



## Effects of GandhPaalashi (*Hedychium spicatum*) on the expression of hepatic genes associated with biotransformation, antioxidant and immune systems in WLH cockerels fed indoxacarb

GOVIND KUMAR CHOUDHARY<sup>1</sup>, SATYA PAL SINGH<sup>2</sup> and AMIT KUMAR<sup>3</sup>

GB Pant University of Agriculture and Technology, Pantnagar, Uttarakhand 263145 India

Received: 15 December 2017; Accepted: 29 January 2018

### ABSTRACT

The objective of the present study was to evaluate the efficacy of *Hedychium spicatum* (HSRP) root powder, to ameliorate changes in gene expression in WLH cockerels fed indoxacarb. After 16 weeks of treatment, all birds from different groups were sacrificed humanly and collected the liver pieces in RNA later for expression study by RT-PCR to evaluate changes in the expression of genes involved in antioxidant function [catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione S-transferase (GST)], biotransformation [cytochrome P450 1A1 (CYP1A1)], and the immune system [interleukins 6 (IL-6)]. Changes in gene expression were determined using the quantitative real-time PCR technique. There were significant upregulation of IL-6, CYP1A1 and GPx genes in indoxacarb treated groups. There was significant down regulation of CAT and SOD genes in indoxacarb treated groups in comparison to control. Simultaneous treatments with HSRP produce ameliorative effect and restore the gene activities at par with control. The current study demonstrates protective effects of HSRP on changes in expression of antioxidant, biotransformation, and immune system genes in cockerels fed indoxacarb.

**Key words:** Antioxidant, Biotransformation, Cockerels, GandhPaalashi, *Hedychium spicatum*, Hepatic genes, Indoxacarb, Immune system

Indoxacarb is a new oxidiazine group of pesticides used as acericides by inhibiting the sodium channels (Lapied *et al.* 2001). It produces toxicity by generating the reactive oxygen species (ROS) in tesis (Mudaraddi *et al.* 2012, Mudaraddi and Kaliwal 2009). Indoxacarb produces toxicities in rat by decline in blood level of FSH and progesterone whereas testosterone levels were increased (Nassar 2016). *Hedychium spicatum* also known as Shati and grown around the Himalayan region of Uttrakhand, Asom and Arunachal Pradesh. The plant contains glycosides, alkaloids, falavonoids, phenolics and other phytoconstituents (Thapliyal *et al.* 2014) and has potent antioxidant capacity (Sravani and Paarakh 2012, Choudhary and Singh 2017), hepatoprotective potency (Thapliyal *et al.* 2014) and had ameliorating potential in indoxacarb treated cockerels (Choudhary and Singh 2016). Several studies have reported that extract of *Hedychium spicatum* have hepatoprotective effect, but to date, no study has been published that reports on the beneficial effects of *Hedychium*

*spicatum* on hepatic gene expression of cockerels fed indoxacarb. Therefore, the objective of the present study was to evaluate the effects of HSRP, on the expression of hepatic genes involved in antioxidant function [catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), biotransformation [cytochrome P450 1A1] and the immune system [interleukin 6 (IL-6)] genes in cockerels fed indoxacarb.

### MATERIALS AND METHODS

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

**Experimental animals:** Forty nine white leghorn cockerels, 6 to 8 weeks old, weighing 300 to 400 g were procured from Instructional Poultry Farm, GBPUAT, Pantnagar and maintained under deep liter system following

Present address: <sup>1</sup>Subject Matter Specialist (Veterinary Science) (drgovindvet@yahoo.co.in), ICAR-KVK, Bhadohi, Uttar Pradesh. <sup>2</sup>Professor and Head (sppharma@rediffmail.com), Department of Pharmacology and Toxicology, College of Veterinary and Animal Sciences. <sup>3</sup>Scientist (SS) (vetamitchandan07@gmail.com), Division of Animal Genetics and Breeding, ICAR-IVRI, Izatnagar, Bareilly, Uttar Pradesh.

standard management practices. Experimental cockerels were randomly divided into seven groups of 7 birds each and were kept for 15 day for adaptation. Feed and drinking (tap) water were given *ad lib.* during the study.

Present study was conducted after approval by the Institutional Ethical Committee (IAEC) vide approval number IAEC/VPT/CVASC/193 dated. 27.02.2015.

Indoxacarb (Avaunt 15.8% EC) used in the study was purchased from local market. Chronic toxicities were induced in WLH cockerels by indoxacarb @ 25 mg/kg body weight. The dose of indoxacarb was selected on the basis of LD<sub>50</sub> in cockerels (250 mg/kg). The experimental plan is detailed in Table 1.

#### Expression of genes by RT-PCR

**Collection of liver tissue:** After 16 weeks of trial, each birds was sacrificed and immediately a small portion (about 2–3 g) of liver tissue was collected and kept in an RNA later® (Thermo Fisher Scientific). After collection of sample in 2 ml ependorff tube and to assure that the tissue cuts were properly dipped in RNA later and kept –20°C for further use.

**RNA isolation and cDNA synthesis:** RNA was isolated from liver tissues of birds by Trizol method. The liver tissues from each bird were grinded/ cut into small pieces and 700 µl Trizol reagent was added (Thermo Fisher Scientific) and

homogenized (Omni, International, USA). The homogenates were incubated for 5 min. Chloroform (200 µl) was added and mixed by vortexing. The tubes were incubated for 5–10 min at room temperature. Then the samples were centrifuged at 1,200 rpm for 15 min at 4°C. The different phases were separated and then, aqueous phases were removed by angling the tube at 4°C and pipetting these solutions into the new tubes. Isopropanol (500 µl) was added in the aqueous phase and mixed by inverting the tube in 5–10 times. Tubes were incubated for 10 min at room temperature and centrifuged the samples at 10,000 rpm for 5 min at 4°C. The supernatant were removed carefully and only saving the RNA pellets. The RNA pellets were washed with 1 ml of 75% ethanol by mixing it and further centrifuged the samples at 10,000 rpm for 15 min at 4°C. The supernatant were removed. The RNA pellets were air dried for 10 min. The RNA pellets were resuspended in 25 µl RNSase free water and incubated in water bath at 55–60°C for 10 min. The purity of the RNA was assessed by Nanodrop measuring the absorbance of RNA solution at 260 nm and 280 nm. The RNA samples showing the OD260:OD280 value in between 1.9–2.2 were considered as good quality and used for further analysis. The Reverse transcription of total RNA was carried out using Quanti Tect Reverse Transcription Kit for cDNA synthesis according to the manufacturer's instructions. The cDNA product was stored at –20°C. Real time RT-PCR was performed using ABI Prism 7500 real time machine (Applied Biosystem). GAPDH was used as an endogenous control. The primer sequences of genes used in the study are given in Table 2.

All the samples were run in triplicates. The amplification was carried out in 20 ml volume reaction mixture containing 10 ml of 2× master mix (Qiagen SYBR Green qPCR Master Mix), 1 ml (10 pmol) each of gene specific forward and reverse primer, 2 ml cDNA template and 6 ml nuclease free water. Negative control was included for the RT-qPCR assay. In negative control (NTC), cDNA was not added. The thermal profile used was as follows: 50°C, 2 min; 95°C for 15 min then 40 cycles of 95°C for 15 sec, AT for 30 sec, and 60°C for 1 min with fluorescence recording at the end of each cycle, followed by denaturation of products from

Table 1. Experimental plan

Group	No. of birds	Treatment
Group I (Control)	7	Standard ration <i>ad lib.</i>
Group II	7	Indoxacarb (250 ppm) in feed
Group III	7	Silymarin (250 ppm) in feed
Group IV	7	Indoxacarb (250 ppm) + Silymarin (250 ppm) in feed
Group V	7	<i>Hedychium spicatum</i> rhizome powder-(HSRP) @ 4000 ppm in feed
Group VI	7	HSRP @ 2000 ppm + indoxacarb @ 250 ppm in feed
Group VII	7	HSRP @ 4000 ppm + indoxacarb @ 250 ppm in feed

Table 2. Real time based analysis of transcript abundance

Name	Symbol	Primer	Product size
Catalase	CAT	GGGGAGCTGTTTACTGCAA-F TTTCCATTGGCTATGGCATT-R	139 bp
Cytochrome P4501A1	CYP1A1	CACTTTCTGCCTGCTCCTG-F GGTCCTTCCTCAGCTCCAG-R	125 bp
Glutathione peroxidase	GPx	TTGTAAACATCAGGGGCAA-F TGGGCCAAGATCTTTCTGTAA-R	140 bp
Superoxide dismutase	SOD	AGGGGGTCATCCACTTCC-F CCCATTGTGTTGTCTCCAA-R	122 bp
Glyceraldehyde-3 phosphate dehydrogenase	GAPDH	CCTCTCTGGCAAAGTCCAAG-F CAACATCAAATGGGCAGATG-R	128 bp
Interleukin-6	IL-6	GACTCGTCCGGAGAGGTTG-F CGCACACGGTGAACCTTCT-R	128 bp

65°C to 95°C with fluorescence recording throughout the step.

**Statistical analysis:** Expression level of genes were analyzed in six ways—control versus indoxacarb @ 250 ppm; control versus silymarin @ 250 ppm; control versus indoxacarb @ 250 ppm + silymarin @ 250 ppm; control versus HSRP @ 4000 ppm; control versus HSRP @ 2000 ppm + indoxacarb @ 250 ppm and control versus HSRP @ 2000 ppm + indoxacarb @ 250 ppm. The relative expression of each sample was calculated using the  $2^{-\Delta\Delta CT}$  method with control group as calibrator (Schmittgen and Livak 2008). One way ANOVA was done in JMP9 (SAS Institute Inc, Cary, USA) and differences between groups were considered significant at  $P < 0.05$ .

## RESULTS AND DISCUSSION

The expression of hepatic genes involved in antioxidant function [catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx)] is depicted in Figs 1, 2, and 3 respectively. The expression of biotransformation [cytochrome P450 1A1] and the immune system [interleukin 6 (IL-6)] genes is listed in Fig. 4 and 5 respectively.

**Antioxidant gene expression:** Antioxidant enzymes catalase (CAT) present within the peroxisomes and cytosolic GPx are involved in the conversion of hydrogen peroxide (a powerful and potentially harmful oxidizing agent) into water and molecular oxygen (Liska 1998). Hydrogen peroxide is unstable and forms hydroxyl free radicals; its break-down on the inner mitochondrial matrix and within the mitochondrial matrix targets. The decomposition of hydrogen peroxide outside the mitochondria may cause damage to the outer mitochondrial membrane itself which suggests that the breakdown of hydrogen peroxide to the hydroxyl radical is highly detrimental.

Compared with controls, the expression of the CAT gene was significantly ( $P < 0.05$ ) down regulated in indoxacarb treated group I and also down regulated in group III treated with silymarin + indoxacarb (Fig. 1). Down regulation in indoxacarb + HSRP high dose group VI was down regulated but it was four fold less than the group I treated with indoxacarb. This, finding suggested that the toxicity of indoxacarb suppressed the gene expression and lowered level of suppression in group III and VI which might be due to the ameliorating effect of silymarin and HSRP by the presence of antioxidants available for neutralizing the ROS/RNS generated inside body. In group II and IV treated with silymarin and HSRP, the expression was nonsignificantly up regulated in comparison to control. The present study on CAT in RBC and tissues is also in agreement with the CAT gene expression in liver tissue as CAT enzyme activity was also decreased in indoxacarb treated group (Choudhary and Singh 2017). This result was in agreement with findings of aflatoxin toxicity in broiler chick causing down regulation of CAT gene expression (Yarru *et al.* 2009).

Superoxide dismutase catalyses the conversion of superoxide anions to hydrogen peroxides and is one of the

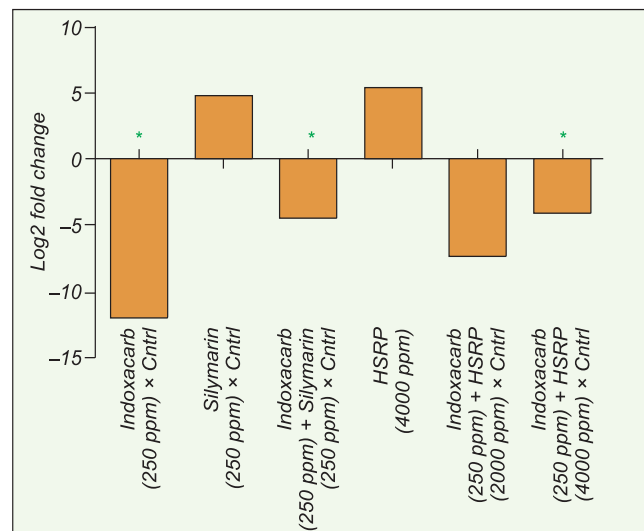


Fig. 1. Log2 fold changes in gene expression of catalase (CAT) gene following administration of HSRP in feed for 16 weeks in indoxacarb treated WLH cockerels.

primary enzymatic defence system against ROS. The hepatic gene expression of SOD in comparison to control was significantly ( $P < 0.05$ ) down regulated in group I, III, V and VI (Fig. 2). The indoxacarb treated group I showed suppression of SOD genes which was 4/3 fold more than the group III and VI. Group II and IV showed up regulation of SOD gene in comparison to control. This finding suggest that the SOD gene was suppressed by indoxacarb and caused down regulation due to the toxicity produced by the indoxacarb but simultaneous feeding of silymarin in group III and HSRP in group V and VI ameliorated the toxicity produced by the indoxacarb and led to up regulation in V and VI groups in comparison to group I down regulation.

Glutathione peroxidase gene expression was significantly up regulated in indoxacarb treated group in comparison to control (Fig. 3). This finding also corroborated with finding of Choudhary and Singh (2017). In group II and group IV, GPx gene was down regulated that might be due to antioxidant action of silymarin and HSRP which combat the ROS/ RNS generated by the indoxacarb. In group III, V and VI, the GPx gene was up regulated in comparison to control but less than the Group I treated with indoxacarb. This might be due to amelioration by simultaneous feeding of silymarin and HSRP in dose dependent manner which directly or indirectly influenced the GPx gene expression.

**Expression of biotransformation genes (CYP1A1):** The main hepatic transformation processes include xenobiotics biotransformation through phase I metabolism and further by the conjugation of the resulting metabolites to convert them into more water soluble conjugates which are rapidly readily removed from the body. The microsomal cytochrome P450 (CYP)- dependent monooxygenase system in the liver plays an essential role in phase I metabolism (Akahori *et al.* 2009). The CYP enzymes are associated with several biological interaction involving

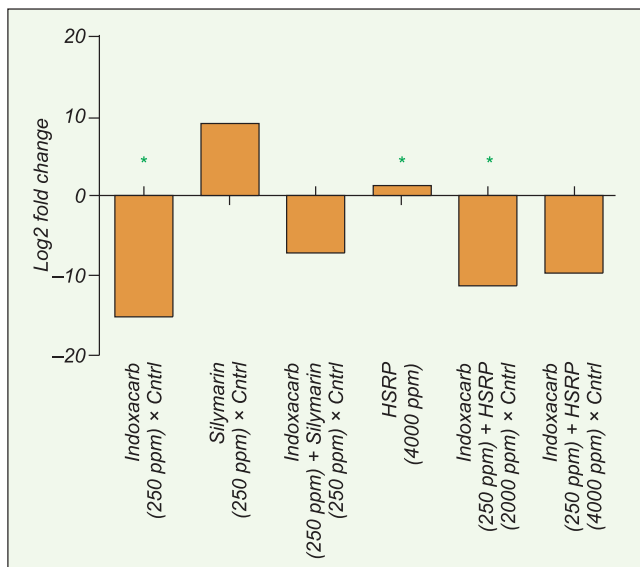


Fig. 2. Log2 fold changes in gene expression of catalase (CAT) gene following administration of HSRP in feed for 16 weeks in indoxacarb treated WLH cockerels.

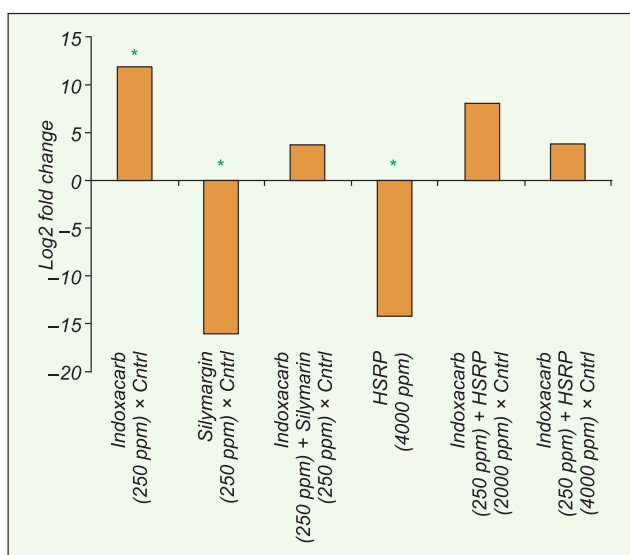


Fig. 3. Log2 fold changes in gene expression of GPx gene following administration of HSRP in feed for 16 weeks in indoxacarb treated WLH cockerels.

hydroxylation, epoxidation, oxygenation, dehydrogenation, nitrogen dealkylation and oxidative deamination (Bruton *et al.* 2011). Cytochrome P450 mediated reactions can also generate ROS. The CYP enzymes are involved in hepatic metabolism of indoxacarb in poultry and other enzymes like AST, ALT, GGT and ALP were elevated in indoxacarb treated poultry and feeding of *H. spicatum* root powder ameliorated the enzymic activities towards normal (Choudhary and Singh 2017).

The hepatic CYP1A1 gene was up regulated in indoxacarb treated group in comparison to control (Fig. 4). Overexpression of these CYP isoforms has been shown to induce chronic oxidative stress by generating more ROS/

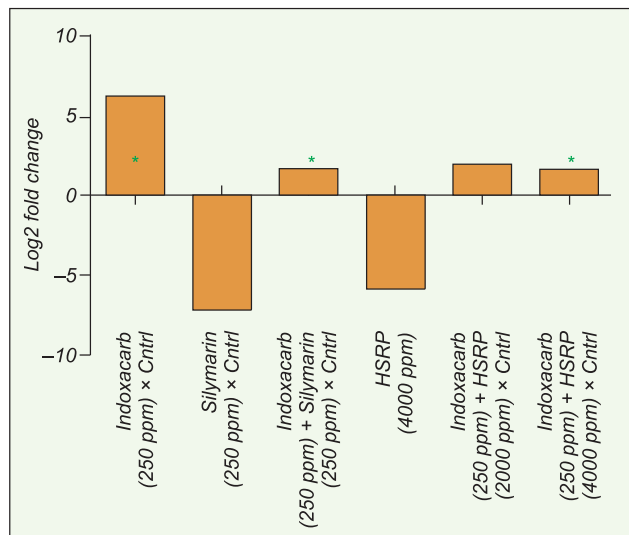


Fig. 4. Log2 fold changes in gene expression of CYP1A1 gene following administration of HSRP in feed for 16 weeks in indoxacarb treated WLH cockerels.

RNS, leading to hepatocellular injury (Kumar and Kuttan 2006). In group II and IV treated with silymarin and HSRP alone, the CYP1A1 gene was down regulated which might be due to suppression of the gene but in group III, V and VI treated with indoxacarb+silymarin and HSRP+indoxacarb with low and high doses, CYP1A1 was up regulated in comparison to control but less than the indoxacarb treated group, which indicated the ameliorative effect of silymarin and HSRP in dose dependent manner. The overexpression of the CYP1A1 gene was also reported by Kumar and Kuttan (2006) following aflatoxin toxicity in broiler chick.

*Expression of cytokine gene (IL-6):* Interleukins form a group of cytokines that are important components of the immune system. They play vital role and are now well recognised in inflammation and a pathological condition in systemic inflammatory states. Any imbalance in cytokine production and dysregulation in circulating cytokines could result into various pathological disorders (Tayaland Kalra 2007).

The IL-6 is a proinflammatory cytokine. The IL-6 gene was significantly ( $P < 0.05$ ) upregulated in indoxacarb treated group in comparison to control. This might be due to the excessive generation of free radicals which damages the cells leading to release of IL-6 and over expression of IL-6 gene (Fig. 5). IL-6 gene was down regulated in group II and IV treated with silymarin and HSRP. In group III, V and VI, the IL-6 genes was significantly ( $P < 0.05$ ) upregulated in comparison to the control but was less than the indoxacarb treated groups. Reduction in up regulation of IL-6 in group V and VI might be due to scavenging the free radicals by HSRP in dose dependent manner by its anti-inflammatory properties (Kunnumakkara *et al.* 2008). Indoxacarb damages the spleen showed moderate depletion of lymphoid cell from white pulp, vacuolation of the tunica media/ tunica muscularis of the many large and small blood vessels of arterial side; hemosiderosis were also seen noticed

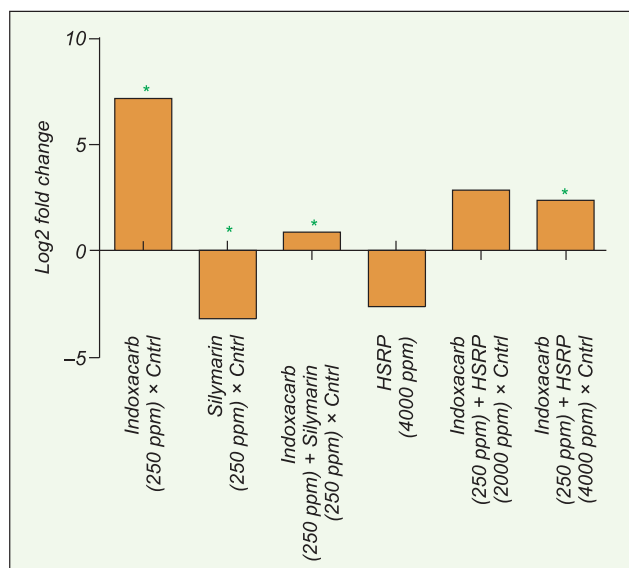


Fig. 5. Log<sub>2</sub> fold changes in gene expression of IL-6 gene following administration of HSRP in feed for 16 weeks in indoxacarb treated WLH cockerels.

but simultaneous feeding of *H. spicatum* rhizome powder the lesion become mild to moderate (Choudhary *et al.* 2017).

Thus it is concluded from the present investigation that the *Hedychium spicatum* produces amelioration on long term intoxication with indoxacarb in WLH cockerel by the up regulation and down regulation of different hepatic genes involved in antioxidant function [catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx)], biotransformation [cytochrome P450 1A1] and the immune system [interleukin 6 (IL-6)] in cockerels fed indoxacarb.

#### ACKNOWLEDGEMENT

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

#### REFERENCES

Akahori M A, Takatori S Itagaki and Yoshikawa Y. 2005. No regional differences of cytochrome P450 expression in the liver of cynomolgus monkey (*Macaca fascicularis*). *Experimental Animal* **54**: 131–36.

Bruton L, Chabner B A and Knollman B. 2011. Goodman & Gillman The pharmacological basis of therapeutics. 12<sup>th</sup> edn. McGraw Hill, New Delhi. pp. 27–28.

Choudhary G K and Singh S P. 2016. Ameliorative efficacy of *Hedychium spicatum* root powder in indoxacarb intoxicated WLH cockerels. *Journal of Veterinary Pharmacology and Toxicology* **15**(2): 61–64.

Choudhary G K and Singh S P. 2017. Chronic toxicity of indoxacarb and its amelioration by rhizome powder of *Hedychium spicatum* in WLH cockerels. *Toxicology International* **24**(2): 203–10.

Choudhary G K and Singh S P. 2017. Ameliorating potential of *Hedychium spicatum* on oxidative stress following chronic exposure to indoxacarb in WLH cockerels. *Indian Journal of Animal Research* **51**(5): 832–36.

Choudhary G K, Singh S P and Batra M. 2017. Ameliorative effect of rhizome powder of Gandhpalasi (*Hedychium spicatum*) in indoxacarb intoxicated WLH cockerels. *Indian Journal of Veterinary Research* **26**(2): 37–41.

Kumar H K B and Kuttan R. 2006. Inhibition of drug metabolising enzymes (cytochrome P450) *in vitro* as well as *in vivo* by *Phyllanthus amarus* Schum and Thonn. *Biological Pharmacological Bulletin* **29**: 1310–13.

Kunnumakkara A B, Ichikawa H, Anand P, Mohankumar C J, Hema P S, Nair M S and Agrawal B B. 2008. Coronarium D, a labdanoid diterpene, inhibits both constitutive and inducible nuclear factor-kappa B pathway activation, leading to potentiation of apoptosis, inhibition of invasion and suppression of osteoclastogenesis. *Molecular Cancer Therapeutics* **7**(10): 3306–17.

Lapied B, Grolleau F and Sattelle D B. 2001. Indoxacarb, an oxadiazine insecticide, blocks insect neuronal sodium channels. *British Journal of Pharmacology* **132**: 587–95.

Liska D J. 1998. The detoxification enzyme system. *Alternative Medical Review* **3**: 187–98.

Mudaraddi T Y and Kaliwal B B. 2009. Indoxacarb induces testes oxidative stress in Swiss Albino Mice. *International Journal of Biotechnology Research* **1**: 40–48.

Mudaraddi T Y, Potadar R R and Kaliwal B B. 2012. Indoxacarb induces liver oxidative stress in Swiss Albino mice. *European Journal of Experimental Biology* **2**(1): 180–86.

Nassar A M K. 2016. Comparative endocrine disrupting effects of abamectin and indoxacarb insecticides. *International Journal of Pharmacology and Toxicology* **4**(1): 89–92.

Schmittgen T D and Livak K J. 2008. Analyzing real-time PCR data by the comparative C(T) method. *Natural Protocols* **3**(6): 1101–08.

Settivari R S, Bhusari S, Evans T E, Eichen P A, Hearne L B, Antoniou E and Spiers D E. 2006. Genomic analysis of the impact of fescuetoxicosis on hepatic function. *Journal of Animal Science* **84**:1279–94.

Sravani T and Paarakh P M. 2012. Antioxidant property of *Hedychium spicatum* Buch.- Ham. rhizomes. *Indian Journal of Natural Product and Resources* **3**(3): 354–58.

Tayal V and B S Kalra. 2007. Cytokines and anti-cytokines as therapeutic- an update. *European Journal of Pharmacology* **579**: 1–12.

Thapliyal S, Juya V and Bhandari A. 2014. Pharmacognostic screening of *Hedychium spicatum* rhizomes. *International Journal of Pharmaceutical and Chemical Sciences* **3**(4): 483–88.

Thapliyal S, Juyal V and Bhandari A. 2014. Hepatoprotective and antioxidant activity of ethanol extract of *Hedychium spicatum* against CCl<sub>4</sub>-induced liver injury in rats. *Research Journal of Pharmaceutical Biology and Chemical Sciences* **5**(2): 1428–37.

Yarru L P, Settivari R S, Gowda N K S, Antoniou E, Ledoux D R and Rottinghaust G E. 2009. Effects of turmeric (*Curcuma longa*) on the expression of hepatic genes associated with biotransformation, antioxidant and immune systems in broiler chicks fed aflatoxin. *Poultry Science* **88**: 2620–27.