kg) (Gaetke & Chow, 2003; JMPR, 2010).

Sample collection

After 90day's of exposure period of xenobiotics, rats were humanely sacrificed by cervical dislocation. Liver was collected, blotted with tissue paper and, minerals level of copper, iron, zinc and manganese in wet-digested liver tissues of the rats of different treatment groups were measured with the help of Atomic Absorption Spectrophotometer.

Microdigestion and estimation of copper, iron, zinc and magnesium in liver tissues

One gram liver tissue was mixed with equal volume of pure nitric acid in 50 ml digestion tube. This mixture was kept overnight at room temperature and next day transferred into sixteen vessels of microdigestion. 10 ml mixture {(containing: 2 ml HNO_3 (sub-boiling, 65 %) + 2 ml H_2O_2 (suprapure, 30 %) + 0.5 ml HCl (suprapure, 30 %) + 5.5 ml H_2O (ultra pure)} was poured into each vessels and digestion was carried out with Multiwave 3000 with high performance rotor 16XF100 (100 ml PTFE-TFM vessels, 60 bar) (Anton Paar GmbH, Graz). After digestion, triple glass distilled water was added to the digested samples to make the final volume of 30 ml. Samples were filtered through Whatman filter paper No. 40 before analysis by Atomic Absorption Spectrometer-400 (Analyst-400, Perkin Elmer, Germany).

Statistical analysis

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

RESULTS

Residual levels of copper, iron, zinc and manganese levels

There was 15.15 and 27.27% increase in copper levels in copper-alone and copper + flubendiamide

treated groups, but it was not statistically significant when compared with control groups or those treated with xenobiotics + α-tocopherol as evident from the data summarized in Table 1, Fig. 1. However, copper + flubendiamide + α-tocopherol-treated group, copper levels was found to be 5.25 ± 0.20 ppm and this value was almost comparable to that detected in rats of control groups (I, II and III, respectively). Iron (Fe) levels in liver were moderately higher in flubendiamide alone (33.28%) and markedly higher (86.68%) in copper + flubendiamide treated groups, respectively but it was not statistically significant. Following simultaneous treatment of rats with α-tocopherol and flubendiamide (VIII) or copper + flubendiamide + α-tocopherol (IX), marked decrease in hepatic iron level and significant decrease in group IX compared to their respective positive controls i.e. groups V and VI was observed (Table 1; Fig. 2). Data on zinc levels did not differ significantly in different treatment groups. Residual levels of manganese (Mn) in copper, flubendiamide and copper + flubendiamide groups were decrease 38.65, 41.25 and 69.92%, respectively and concurrent treatment with α-tocopherol and xenobiotics of the groups VII, VIII and IX, the values of manganese increase almost comparable towards control groups (Table 1, Fig. 1).

DISCUSSION

Copper is an essential trace element of all cells, but even a modest excess of copper can be life-threatening due to liver and kidneys damage. Maintenance of normal copper homeostasis depends on the balance between gastrointestinal absorption, transported to liver and excretion via biliary excretion. Several factors influence absorption and bioavailability of copper such as interaction with divalent cations (calcium, iron and zinc etc), carbohydrate, phytate and fructose (EC SCF, 2003) content in the diet. Chronic copper exposure primarily affects the liver because it is the first site of copper deposition after it enters the blood. Copper absorption in body ranges from 25-60% of the intake which depends on other dietary components (Turnlund *et al.,* 1989; Lönnerdal, 1996). In the present study, level of copper (ppm) was markedly higher in liver of copper-alone and copper + flubendiamide exposed rats compared to other xenobiotics treated groups. It may be due to involvement of Menkes protein (MNK) which transport copper across the basolateral membrane of intestinal cells (Pena *et al.,* 1999), delivery of copper to copper-dependent enzymes and efflux of copper from the cell. In-addition copper transporters (hCtr1 and hCtr2) play an important role in regulation of copper uptake also. Divalent metal transporter 1 (DMT1) and copper transporter 1 (Ctr1) are thought to be responsible for absorption of dietary copper. DMT1 transports copper (Cu^{2+}) directly from diet and high copper levels modify the expression of DMT1; thus suggesting that DMT1 acts as a major intestinal copper transporter (Puig and Thiele, 2002; Sharp, 2003; Handy *et al.,* 2002). Ctr1 is a high-affinity membrane-spanning transport protein which is highly expressed in liver and kidneys. In addition, it is also expressed at high concentrations in small intestine, where Ctr1 is localized on the apical plasma membrane. Ctr1 is a transporter of Cu+, which is reduced by endogenous plasma membrane reductase and dietary

Table1:
Effect of α-tocopherol on copper, iron, zinc and manganese levels in liver of rats of different treatment groups following once daily oral exposure to copper (33mg/kg), flubendiamide (200 mg/kg) and both in combination (copper 33 mg/kg + flubendiamide 200 mg/kg) for 90 days.

Groups	Treatment	Hepatic Minerals profile (ppm)			
		Copper	Iron	Zinc	Manganese
I	Control	5.61 ± 1.04^{a}	170.52 ± 30.22^{ab}	42.50 ± 3.90^{a}	2.26 ± 0.35^{a}
II	Vehicle control (Corn oil)	5.29 ± 0.48^{a}	159.74 ± 47.18^{ab}	42.10 ± 2.55^{a}	2.22 ± 0.66^{a}
III	α-tocopherol (100 mg/kg)	5.44 ± 0.19^{a}	132.24 ± 9.95^{ab}	41.80 ± 2.36^{a}	2.17 ± 0.14^{a}
IV	Copper sulphate (33 mg/kg)	6.46 ± 0.58^{a} (15.15%)	176.86 ± 10.09^{ab}	40.27 ± 2.18^{a}	1.63±0.05^{a} (38.65%)
V	Flubendiamide (200 mg/kg)	6.08 ± 0.48^{a} (8.37%)	227.27 ± 37.43^{ab}	41.17 ± 3.83^{a} (33.28%)	1.60±0.33^{a} (41.25%)
VI	Copper sulphate (33 mg/kg) + Flubendiamide (200 mg/kg)	7.14 ± .71^{a} (27.27%)	318.33 ± 76.38^{b} (86.68%)	41.35 ± 4.61^{a}	1.33±0.35^{a} (69.92%)
VII	Copper sulphate (33 mg/kg) + α- tocopherol (100 mg/kg)	5.82 ± 0.31^{a}	266.89 ± 61.96^{ab} (56.49%)	40.70 ± 1.95^{a}	2.32±0.61^{a}
VIII	Flubendiamide (200 mg/kg) + α-tocopherol (100 mg/kg)	4.81 ± 0.81^{a} (16.63%)	141.44 ± 13.02^{ab}	39.95 ± 3.49^{a}	2.08±0.39^{a}
IX	Copper sulphate (33 mg/kg) + Flubendiamide (200 mg/kg)) + α-tocopherol (100 mg/kg)	5.25 ± 0.20^{a}	126.25 ± 18.86^{a}	40.74 ± 1.59^{a}	2.11±0.28^{a}

Values (mean ± SEM; n=6) bearing different superscripts in the same column differed significantly (*P<0.05*).
Figures in parentheses indicate per cent changes as compared to control group I.

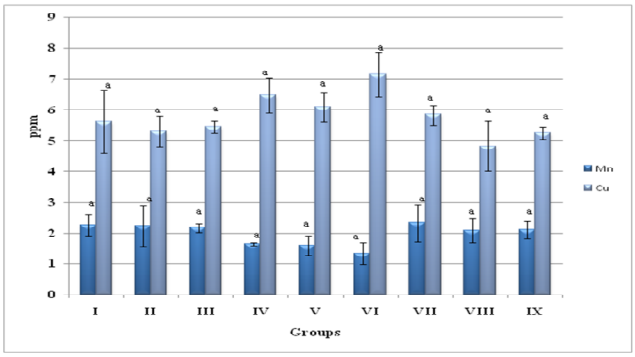

Fig. 1:
Effect of α-tocopherol on hepatic copper and manganese levels in rats of different treatment groups following once daily oral exposure for 90 days to copper (33 mg/kg), flubendiamide (200 mg/kg) and both in combination (copper 33 mg/kg + flubendiamide 200 mg/kg).
• Different superscripts in histograms show significant *(P<0.05)* difference.

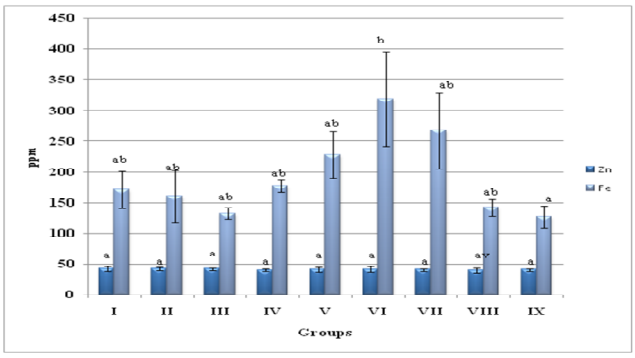

Fig.2:
Effect of α-tocopherol on hepatic zinc and iron levels in rats of different treatment groups following once daily oral exposure for 90 days to copper (33 mg/kg), flubendiamide (200 mg/kg) and both in combination (copper 33 mg/kg + flubendiamide 200 mg/kg).
• Different superscripts in histograms show significant (P<0.05) differences.

components such as ascorbate (Sharp, 2003). Toxicity of copper is due to its transitions between Cu (II) and Cu (I) and resultant generation of the superoxide and hydroxyl radicals (OH^+) (Camakaris *et al.,* 1999) via *Haber-Weiss* reaction (Kadiiska *et al.,* 1993; Bremner, 1998).

In this study daily exposure of copper-sulphate resulted uptake into intestinal cells is saturable and, its uptake into intestinal cell and efflux is influenced by intracellular copper concentrations (Arredondo *et al.,* 2000). Thus suggested nonsignificant increase copper level in copper-exposed groups may be due to decreased copper absorption following increased copper intake as suggested by Ehrenkranz *et al.* (1989) and Turnlund *et al.* (1989). Within the hepatocytes, copper is bound to metallothionein, superoxide dismutase and other binding proteins (Luza and Speisky, 1996). Copper is secreted from liver into blood and predominantly bound to ceruloplasmin or complexed with low molecular weight compounds (Groff *et al.,* 1995; Harris, 2001). In the bloodstream, copper is bound to albumin (not specific binding), ceruloplasmin or transcuprein (specific binding) (Puig and Thiele, 2002; Zakim and Boyer, 2002). Expression of transcuprein suggested to be modulated by copper and iron availability (Zakim and Boyer, 2002). In addition to the role of ceruloplasmin as a transport protein, it also acts as an enzyme; catalyzing the oxidation of minerals, most notably iron (Adelstein and Vallee, 1961). On simultaneous treatment of rats with α-tocopherol and xenobiotics of the groups VII-IX, there was decrease in level of copper in liver compared to those in rats of groups IV, V and VI thus suggesting that α-tocopherol offers regulatory activity and interferes or alter copper accumulation in liver.

Accumulation of iron in liver of flubendiamide-treated (86.68 %) and flubendiamide + copper-treated (56.49%) groups was more compared to copper alone-treated (33.28%) group compared to control group. α-tocopherol treatment (VIII and IX groups) decreased the Fe levels towards values in control groups and this might be due activation of ferroxidase I (ceruloplasmin) by copper which is necessary for iron absorption and mobilization from storage in liver (Linder, 1996; Turnlund, 1999; EC SCF, 2003). Oxidation of iron by ceruloplasmin is necessary for iron to be bound to its transport protein-transferrin, so iron deficiency anemias may be a symptom of copper deficiency (Groff *et al.,* 1995; Araya *et al.,* 2006). Whittaker (1998) reported that large supplemental iron decreases zinc absorption. But copper and iron accumulation were accompanied with decrease in manganese levels in liver of xenobiotics-treated groups. Copper reacts with a variety of nutrients, including iron, zinc, molybdenum, sulfur, selenium and vitamin C (Araya *et al.,* 2006). Excess copper, like excess iron, can lead to the production of reactive oxygen species that initiate destructive lipid peroxidation reactions (Mandil *et al.,* 2016) that affect the mitochondria and other cellular membranes (Danielle *et al.,* 2017). Histopathological changes in liver and kidneys have also been reported in the present study (data not published) following exposure of copper and/or flubendiamide.

Zinc (Zn) level in liver was found to be slightly decreased in xenobiotics (IV, V and VI) and α-tocopherol + xenobiotics treated (VII, VIII and IX) groups but it was not statistically significant. Elevated copper levels are especially due to low level of zinc (Broun *et al.,* 1990; Evans, 2006). Similarly, manganese (Mn) was also estimated to be appreciably lower in copper, flubendiamide and copper + flubendiamide groups compared to the control groups. Co-treatment of rats with α-tocopherol and xenobiotics (VII, VIII and IX)

resulted in increase in Mn levels. Proper absorption and metabolism of copper requires an appropriate balance between zinc and manganese. In the present study manganese has inverse relation with copper; thus decrease levels of zinc and manganese increase the copper levels in liver. Because zinc can compete with copper in small intestine and interferes with its absorption (Harris, 2001). Groff *et al.* (1995) reported that high levels of zinc in diet may increase the risk of copper deficiency. Zinc is used to reduce copper toxicity and copper-associated hepatopathies. Zinc inhibits the absorption of copper in the gastrointestinal tract. Thus zinc supplementation is often suggested for liver disease as part of a prescription diet (Alexander *et al.,* 2008). Therefore, result of present study concluded that 90 days of copper and/or flubendiamide-treatment promote accumulation of copper and iron levels and decrease the levels of zinc and manganese in liver and alpha-tocopherol possess antioxidant potential to normalized the values of these minerals towards control groups.

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REFERENCES

Adelstein, S.J. and Vallee, B.L. (1961). Copper metabolism in man. *N. Eng. J. Med*. **265**: 892-897

Aldana, L., Tsutsumi, V., Craigmill, A., Silveira, M.I. and Gonzalez de Mejia, E. (2001). alpha-Tocopherol modulates liver toxicity of the pyrethroid cypermethrin. *Toxicol. Lett.* **125**: 107-116.

Alexander J., GermanJill E., Maddison., Grant Guilford. (2008). Chapter 19 - Gastrointestinal drugs. Small Animal Clinical Pharmacology (Second Edition). 469-497

Araya, M., Pizarro, F., Olivares, M., Arredondo, M., Gonzalez, M., *et al.* (2006). Understanding copper homeostasis in humans and copper effects on health. *Biol. Res*. **39**: 183-187

Arredondo, M., Uauy, R. and Gonzalez, M. (2000). Regulation of copper uptake and transport in intestinal cell monolayers by acute and chronic copper exposure. *Biochim. Biophys. Acta*. **1474**: 169–76

Baker, D., Karalliedde, L., Murray, V., Maynard, R.L. and Parkinson, N.H.T. (2012). Essentials of Toxicology for Health Protection. A Handbook for Field. 2nd. (ed.) Oxford University Press. U.K, 184.

Bayer, A.G. (2003). Report MR-202/03, Laboratory Project ID: P601030020, Leverkusen.

Bremner, I. (1998). Manifestations of copper excess. *Am. J. Clin. Nutr.* **67**: 1069S-1073S.

Broun, E.R., Greist, A., Tricot, G. and Hoffman, R. (1990). Excessive zinc ingestion. *JAMA*. **264**: 1441-1443.

Camakaris, J., Voskoboinik, I. and Mercer, J.F. (1999). Breakthroughs and views: molecular mechanisms of copper homeostasis. *Biochem. Biophys. Res. Commun*. **261**: 225-232.

Danielle L. B., Arnaud J., Van Wettere. and John, M.C. (2017). Chapter 8 - Hepatobiliary System and Exocrine Pancreas. Pathologic Basis of Veterinary Disease (Sixth Edition). 412-470.

Durak, D., Uzun, F.G., Kalender, S., Ogutcu, A., Uzunhisarcikli, M. and Kalender, Y. (2008). Malathion-induced oxidative stress in human erythrocytes and the protective effect of vitamins C and E *in vitro*. *Environ. Toxicol*. **24:** 235-242.

Ehrenkranz, R.A., Gettner, P.A., Nelli, C.M., Sherwonit, E.A., Williams, J.E., Ting, B.T.G. and Janghorbani, M. (1989). Zinc and copper nutritional studies in very low birth weight infants: comparison of stable isotopic extrinsic tag and chemical balance methods. *Pediatr Res*. **26**: 298-307

European Commission, Scientific Committee on Food (EC SCF 2003). Opinion of the Scientific Committee on Food on the Tolerable Upper Intake Level of Copper. European Commission, SCF/CS/NUT/UPPLEV/57 Final Report. Brussels.

Evans, J.R. (2006). Antioxidant vitamin and mineral supplements for slowing the progression of age-related macular degeneration. *Cochrane Database Syst. Rev*. **2**: CD000254.

Gaetke, L.M. and Chow, C.K. (2003). Copper toxicity, oxidative stress, and antioxidant nutrients. *Toxicology*. **189**: 147-163

Groff, J.L., Gropper, S.S. and Hunt, S.M. (1995). Advanced Nutrition and Human Metabolism. West Publishing Company, New York.

Handy, R.D., Eddy, F.B. and Baines, H. (2002). Sodium-dependent copper uptake across epithelia: a review of rationale with experimental evidence from gill and intestine. *Biochim. Biophys. Acta*. **1566**: 104-15

Harris, E.D. (2001). Copper homeostasis: the role of cellular transporters. *Nutr. Rev*. **59**: 281-285

Hirooka, T., Nishimatsu, T., Kodama, H., Reckmann, U. and Nauen, R. (2007). The biological profile of flubendiamide, a new benzenedicarboxamide

insecticide. *Pflanzenschutz-Nachrichten Bayer.* **60:** 183-202

INSA. (2011). Hazardous metals and minerals pollution in India:Source, toxicity and management: a position paper. Indian National Science Academy, New Delhi

JMPR (Joint Meeting on Pesticide Residues), (2010). Report of the joint meeting of the FAO panel of experts on pesticide residues in food and the environment and the WHO core assessment group on pesticide residues at the request of codex committee on pesticide residues Italy. 345-382

Kadiiska, M.B., Hanna, P.M., Jordan, S.J. and Mason, R.P. (1993). Electron spin resonance for free radical generation in copper-treated vitamin-E and selenium-deficient rats*: in vivo* spin-trapping investigation. *Mol. Pharmacol*. **44** : 222-227.

Kalender, S., Ogutcu, A., Uzunhisarcikli, M., Acikgoz, F., Durak, D., Ulusoy, Y. & Kalender, Y. (2005). Diazinon-induced hepatotoxicity and protective effect of vitamin E on some biochemical indices and ultrastructural changes. *Toxicology*. **211**: 197-206.

Linder, M.C. (1996). Copper. In: Ziegler EE, Filer LJ, eds. Present Knowledge of Nutrition. 7th ed. Washington, DC: ILSI Press. 307-319

Lönnerdal, B. (1996). Bioavailability of copper. *Am. J. Clin Nutr*. **63**: 821S-9S

Luza, S.C. and Speisky, H.C.(1996). Liver copper storage and transport during development: implications for cytotoxicity. *Am J Clin Nutr*. **63(5):**812S-20S.

Mandil, R., Rahal, A., Prakash, A., Garg, S.K., Ganagwar, N.K., & Swain, D. (2016). Ameliorative potential of α-tocopherol against flubendiamide and copper-induced testicular-insult in Wistar rats. *Chemico-biological interactions*. **260**. 91-101.

Mandil, R., Rahal,A., Koli, S., Prakash, A., Kumar, R. and Garg. S. K. 2017. Protective role of α-tocopherol against flubendiamide and copper-induced chronic toxicity in Wistar rats. *Journal of Veterinary Pharmacology and Toxicology*.**16 (2)**.18-27.

Motoba, K. (2004). Absorption, distribution, metabolism and excretion of [phthalic ring (U)-14C]NNI-0001 following 14 repetitive oral administration to male and female rats. Unpublished report No. LSRC-M04-114A from Nihon Nohyaku Co., Ltd, Osaka, Japan. Submitted to WHO by Bayer CropScience AG, M onheim, Germany.

Nauen, R. (2006). Insecticide mode of action: return of the ryanodine receptor. *PestManag. Sci.* **62**: 690-692.

Pena, M.M., Lee, J. and Thiele, D.J. (1999). A delicate balance: homeostatic control of copper uptake and distribution. *J Nutr*. **129**: 1251-1260

Pessah, I.N. (1989). Recent advances in the chemistry of insect control. In: Crombie L (ed) Royal Society of Chemistry, Special Publication, vol 79. RSC, Cambridge, pp 278–296.

Puig, S. and Thiele, D.J. (2002). Molecular mechanisms of copper uptake and distribution. *Curr. Opin. Chem. Biol*. **6**: 171- 80

Sharp, P.A. (2003). Ctr1 and its role in body copper homeostasis. *Int. J. Biochem. Cell Biol*. **35**: 288-91

Sodhi, S., Sharma, A., Brar, A.P.S. and Brar. R.S. (2008). Effect of α-tocopherol and selenium on antioxidant status, lipid peroxidation and hepatopathy induced by malathion in chicks. *Pestic. Biochem. Phys*. **90**: 82-86.

Swarup. D and Dwivedi, S.K. (2002) Environmental pollution and effects of lead and fluoride on animal health. Indian Council of Agricultural Research, New Delhi.

Tohnishi, M., Nakao, H., Furuya, T., Seo, A., Kodama, H., Tsubata, K., Fujioka, S., Kodama, H., Hirooka, T. and Nishimatsu, T. (2005). Flubendiamide, A novel insecticide highly active against lepidopterous insect pests. *J. Pestic. Sci.* **30**: 354-360.

Turnlund, J.R. (1999). Copper. In: Shils ME, Olson JA, Shike M, eds. Modern Nutrition in Health and Disease. 9th ed. Philadelphia: Lea and Fibiger. 241-264

Turnlund, J.R.; Keyes, W.R.; Anderson, H.L. and Acord, L.L. (1989). Copper absorption and retention in young men at three levels of dietary copper by use of the stable isotope 65Cu. *Am. J. Clin. Nutr*. **49**: 870-8

Whittaker, P. (1998). Iron and zinc interactions in humans. *Am. J. Clin Nutr*. **68**: 442-446.

Yavuz, T., Delibas, N., Yildirim, B., Altuntas, I., Candir, O., Cora, A., Karaman, N., Ibrisim, E. & Kutsal, A. (2004). Vascular wall damage in rats induced bymethidathion and ameliorating effect of vitamins E and C. *Arch. Toxicol*. **78** : 655-659.

Zakim, D. and Boyer, T.D. (2002). Hepatology: A Textbook of Liver Disease, (Elsevier Health Sciences Philadelphia.

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Research Article

IN VITRO ANTIBACTERIAL SENSITIVITY OF CINNAMON AND CLOVE OILS AGAINST GRAM POSITIVE AND GRAM NEGATIVE BACTERIA

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ABSTRACT

The study was planned to evaluate *in vitro* antibacterial activity of cinnamon and cove oils. Screening of cinnamon and clove oils for antibacterial activity was done by the disc diffusion method against *Streptococcus agalactiae* (ATCC 13813), *Listeria Monocytogenes* (ATCC 1911), *Staphylococcus aureus* (ATCC 6538P), *Pseudomonas aeruginosa* (ATCC 19154), *Escherichia coli* (ATCC 10799) and *Salmonella typhimurium* (ATCC 23564). It was performed using an 18 h culture at 37°C in 10 ml of Muller Hinton Agar (for *S. agalactiae* 5% defibrinated sheep blood was added). The test suspension was standardized to match 0.5 McFarland turbidity standards. The cinnamon and clove oil were suspended in a solution containing 10% dimethyl sulfoxide and 0.5% tween 80. Under aseptic condition, empty sterilized discs were impregnated with 50 µl of different concentrations (1:1, 1:2, 1:5, 1:10 and 1:20) of cinnamon and clove oils and placed on the agar plate surface. Paper disc moistened with vehicle (DMSO plus tween 80) was placed on the seeded petriplate as a vehicle control. Standard disc containing antibacterial drugs (cefotaxime, ampicillin, tetracycline and gentamicin) were used as reference control. The petri plates were incubated at 37°C for 18 h. After the incubation period, the zone of inhibition was measured. The results of the present study revealed that the cinnamon and clove oils showed antibacterial activity. Both gram positive (*Staphylococcus aureus, Listeria monocytogenes* and *Streptococcus agalactiae*) and gram negative (*Salmonella typhimurium* and *Escherichia coli*) bacteria were sensitive to the cinnamon and clove oils. *Pseudomonas aeruginosa* was also found sensitive to the clove oil but resistant to cinnamon oil. There was no inhibition in growth of bacteria with the vehicle control. Four antibacterial drugs (cefotaxime, ampicillin, tetracycline and gentamicin) were also tested against all six organisms and were found active against test bacteria.

Key words: Cinnamon oil, Clove oil, Antibacterial sensitivity, gram positive, gram negative bacteria

INTRODUCTION

Essential oils (EOs) also called volatile or ethereal oils are aromatic oily liquids obtained from plant parts (flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits and roots). It can be obtained by expression, fermentation, enfleurage or extraction but the method of steam distillation is most commonly used for commercial production of EOs. As estimated 3000 EOs are known, of which about 300 are commercially important destined chiefly for the flavors and fragrances market (Van de Braak and Leijten, 1999). It has long been recognized that some EOs have antimicrobial properties (Nychas, 1995). Cinnamon oil is extracted from *Cinnamomum zeylanicum*, also called Cinnamon in English, Dalchini in Hindi, Taj in Guajarati (Indian spices board). *Cinnamomum zeylanicum*, the source of cinnamon bark and leaf oil, is an indigenous tree of Sri Lanka, although most oil now comes from cultivated areas. Smaller areas of wild trees are also found in the south-western parts of India (The Wealth of India, 1992).

The clove is aromatic, dry, fully grown, but unopened flower bud of clove tree. Scientific name of clove is *Syzygium aromaticum* (*Eugenia caryophyllata*) belonging to Myrtaceae family. Clove is commonly called as "laving" in Gujarati and "laung" in Hindi (Charles, 2013). Clove represents one of the major vegetal sources of phenolic compounds as flavanoids, hydroxybenzoic acids, hydroxycinamic acids and hydroxyphenylpropens. Clove oil can be obtained from distillation of buds, leaf or stem, each resulting in oil having different characteristics of oil (Anderson *et al.*, 1997). Clove bud oil, a colourless or yellow liquid. Clove buds contain 15 to 20 % of oil by weight. The main oil constituents are eugenol (70–95 %), eugenol acetate (up to 20 %) and β-caryophyllene (12–17 %) (Neveu *et al.*, 2010).

MATERIALS AND METHODS

Sources of Test Organisms/ Reference Strain

The test bacterial organisms were procured from National Chemical Laboratory, Pune. Bacterial strains of six bacterial species, namely, *Streptococcus agalactiae* (ATCC 13813), *Listeria Monocytogenes* (ATCC 1911), *Staphylococcus aureus* (ATCC 6538P), *Pseudomonas aeruginosa* (ATCC 19154), *Escherichia coli* (ATCC 10799) and *Salmonella typhimurium* (ATCC 23564) were procured. Purity and viability of the

organisms were checked by morphological, cultural and biochemical tests and maintained by periodical subculture.

Preparation of Diffusion Solution

For the preparation of 10% dimethylsulfoxide (DMSO), 10 ml of DMSO was dissolved in 90 ml of distilled water in measuring cylinder. For the preparation of diffusion solution, 0.5 ml of tween 80 was dissolved in 99.5 ml of 10% DMSO.

Preparation of Different Concentration of Cinnamon and Clove Oils

Appropriate quantity of pure cinnamon and clove oil was mixed with diffusion solution to obtain dilution of 1:1 (100%), 1:2 (50%), 1:5 (20%), 1:10 (10%) and 1:20 (5%) of cinnamon and clove oil.

Antibacterial sensitivity testing/ disc diffusion method

Screening of cinnamon oil for antibacterial activity was done by the disc diffusion method. Three gram-positive and three gram-negative strains of bacteria were tested. It was performed using an 18 h culture at 37°C in 10 ml of Mueller Hinton Agar. The test suspension was standardized to match 0.5 McFarland turbidity standard which corresponds to approximately 10^5CFU/ml with sterile saline solution. Five hundred microliters of the suspensions were spread over the plates containing Mueller Hinton Agar (for *S. agalactiae* 5% defibrinated sheep blood was added) using a sterile cotton swab in order to get a uniform microbial growth on both control and test plates. The cinnamon oil and clove oil dilution prepared in diffusion solution were sterilized by filtration through a 0.45 µm membrane filter. Under aseptic condition, empty sterilized discs (Whatman no. 5, 6 mm diameters) were impregnated with 50 µl of different concentrations (1:1, 1:2, 1:5, 1:10, 1:20) of the respective cinnamon and clove oils and placed on the agar plate surface (Wayne, 2002). Paper disc moistened with diffusion solution containing DMSO and tween 80 was placed on the seeded petriplate as a vehicle control. Standard disc containing antibacterial drugs were used as reference control. All petridishes were sealed with sterile laboratory parafilm to avoid eventual evaporation of the test samples. The plates were left for 30 minutes at room temperature to allow the diffusion of oil, and then they were incubated at 37°C for 18 h. After the incubation period, the zone of inhibition was measured with a verniercalliper. All the dilutions of cinnamon and clove oils were tested in triplicate against each bacteria.

RESULTS

The results of the present study revealed that the cinnamon and clove oils showed antibacterial activity. The zone of inhibition above 10 mm in diameter was taken as positive. Both gram positive and gram negative bacteria were sensitive to the cinnamon and clove oils, except *Pseudomonas aeruginosa* which was found resistant to cinnamon oil. There was no inhibition in growth of bacteria with the vehicle control (10% DMSO with 0.5% tween 80). Four antibacterial drugs (cefotaxime, ampicillin, tetracycline and gentamicin) were also tested against all six organisms and were found active against test bacteria. The anti-bacterial activity of 5 different concentrations of cinnamon and clove oils and four standard antibiotics is summarized in Table 1.

Cinnamon oil (1:1) and gentamicin have been found equally active against *S. typhimurium*, while 1:2, 1:5, 1:10 concentrations of cinnamon oil were less active than cefotaxime, ampicillin and tetracycline. Against *E. coli* 1:1, 1:2 and 1:5 concentrations of cinnamon oil have been found more active than gentamicin, while 1:1 concentration of cinnamon oil, ampicillin and tetracycline were equally active against *E. coli*. Against *S. aureus* 1:1 and 1:2 concentrations of cinnamon oil have been found more active than gentamicin. Against *L. monocytogenes* all concentrations of cinnamon oil were less active than standard antibacterial drugs. Against *S. agalactiae*1:1, 1:2 concentrations of cinnamon oil and tetracycline were equally active, while cinnamon oildid not show activity against *Pseudomonas aeruginosa*. The Vehicle (10% DMSO with 0.5% tween 80) used for preparation of various concentrations of cinnamon oil did not show antibacterial activity.

Clove oil (*Syzygium aromaticum*) at 1:1, 1:2, 1:5 and 1:10 concentartions were less active against *Salmonella typhimurium* than cefotaxime, ampicillin, tetracycline and gentamicin. Against *P. aeruginosa* 1:1, 1:2 and 1:5 concentrations of clove oil, tetracycline and gentamicin were equally active, while all concentrations of clove oil were less active than cefotaxime and ampicillin. Against *E. coli* 1:1, 1:2, 1:5 and 1:10 concentrations of clove oil and gentamicin were equally active, while all concentrations of clove oil were less active than cefotaxime, ampicillin and tetracycline. Against *S. aureus*1:1, 1:2 and 1:5 concentrations of clove oil and gentamicin were equally active, while all concentrations of clove oil were less active than cefotaxime, ampicillin and tetracycline. Against *L. monocytogenes* all concentrations of clove oil were less active than cefotaxime, ampicillin and tetracycline. Against *S. agalactiae* 1:1, 1:2, 1:5 and 1:10 concentrations of clove oil and tetracycline were equally active, while all concentrations of clove oil were less active than cefotaxime, ampicillin and gentamicin.

DISCUSSION

In the present study, cinnamon oil was found to

have *in vitro* antibacterial activity against test bacteria except *P. aeruginosa.* Similar results were reported by Prabuseenivasan *et al.* (2006). They reported significant inhibitory effect of cinnamon oil against *E. coli* (29.8 mm) and *S. aureus* (20.8 mm) at four different concentrations (1:1, 1:5, 1:10 and 1:20). Goni *et al.* (2009) have reported inhibition and growth reduction by cinnamon essential oil by vapour diffusion tests against *Escherichia coli* (22 ± 3 mm), *Staphylococcus aureus* (26 ± 4 mm) and *Listeria monocytogenes* (26 ± 2 mm). They also reported that cinnamon essential oil by solid disc diffusion test is active against *E. coli* (18 ± 1 mm), *S. choleraesuis* (12 ± 1 mm) and *L. monocytogenes* (19 ± 1 mm).

Similarly, Mukhtar and Ghori (2012) also reported antibacterial activity of aqueous and ethanolic extracts of cinnamon against *Escherichia coli* (ATCC 25922) by using disc diffusion method. Mean diameter of inhibition zone (mm) of cinnamon aqueous extract at different concentration against *E.coli* was 9.3 ± 0.38 mm at 60%, 10 ± 0.40 mm at 80%, 10.3 ± 0.41 mm at 100% and for cinnamon ethanolic extract 8 ± 0.32 mm at 10%, 11 ± 0.28 mm at 20%, 12 ± 0.50 mm at 40%, 14 ± 0.52 mm at 60%, 16 ± 0.52 mm at 80%, 17 ± 0.52 mm at 100% respectively. Bayati and Mohammed (2009) also reported antibacterial activity of cinnamon oil against selected pathogenic bacteria. Cinnamaldehyde at different concentrations (1:1, 1:5, 1:10 and 1:20) was active against all tested bacteria and the highest inhibitory effect was observed against *S. aureus* (24.1 mm) and for *Escherichia coli* (23.2 mm) using the disk diffusion method. Rohraff and Morgan (2014) also reported antimicrobial activity of cinnamon bark essential oil. Cinnamon bark oil showed zone of Inhibition of 27.5 mm and 12.5 mm against *S. aureus* and *E. coli*, respectively. Aumeeruddy-Elalfi *et al.* (2015) reported antibacterial activity of *Cinnamomum zeylanicum* essential oil against *E. Coli* (24.4 ± 0.10 mm) and *S. aureus* (22.6 ± 0.06 mm). On the contrary, Shan *et al.* (2007) reported less antibacterial activity of *cinnamomum burmannii* against common food borne pathogenic bacteria like *S. aureus* was (15.7 mm), *B. cereus* (15.4 mm), *L. monocytogenes* (11.5 mm), *S.anatum* (12.1 mm) and *E. coli* (8.7 mm).

Clove oil was found to have good *in vitro* antibacterial activity against both gram positive and gram negative bacteria. Similar results were reported by Dorman and Deans (2000). They reported significant inhibitory effect of *Syzigium aromaticum* oil against *E. coli* (13.6 mm), *S. aureous* (14.9 mm) and *P. aeruginosa* (14.0 mm). Similarly, Fu *et al.* (2007) reported antibacterial activity of clove oil against *E. coli* (16.3 mm), *S. aureous* (16.3 mm) and *P. aeruginosa* (9.5 mm). Sofia *et al.* (2007) evaluated antibacterial activity of clove extract against common foodborne pathogens and found effective against *E. coli* and *S.aureous* with 23.3 mm and 25.6 mm zone of inhibition, respectively at 3% concentration. Ayoola *et al.* (2008) also reported 23.00 mm zone of inhibition against *Escherichia coli* (ATCC 35218) and 21.00 mm zone of inhibition against *Staphylococcus aureus* (ATCC 25923) at 445 mg/ml concentration of *Syzigium aromaticum* oil. Saeed and Tariq (2008) reported *in vitro* antibacterial activity of clove oil against gram negative bacteria and they reported 11.87 mm zone of inhibition against *E. coli*, 16.50 mm against *S. typhimurium* and 18.86 mm against *P. aeruginosa*. Kumar *et al.* (2014) also reported antibacterial activity of Clove oil on different pathogenic bacteria and they reported 13 mm zone of inhibition against *E. coli*, 19.5 mm against *S. aureus* and 23 mm against

Table 1:
Antibacterial activity of cinnamon and clove oils against *Salmonella typhimurium, E. coli, S. aureus, L. monocytogenes, S.agalactiae* and *P. aeruginosa* using antibacterial sensitivity test

	Salmonella typhimurium	*E.coli*	*S.aureus*	*L.monocytogenes*	*S.agalactiae*	*P. aeruginosa*
CZ (1:1) (100%)	19.00 ± 1.00^{cd}	24.67 ± 0.67^{e}	22.33 ± 0.33^{a}	21.00 ± 1.00^{b}	17.33 ± 0.33^{b}	0.00
CZ (1:2) (50%)	17.66 ± 0.33^{bc}	21.00 ± 1.15^{d}	20.00 ± 0.00^{cd}	20.33 ± 0.88^{b}	16.33 ± 0.33^{b}	0.00
CZ (1:5) (20%)	15.66 ± 0.88^{b}	20.00 ± 1.53^{cd}	17.33 ± 1.45^{bc}	19.00 ± 1.53^{ab}	12.33 ± 1.86^{a}	0.00
CZ (1:10) (10%)	10.00 ± 0.00^{a}	16.00 ± 0.58^{ab}	14.33 ± 1.33ab	14.00 ± 2.08^{a}	10.33 ± 0.33^{a}	0.00
CZ (1:20) (5%)	0.00	14.67 ± 0.67^{a}	10.67 ± 0.67^{a}	0.00	0.00	0.00
SA (1:1) (100%)	16.67 ± 0.33cd	20.00 ± 0.00b	19.67 ± 1.20b	25.00 ± 2.00bcd	17.67 ± 1.76b	18.33 ± 0.33^{b}
SA (1:2) (50%)	16.33 ± 0.33cd	19.00 ± 0.00b	18.67 ± 1.20b	25.33± 1.33bcde	17.00 ± 2.08b	17.33 ± 0.88^{b}
SA (1:5) (20%)	15.33 ± 0.67c	19.33 ± 0.67b	18.33 ± 1.86b	24.00± 0.00bc	15.33 ± 2.40b	15.33 ± 0.88^{b}
SA (1:10) (10%)	12.00 ± 0.00b	16.67 ± 0.88b	15.33 ± 1.86ab	20.67± 0.88b	13.67 ± 2.1b	0.00
SA (1:20) (5%)	0.00	8.67 ± 4.48a	13.00 ± 1.1a	11.33± 5.69a	0.00	0.00
Cefotaxime	27.33 ± 0.33^{e}	29.00 ± 1.00^{f}	28.33 ± 0.88^{e}	30.67 ± 0.67^{c}	30.67 ± 0.88^{d}	27.00 ± 0.58^{b}
Ampicillin	26.67 ± 0.33^{e}	25.33 ± 1.20^{e}	31.67 ± 2.40^{e}	33.67 ± 2.85^{c}	23.33 ± 0.88^{c}	30.33 ± 1.45^{b}
Tetracycline	21.33 ± 0.88^{d}	25.00 ± 0.58^{e}	28.67 ± 1.86^{e}	34.33 ± 2.19^{c}	16.00 ± 1.00^{b}	17.33 ± 5.3^{a}
Gentamicin	19.00 ± 1.15^{cd}	18.00 ± 0.58^{bc}	19.00 ± 0.58^{bc}	31.67 ± 1.20^{c}	29.67 ± 0.88^{d}	20.33 ± 1.86^{a}

Values expressed are zone of inhibition in milimeter (Mean ± SE, n=3) Mean values with dissimilar superscript in a column vary significantly at p<0.05
CO = *Cinnamomum zeylanicum* oil (cinnamon oil)
SA = *Syzygium aromaticum* oil (clove oil)

S.typhimurium at 2000 ppm concentration. They concluded that clove has antibacterial activity against the pathogens tested and can be used as an alternative to standard antibacterial drugs. Vanin *et al.* (2014) also reported 21.16 mm zone of inhibition against *E. coli*, 14.16 mm against *S. aureous*, 14.5 mm against *L. monocytogenes* and 15 mm against *P. aeruginosa* of clove oil. Abdullah *et al.* (2015) studied antibacterial activity of clove oil on multidrug resistant bacteria and reported zone of inhibition 25±1.4 against *P. aeruginosa* (ATCC 27853) and 22±1.6 against *S. aureous* (ATCC 29213) at 10% concentration. Similarly, Soman *et al.* (2017) also reported antibacterial activity of endophytic bacteria (*Staphylococcus arlettae)* isolated from babool against a panel of human pathogenic microorganisms (*Staphylococcus aureus*, *Streptococcus pyogenes*, *Bacillus cereus*, *Klebsiella pneumoniae, SalmonellaTyphimurium* and *Escherichia coli*). Likewise, in other report on the antibacterial activity of endophytic bacteria isolated from *Aloe barbadensis* (aloe vera) showed good antibacterial activity against *Staphylococcus aureus* and *Escherichia coli* (Singh *et al.*, 2016). Similarly, Shrivastav *et al.* (2016) reported *in vitro* effect of *Punica granatum* and *Ocimum sanctum* on extended spectrum beta lactamase enzyme of *E.coli.* In case of fruit peel juice of *Punica granatum* and fresh leaves juice of *Ocimum sanctum,* 11.8 ± 0.31 (Mean±S.E.) with 13.4%, and 13.0± 0.45 (Mean±S.E.) with 10.2% of zone of inhibition and inhibition per cent,($p<0.05$) were observed. *Punica granatum and Ocimum sanctum in* combination gave 13.0± 0.37 to 11.0±0.21 of inhibition zone with per cent inhibition of 27 to 19%. The present study results demonstrated that cinnamon and clove oils possesses varying degree of antibacterial activity and it may be evaluated as alternative to standard antibacterial drugs.

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REFERENCES

Abdullah, B. H., Hatem, S. F., and Jumaa, W. (2015). A comparative study of the antibacterial activity of clove and rosemary essential oils on multidrug resistant bacteria. *UKJPB.* **3(1)***: 1*8-22.

Anderson, W. G., McKinley, R. S., and Colavecchia, M. (1997). The use of clove oil as an anesthetic for rainbow trout and its effects on swimming performance. *N. Am. J. Fish. Manag*. **17(2)**: 301-307.

Aumeeruddy-Elalfi, Z., Gurib-Fakim, A., and Mahomoodally, F. (2015). Antimicrobial, antibiotic potentiating activity and phytochemical profile of essential oils from exotic and endemic medicinal plants of Mauritius. Ind. Crops Prod.**71**: 197-204.

Ayoola, G. A., Lawore, F. M., Adelowotan, T., Aibinu, I. E., Adenipekun, E., Coker, H. A. B., and Odugbemi, T. O. (2008). Chemical analysis and antimicrobial activity of the essential oil of *Syzigiumaromaticum* (clove). *Afr. J. Microbiol. Res.* **2(7)**: 162-166.

Bayati, F. A., and Mohammed, M. J. (2009). Isolation, identification, and purification of cinnamaldehyde from *Cinnamomumzeylanicum* bark oil. An antibacterial study. *Pharm. Biol.* **47(1):** 61-66.

Charles, D. J. (2013). Saffron. In antioxidant properties of spices, herbs and other sources, pp. 509–520.

Dorman, H. J. D., and Deans, S. G. (2000). Antimicrobial agents from plants: antibacterial activity of plant volatile oils. *J. Appl. Microbiol.* **88(2)**: 308-316.

Fu, Y., Zu, Y., Chen, L., Shi, X., Wang, Z., Sun, S., and Efferth, T. (2007). Antimicrobial activity of clove and rosemary essential oils alone and in combination. *Phytother. Res.* **21(10)**: 989-994.

Goni, P., López, P., Sαnchez, C., Gómez-Lus, R., Becerril, R., and Nerín, C. (2009). Antimicrobial activity in the vapour phase of a combination of cinnamon and clove essential oils. *Food Chem.***116(**4): 982-989.

Kumar, Y., Agarwal, S., Srivastava, A., Kumar, S., Agarwal, G., and Khan, M. Z. A. (2014). Antibacterial activity of Clove (*Syzygium aromaticum*) and Garlic (*Allium sativum*) on different pathogenic bacteria. *Int. J. Pure Appl. Biosci.* **2(3)**: 305-311.

Mukhtar, S., and Ghori, I. (2012). Antibacterial activity of aqueous and ethanolic extracts of garlic, cinnamon and turmeric against *Escherichia coli* ATCC 25922 and *Bacillus subtilis* DSM 3256. *Int. J. Appl. Biol. Pharm. Tech.* **3(2)**: 131-136.

Neveu, V., Perez-Jimenez, J., Vos, F., Crespy, V., Du Chaffaut, L., Mennen, L. and Scalbert, A. (2010). Phenol-Explorer: an online comprehensive database on polyphenol contents in foods. *Database*-2010.

Nychas, G. J. E. (1995). Natural antimicrobials from plants. In *New methods of food preservation* (pp. 58-89). Springer, Boston, MA.

Prabuseenivasan, S., Jayakumar, M., and Ignacimuthu, S. (2006). *In vitro* antibacterial activity of some plant essential oils. *BMC Complement. Altern.*

Med. **6(1)**: 39-46.

Rohraff, D., and Morgan, R. (2014). The evaluation of essential oils for antimicrobial activity. Grand Valley State University, Undergraduate Research and Creative Practice, pp. 1-8.

Saeed, S., and Tariq, P. (2008). *In vitro* antibacterial activity of clove against Gram negative bacteria. *Pak. J. Bot.* **40(5)**: 2157-2160.

Shrivastav, A., Sharma R.K., Sahni, Y. P., Shrivastav, N., Vidhi Gautam and Jain, S.K. (2016). *In vitro* effect of *punica granatum* and *ocimum sanctum* on extended spectrum beta lactamase enzyme of *e.coli. J. Vet. Pharmacol. Toxicol.***15 (2)**: 8-10.

Singh, A.K., Sharma, R.K., Sahni, Y.P., Sharma V. and Singh T. (2016). Iisolation, morphological identification and *in vitro* antibacterial activity of endophytic bacteria isolated from *Aloe barbadensis* (*Aloe vera*) leaves. *J. Vet. Pharmacol. Toxicol.***15(2)**:11-14.

Sofia, P. K., Prasad, R., Vijay, V. K., and Srivastava, A. K. (2007). Evaluation of antibacterial activity of Indian spices against common foodborne pathogens. *Int. J. Food Sci. Technol*. **42(8)**: 910-915.

Soman, S., Sharma, R.K., Sharma, V., Gautam, V., Shrman K., Sawarkar, A., Talpade, J. Meena, N.S. and Kewat R. (2017). Isolation, identification and antibacterial activity of endophytic bacteria isolated from babool leaves. *J. Vet. Pharmacol. Toxicol.***16 (2)**: 64-68.

The Wealth of India (1992). A Dictionary of Indian Raw Materials and Industrial Products, Publications and Information Directorate, NewDelhi, India, p. 582.

Van de Braak, S. A. A. J., and Leijten, G. C. J. J. (1999). Essential oils and oleoresins: a survey in the Netherlands and other major markets in the European Union. CBI, Centre for the Promotion of Imports from Developing Countries, Rotterdam, 116.

Vanin, A. B., Orlando, T., Piazza, S. P., Puton, B. M., Cansian, R. L., Oliveira, D., and Paroul, N. (2014). Antimicrobial and antioxidant activities of clove essential oil and eugenyl acetate produced by enzymatic esterification. *Appl. Biochem. Biotech*. **174(4)**: 1286-1298.

Wayne, P. A. (2002). National committee for clinical laboratory standards. Performance standards for antimicrobial disc susceptibility testing, **12:** 01-53.

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Research Article

STORAGE RESISTANCE OF BUBALINE EPIDIDYMAL SPERMATOZOA AT 5°C IN MODIFIED EGG YOLK CITRATE DILUTORS

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ABSTRACT

This study was designed to evaluate the effect of alanine, cysteine and glutamine on structural aspect and storage resistance of buffalo bull epididymal spermatozoa. The 48 testicles from slaughtered buffalo bulls were collected in 0.9% normal saline solution from slaughter house and were stored at 5^{0}C. The epididymal semen was collected from these testicles at 0 hours of storage and spermatozoa were evaluated for their initial motility, livability, abnormality and HOS reactivity. A total of four groups were studied. In group 1 epididymal semen sample were diluted with Egg Yolk Citrate (EYC) as extender and kept as control and in rest 3 groups epididymal semen samples extended with Egg Yolk Citrate (EYC) were additionally supplemented with 25mM alanine (D1), 5 mM cysteine (D2) and 25 mM glutamine (D3) and stored at 5 ºC. Semen quality parameters were examined post dilution and post-thawing at 0, 24 48 and 72 hours of refrigeration storage. Results demonstrated that both cysteine (D2) and glutamine (D3) supplimentation showed significant ($P<0.05$) positive effects on post-thaw motility, live sperms concentration, membrane integrity and abnormality when added to EYC extender at 5 mM and 25Mm concentration respectively compared to control and alanine supplemented group (D1). Thus, it may be concluded that addition of 5mM cysteine and 25 mM glutamine in conventional storage medium enhanced post-thaw motility, percent live sperms, improved membrane integrity and significantly reduced percent abnormal sperms in buffalo bull semen.

Key Words- Buffalo bull, Epididymal semen, Refrigeration, Alanine, Cystiene and Glutamine

INTRODUCTION

Buffaloes are an important component of animal production in India. Artificial insemination (AI) has been used for increasing genetic production potential of animals but AI with frozen thawed semen has been restricted in buffalo due to low freezing ability and fertility of buffalo spermatozoa. Storage at lower temperatures and thawing of spermatozoa is associated with oxidative stress which accelerates the production of Reactive Oxygen Species (ROS) molecules due to plasma membrane lipid peroxidation. It has also been observed that buffalo sperm are more sensitive to oxidative stress as compared to cattle spermatozoa due to high content of polyunsaturated phospholipids present in sperm membrane. Therefore, to protect the sperm integrity and to improve the sperm viability during storage at lower temperatures of buffalo spermatozoa, an extra antioxidant supplementation is recommended. It has been reported that some amino acids protect several type of animal cells against freezing stress including sperm (Trimeche *et al.,* 1999, Kruuv and Glofcheski, 1991). Several amino acids (glutamine, glycine, proline and histidine) has been detected in seminal plasma and used successfully as non-permeating cryoprotectants to cryopreserve spermatozoa of many mammalian species including goat, ram and stallion (Kundu *et al.,* 2001), however, the exact role of these amino acids in sperm physiology is still unclear. The main goal of this study was to asses and compares the effect of alanine, cysteine and glutamine amino acids on characteristics of epididymal bubaline spermatozao that could be used in bovine semen extender for preserving buffalo-bull sperm motility and viability during freezing and thawing.

MATERIAL AND METHODS

A total of 48 pair of testicles with intact scrotal sac were collected from mature buffalo bulls immediately after their slaughter at Slaughter house, Haldwani and Marya Frozen Agro-Food (Pvt.) limited Bareilly (U.P.). To maintain the holding temperature during transport, the testes were placed on a styropore box previously calibrated with the target storage temperature.

The epididymal semen was collected by incising and squeezing the caput, corpus and cauda epididymis with 16 G hypodermic needle and diluted with EYC extender to gain the motility at 37°C. The diluted spermatozoa were kept at 37°C for 10 min for diluting the epididymal motility inhibitor factor and subsequently stored at 5°C up to 72 hours. The amino acids, alanine, cysteine and glutamine (Sigma Chemical Co. India) were added to the EYC extender at the concentrations of 25 mM, 5Mm and 25mM respectively.

The assessment was undertaken on neat semen and extended semen stored for 0, 24, 48, and 72 hours at 5^{o}C.The parameters studied were subjective initial motility (%), livability (%), abnormality and HOS

reactivity as per standard protocols (Mamuad *et al.,* 2004).

The data were processed using statistical analysis programme. The analysis of variance (two way ANOVA) followed by Tukey, Column statistics and correlation was tested for significance by the Graph pad prism. The version 5.00 software was used for all statistical analysis. Difference with values of $P<0.05$ was considered to be statistically significant.

RESULTS

Effect of modified extenders on percentage of initial motility

The overall mean±SE of percent initial motility just after dilution of epididymal semen in control (EYC), D1 (EYC+ alanine), D2 (EYC+ cysteine) and D3 (EYC+ glutamine) dilutors at 0, 24, 48 and 72 hrs of refrigeration is presented in Table 1.

Effect of modified extenders on percentage of live spermatozoa

The overall mean±SE of percent livability just after dilution of epididymal semen in control (EYC), D1 (EYC+ alanine), D2 (EYC+ cysteine) and D3 (EYC+ glutamine) dilutors at 0, 24, 48 and 72 hrs of refrigeration is presented in Table 2.

Effect of modified extenders on percent spermatozoal abnormality

The overall mean±SE of percent abnormality just after dilution of epididymal semen in control (EYC), D1 (EYC+ alanine), D2 (EYC+ cysteine) and D3 (EYC+ glutamine) dilutors at 0, 24, 48 and 72 hrs of refrigeration is presented in Table 3.

Effect of modified extenders on percentage of HOST reactive spermatozoa.

The overall mean±SE of percent abnormality just after dilution of epididymal semen in control (EYC), D1 (EYC+ alanine), D2 (EYC+ cysteine) and D3 (EYC+ glutamine) dilutors at 0, 24, 48 and 72 hrs of refrigeration is presented in Table 4.

DISCUSSION

The increase in sperm sensibility to oxidative stress and cryodamage affects spermatozoa quality, shortening their life span resulting in a reduction in sperm motility, viability, antioxidant enzyme activity and fertilizing capacity during extending, freezing and thawing process. Frozen-thawed bull semen is more easily peroxidized than fresh semen. Additionally, intracellular antioxidant capacity in sperm decreases following freezing-thawing process (Hammerstedt, 1993). Pattern of sperm motility is affected by physico-chemical properties of the diluents (Akhter *et al.,* 2008). In present study, post thaw sperm progressive motility was significantly higher in extender containing cysteine (5mM) and glutamine (25 mM) as compared to control and extender conataining alanine at 0, 24, 48 and 72 hours of post thawing. However, glutamine showed better percent sperm motility compared to cysteine at 72 hours of post thaw. Our results are in agreement with the findings of previous studies in which cysteine addition in extender improved the sperm motility in liquid (Dhami *et al.,* 1994) and cryopreserved semen (Singh *et al.,* 1990; Dhami and Sahni, 1993) of Indian Murrah and Italian buffalo bulls (Del Sorbo *et al.,* 1995). Our results are also in agreement with Sheshtawy *et al.* (2008) who documented significant increase in progreesive motile sperms after the addition of glutamine (25mM) and cysteinine (5mM) in extender of buffalo bull semen.

The number of viable sperms after storage at lower temperatures significantly affects the fertility rates in the field (Andrabi *et al.,* 2006). In present study, post thaw percent live sperms were significantly higher in extender containing cysteine (5mM) and glutamine (25 mM) as compared to control and extender containing alanine (25mM) at 0, 24, 48 and 72 hours of post thawing. Our results are in agreement with Bucak *et al.* (2009) who reported a significant higher percentage of viable sperm after the addition of cysteine in extender in ovine semen. Our results are also in agreement with the study conducted by El-Sheshtawy *et al.* (2008) who documented significant increase in sperm viability after the addition of glutamine (25mM) and cysteinine (5mM) after cryopreservation of buffalo bull spermatozoa. However, Bucak *et al.* (2008) observed a non-significant increase in the percentage of viable spermatozoa after the addition of cysteine in post thawed ovine semen with a significant increase in catalase activity.

According to Ax *et al.* (2000), normal fertile semen samples should contain no more than 20% abnormal spermatozoa. Our findings revealed that the epididymal sperm samples registered acceptable number of abnormal spermatozoa below 20% which is reflective of the quality of the sperm from the buffalo. In present study, percent abnormality of epididymal semen

Table 1:
Percent motility of cauda epididymal spermatozoa in diluted semen of buffalo bull at 0, 24, 48 and 72 hrs of refrigeration.

	0 hour	24 hour	48 hour	72 hour
Control	75.50±0.53bA	53.00±0.48cB	39.50±1.31bC	32.25±0.92cD
D1	76.50±1.37bA	53.00±0.41bB	40.75±1.54bC	32.50±0.83bD
D2	80.25±0.75aA	58.75±1.56aB	43.75±1.16aC	36.50±1.01aD

Table 2:
Percent livability of cauda epididymal spermatozoa in diluted semen of buffalo bull at 0, 24, 48 and 72 hrs of refrigeration.

	0 hour	24 hour	48 hour	72 hour
Control	80.00±0.83bA	69.00±0.89cB	55.25±1.62bC	44.25±1.28bD
D1	81.50±1.20bA	71.50±1.10bB	56.75±1.60bC	45.75±1.11bD
D2	84.25±0.85aA	74.25±0.95aB	59.00±1.64aC	48.50±1.10aD
D3	85.25±0.57aA	75.25±0.89aB	60.00±1.41aC	49.50±1.15aD

Table 3:
Percent abnormality of cauda epididymal spermatozoa in diluted semen of buffalo bull at 0, 24, 48 and 72 hrs of refrigeiration.

	0 hour	24 hour	48 hour	72 hour
Control	18.00±0.41aA	22.25±0.57aB	28.50±0.41aC	32.25±0.62aD
D1	17.75±0.62aA	21.75±0.46aB	28.25±0.78aC	31.25±0.46bD
D2	16.25±0.46bA	18.00±0.59bB	26.25±0.62bC	26.50±0.63cD
D3	16.00±0.76bA	18.50±1.01bB	26.00±0.53bC	25.75±0.52cD

Table 4:
Percent HOS reactive cauda epididymal spermatozoa in diluted semen of buffalo bull at 0, 24, 48 and 72 hrs of refrigeration.

	0 hour	24 hour	48 hour	72 hour
Control	30.25±0.62cA	28.00±0.59bB	23.25±0.40bC	17.00±0.34bD
D1	31.25±0.52cA	28.25±1.06bB	23.50±0.34bC	17.25±0.40bD
D2	33.25±0.78bA	31.00±0.59aB	25.25±0.52aC	18.00±0.63aD
D3	34.25±0.71aA	31.25±0.62aB	25.75±0.57aC	18.00±0.53aD

was significantly lower (<20%) in extender containing cysteine (5mM) and glutamine (25 mM) as compared to control and extender containing alanine (25mM) at 0 and 24 hours of post refrigeration. However, the percent abnormality was 20-27% at 48 and 72 hours of refrigeration after the addition of glutamine and cysteine which was again significantly lower compared to alanine and control.

Structural and functional sperm plasma membrane integrity is essential for the fertilization process and its evaluation has particular importance. Furthermore, the process of capacitation, acrosome reaction and the oocyte penetration requires a biochemically active plasmalemma .In our study, percentage of sperm with intact functional plasma membrane was significantly higher in extender containing cysteine (5mM) and glutamine (25 mM) as compared to control and alanine (25mM) at 0, 24, 48 and 72 hours of post refrigeration. However, glutamine showed better percentage of HOST reactive sperms at 0 hours of post refrigeration compared to cysteine. Our results are in agreement with El-Sheshtawy *et al.* (2008) who documented significant increase in HOST reactive sperms after the addition of glutamine (25mM) and cysteinine (5mM) in extender of buffalo bull spermatozoa. In similar studies on ovine semen, addition of cysteine resulted in higher percentage of sperm with functional plasma membrane (Bucak *et al.* 2008 and Özkan *et al.,* 1990). Similarly, addition of 20 or 40 mM of glutamine significantly increased post thaw plasma membrane integrity in Egyptian buffalo bull. It is suggested that glutamine and cysteine supplementation in extender protected the membrane integrity by scavenging the ROS molecules (Alvarez and Storey, 1983) directly and/ or indirectly in the semen-extender complex (Aruoma *et al.,* 1989; Bucak *et al.,*1992) which can destroy the sperm cell membrane (Cotran *et al.,* 1989).

In conclusion the current results demonstrated that addition of cysteine (5mM) and glutamine (25 mM) increase the storage resistance of epididymal spermatozoa of buffalo bull in EYC extender stored at 5 ^{0}C up to 72 hours. This will help us to define the suitable extender for freezing of buffalo bull sperm. However, further studies still needed to recognize the effect of such addition on *in vitro* and *in vivo* fertility in farm animals.

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REFERENCES

Andrabi, S.M.H. (2009). Factors affecting the quality of cryopreserved buffalo *(Bubalus bubalis)* bull spermatozoa. *Reprod. Domestic Anim.* **44**: 552-569.

Alvarez, G. and Storey, B. T. (1992). Evidence for increased lipid peroxidation damage and loss of superoxide dismutase activity as a mode of sublethal cryodamage to human sperm during cryopreseivation. *J. Androl.,* 13: 232—234

Akhter, S.; Ansari, M. S.; Andrabi, S. M. H.; ULLAH, N. and Qayyum, M. (2008). Effect of antibiotics in

extender on bacterial control and spermatozoal quality of cooled buffalo (Bubalus bubalis) bull semen. *Reprod. Dom. Anim*. **43**: 272-278.

Aruoma, O.I.; Halliwell, B.; Hoey, B.M. and Butler, J. (1989). The antioxidant action of Nacetylcysteine: Its reaction with hydrogen peroxide, hydroxyl radical, superoxide and hypochlorous acid. *Free Radic. Biol. Med*. **6:** 593–597.

Ax, R.L.; Dally, M.; Didion, B.A.; Lenz, R.W.; Love, C.C. and Varner, D.D. (2000). Semen Evaluation. *In:* Hafez B and Hafez ESE. Reproduction in Farm Animals.7th Ed. Philadelphia: Lea and Febiger. p 365-375

Bucak, M. N.; Atessahin, A. and Yuce, A. (2008). Effect of anti-oxidants and oxidative stress parameters on ram semen after the freeze-thawing process. *Small Rum. Res.* **75:** 128-134.

Bucak, M. N.; Tuncer, P. B.; Sariozkan, S. and Ulutaº, P. A. (2009). Comparison of the effects of glutamine aminoacid solution on post-thawed ram sperm parameters, lipid peroxidation and anti-oxidant activities. *Small Ruminant Res.* **81**(1): 13-17.

Cotran, R. S.; Kumar, V. and Robins, S. L. (1989). Pathologic basis of diseases, 4th edn, W. B. Saunders Co., Philadelphia, pp. 9-16.

Dhami, A. J.; Jani, V. R.; Mohan, G. and Sahni, K. L. (1994). Effect of extenders and additives on freezability, post thaw thermo resistance and fertility of frozen Murrah buffalo semen under tropical climate. *Buffalo J.* **1**: 35-45.

Dhami, A.J. and Sahni, K.L.(1993). Effect of extenders, additives and deep freezing on the leakage of transaminases from Murrah buffalo spermatozoa. *Buffalo J*. 1: 55-64.

El-Sheshtawy, R. I.,; El-Sisy, G.A. and El-Nattat. W.S. (2008). Use of selected amino acids to improve buffalo bull semen cryopreservation. *Glob. Vet*. **2:** 146-150.

Hammerstedt, R.H., (1993). Maintenance of bioenergetic balance in sperm and prevention of lipid peroxidation: A review of the effect on design of storage preservation systems. *Reprod. Fertil. Dev.* **5:** 675-690.

Kruuv, J. and Glofcheski D.J., (1992). Protective effect of amino acids against freeze-thaw damage in mammalian cells. *Cryobiol.* **29**: 291-295.

Kundu, C.N.; Das, K. and Majumder, G.C. (2001). Effect of amino acids on cauda epididymal sperm cryopreservation using a chemically defined model system. *Cryobiol*. **41**: 21-27.

Mamuad, F.V.; Venturina, E.V. and Saito, H. (2004). Collection, Processing and Handling Buffalo Semen. Water Buffaloes and Beef Cattle Improvement Project (WBBCIP). A Joint-JICA Assisted Project of the Philippine Carabao Center and the Bureau of Animal Industry Philippines.

Ozkan, S. S.; Bucak, M. N.; Tuncer, P. B.; Ulutas, P.; Raizada. B. C.; Sattar, A. and Pandey, M. D. (1990). A comparative study of freezing buffalo semen in two diluters. *Adv. Buffalo.Res.* **3**: 66-74.

Singh, N.P.; Manik, R.S. and Raina, V.S. (1990). Effect of cysteine fortification on Cryopreservation of buffalo semen in milk-whey extenders. *Rec. Adv. Buffalo Res*. **3:** 63-65.

Trimeche, A.; Yvon, J.M.; Vidament, M.; Palmer E .and Magistrini, M. (1999). Effects of glutamnine, proline, histidine and betaine on post-thaw motility of stallion spermatozoa Theriogenology. **52:** 181-191.

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Short Communication

ANTIBIOTIC RESISTANCE AND A WAY FORWARD FOR VETERINARIANS

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ABSTRACT

In 1928, Alexander Fleming, a Scottish Physician, discovered penicillin 'quite by accident', revolutionising medicine and the human relationship with microbial diseases forever. Diseases such as scarlet fever, pneumonia, meningitis and diphtheria which were devastating in Britain were suddenly curable.Today however, less than 100 years later, we are once again threatened by diseases that for the last century been curable. As antimicrobial resistance becomes a global concern, as Veterinarians our role in the current and future use remains of utmost importance.

The role of veterinarians in the healing of animals has occurred alongside the healing of humans for much of human history. Modern veterinary medicine as a production animal science, originated as a production animal subject between the two World Wars in Europe, to feed armies and populations in ever increasing numbers.

Since then, veterinary medicine has evolved rapidly in the last century and now ranges from production animal and livestock rearing, companion animal medicine, wildlife medicine, research and vaccine production etc. The role of a Veterinarian ranges from surgeon and diagnostician, to researcher, to policy advisor and Veterinarians are found in spaces as diverse as government offices, clinics, slaughterhouses, animal shelters and laboratories.

Few other professions offer the insight that Veterinary medicine offers, whether it is in microbiology, pharmacology, nutrition, medicine and surgery, food hygiene, animal welfare, disease control etc. And it is from this position that as Veterinarians, taking in an all-encompassing view that our decision theory should stem from.

As a Veterinary student in Europe, I remember being quite surprised the first time I stepped into a pharmacy to buy chloramphenicol eye drops and being asked for a prescription. I was prone to getting '*stye's*' from stress, and thus with alarming regularity, right before a critical midterm or exam, one of my eyelids would start swelling. In India I was so accustomed to walking over and picking up they eye drops without a prescription, that it had never occurred to me that it wouldn't be the same in other countries. Faced with the dual pressure of an increasingly swollen eye lid and a big exam the following day, I went home and bathed my eye with warm water and cold tea bags, hoping my appearance wouldn't scare of my professor the next day. After the exam, I went to a doctor who duly examined my eye, prescribed drops and a topical cream and it was only with this prescription was I then allowed to purchase the eye drops. This was my first experience in Europe with the regulated use of antibiotics and it was something that I began to observe more and more carefully.

By the time I started studying pharmacology, withdrawal periods and prudent use were words that would flit through my dreams. Stories of Veterinarians having their licences suspended for failing to observe or advice farmers about adequate withdrawal periods were told to us, alongside stories of discoloured milk and discarded eggs. The EU standards of food traceability along with the increased consumer pressure both for better animal welfare, more organic and local production meant that the legislation for the use of antibiotics became more and more strict.

The EU laws on animal keeping thus ranged from the number of animals permitted, the antibiotic usage, and welfare at the farm – both in terms of disease and enrichment for the animals, (starting with the rules for poultry in 2012 with regards to cage sizes and now extending to swine and cattle), limits on travel for transport and slaughter and humane slaughter techniques.

A summer internship in the USAdemonstrated similar tendencies, but with less strict withdrawal periods and a greater emphasis on animal production than animal welfare; but growing awareness across Europe, the America's, Australia, New Zealand etc. where *historically* a bulk of the world's livestock was produced and consumed shows a growing trend towards the concept that increased animal welfare such as extensive farming techniques and the growing research and consensus that demonstrates that healthy, happy animals are actually economically more effective is rapidly changing the way animals are reared, kept and ultimately slaughtered.

While working in Africa, I observed practices range from extremely modern techniques in South Africa to the traditional keeping methods of the nomadic Masaii

tribes. The widespread dissemination of antibiotic usage and subsequent mis-usage, have already given cause for concern.

Decades of specialization by both Veterinarians and human doctors, resulted in the notion that human and animal health were separate topics that required separate approaches. However, increasingly, even large organizations such as the FAO and the OIE have adopted the "one health" approach in understanding that human health, animal health and the environment are all intricately linked to one another, and in the field, the laboratory like isolation of disease, causation, factor and treatment can have long lasting repercussions for everyone.

Countries such as Sweden and Netherlands now produce dedicated formularies annually based on research conducted in the country and all antibiotics for both farm animals and companion animals are prescribed in there. Thus across the country the first treatment plan for a dog with an ear infection will be a specific product that will be prescribed by every clinic in the country, and only if it doesn't work will a second recommended product be prescribed. Additionally independent audits conducted in each practice govern the prescription of the antibiotics. The use of the antibiotics is thus highly regulated and companion animal, production animal and human antibiotics almost don't overlap. The Netherlands is also investing heavily in finding alternative therapies – herbal, holistic to incorporate them into the Veterinary sector.

In Sweden, diseases like Mastitis aren't treated with antibiotics anymore. All veterinarians are mandated by law to send samples to State labs where the pathogens are tested for antibiotic resistance. Based on the results, the lab informs the veterinarian about which antibiotic can be used (if required). In the meantime, conscious of the discomfort the animal might be in, the farmers are taught to apply holistic remedies such as heating and cooling pads on the inflamed udder, to massage it and release the milk – therapies that are known to work and treat the underlying inflammation and pain.

India stands at a unique crux of combining older, traditional systems of animal rearing and healing and newer, more modern techniques particularly in the poultry industry. India also stands a chance at learning from the mistakes of our western counterparts, where decades of systematic antibiotic use and abuse have now given way to extremely stringent use and antibiotic vigilance.

Unlike so many other countries, in India we have the advantage of centuries of traditional knowledge, which if properly scrutinized and harnessed, might also pave the way for those countries with fledging livestock sectors that are looking for an alternative path.

While highly regulated audits to control antibiotic use might be difficult to adapt to a country across the size and scope of India, given our strong links with traditional knowledge, we can combat the emerging threat of antibiotic resistance by advocating for localized production, better animal welfare, thus combing our philosophies of animal sentience along with the best of modern veterinary medicine from the west. Learning from countries such as Sweden and Netherlands we can aim to use antibiotics strictly where needed and thus take that first crucial step in a concerted effort towards combating antibiotic resistance.

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CORRIGENDUM

Research paper entitled "PROTECTIVE ROLE OF β-TOCOPHEROL AGAINST FLUBENDIAMIDE AND COPPER-INDUCED CHRONIC TOXICITY IN WISTAR RATS published in Dec. 2017/Vol.16/ Issue 2/18-27 of Journal of Veterinary Pharmacology and Toxocology, symbol 'α' should be read in place of symbol 'β' in title and whole running text in the paper. Inconvenience is deeply regreted.

INDIAN SOCIETY OF VETERINARY PHARMACOLOGY & TOXICOLOGY

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5. Membership fee along with Admission fee of Rs.20/- be sent by Bank Draft drawn in favour of Indian Society of Veterinary Pharmacology & Toxicology payable at the Central bank of India, Anand Branch. Personal cheques are not acceptable. The completed Application form along with the draft be sent to ***Dr. A. M. Thaker, Treasurer, ISVPT, Department of Pharmacology, Veterinary College, Anand Agricultural University, Anand-388001 (Gujarat).***

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Journal of Veterinary Pharmacology And Toxicology

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Bywater, R.J. (1991). Tetracycline. In Veterinary Applied Pharmacology and Therapeutics. 5th eds. Brander, G.C.. Puqh, D.M., Bywater, R.J. and Jenkins, W.L. pp. 467-473. Bailliere Tindall, London

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