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Elucidation of Immunomodulating Potential of *Morus alba* against Sub Acute Exposure of Fipronil in Rats

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ABSTRACT

Immunotoxicity is defined as adverse effects on the functioning of the immune system that results from exposure to chemical substances. The present investigation was carried out to evaluate immunomodulating effect of *Morus alba* (500mg/kg B.w.) against immunotoxicity induced by sub-acute exposure of Fipronil (10mg/kg B.w.) in rats. Sub-acute immunotoxicity was conducted in adult male wistar rats. Rats were randomly divided into four groups (6 rats/group). Group I served as control in which corn oil (acting as a vehicle of Fipronil) was administered @10 ml/kg B.w. Group II served as Fipronil treated group @10 mg/kg B.w. In Group III Fipronil along with *Morus alba* fruits extract @ 300 mg/kg B.w. was administered and in Group IV *Morus alba* fruits extract @ 300 mg/kg B.w. was administered. Vehicle, Fipronil and *Morus alba* were administered daily to the rats by oral gavage for 28 days. The dose of fipronil was selected on the basis of LD_{50} in rats. TLC, DLC, serum total protein, albumin, globulin, A:G ratio, serum antibody titer/haemagglutination (HA) titer and delayed type hypersensitivity (DTH) response were estimated. Fipronil produced immunotoxicity in the form of alteration from normal values in these parameters. *Morus alba* was significantly effective in restoration of these parameters towards normal**.** The study suggested that *Morus alba* has immunomodulating potential against toxicity induced by fipronil in rats.

Keywords: Immunotoxicity, fipronil, *Morus alba*, rat

The wide spread use of pesticide in agriculture, veterinary, horticulture and public health for the control of pests such as insects and rodents, disease organisms and disease vectors poses great risk and hazard to human and animal health. It has been observed that the pesticides exposure are increasingly linked to immune suppression, genetic abnormality, hormone disruption, diminished intelligence, reproductive abnormalities and cancer. The importance of pesticides in India can be understood from the fact that agriculture is a major component of the Indian economy.

Phenylpyrazole insecticides are a class of chemically-related broad-spectrum insecticides. The phenylpyrazole derivatives show extensive biological activity, including insecticidal, herbicidal, and miticidal properties. Fipronil is phenylpyrazole insecticide which is active ingredient of one of the popular ectoparasiticides products Frontline™. The principle mechanism of fipronil in both insects and mammals is non-competitively inhibition of γ-aminobutyric acid (GABA)-induced ion influx by targeting the GABA-regulated chloride channels which lead to hyperexcitation of CNS, paralysis, convulsion and death.

The immune system play important role in maintenance of health status of animal and human. It can be the target of many chemicals, xenobiotics, with potentially severe adverse effects such as immunosuppression. In recent years the effects of pesticides on immune response have received great attention. It is now clear that important changes in host immunity may occur after pesticide ingestion.

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The adverse effects caused by the pesticides on the immune system can be reversed by the use of herbal immunomodulatory agents. These agents may prevent the damage caused by pesticides to the immune system by their immune stimulant properties and thus can be an area of interest for further research.

In the ancient times, the Indian sages believed that medicinal herbs are one-stop solutions to cure a number of health related problems and diseases. Many herbs are used to alter or change a long-standing condition by eliminating the metabolic toxins. Plants as a source of medicinal compounds have continued to play a crucial role in the maintenance of human as well as animal health.

White mulberry (*M. alba*) is belonging to Moraceae family & also known as, silkworm mulberry and commonly called as *shahtoot* in Hindi. The mulberry leaves are used as infusion in Asian countries most common in Japan and Korea. This is due to the presence of steroids, flavonoids, amino acids, vitamins, triterpenes and other trace elements which show valuable effects (Shoaib *et al.,* 2013). Mulberry leaves contained prenylflavanes and prenylflavane glycoside, quercetin 3-O-β-D glucopyranosyl-(1/6)-β-D-glucopyranoside and quercetin which are having antioxidant property. The shahtoot leaves also possess antimicrobial, hepatoprotective activity (Singh *et al.,* 2013).

The ameliorating potential of extracts of *M. alba* leaves against insecticide induced toxicity is scarcely available in the literature. Keeping these things in view, it is essential to explore the immunomodulatory potential of this plant against fipronil so the present work was undertaken with the objective to evaluate immunomodulatory potential of the extracts of *Morus alba* following immunotoxicity induced by sub-acute exposure of fipronil in rats.

MATERIALS AND METHODS

Experimental animals

The study was conducted in wistar rats weighing 120-140 g procured from Rajendra Bird Park, Meerut (U.P.). Twenty four rats were housed in polyacrylic cages in groups of six animals per cage in Department of Veterinary Pharmacology and Toxicology, College of Veterinary and Animal Science Navania, Udaipur and maintained under controlled conditions of temperature (22-25°C), humidity (40-60%), and light (12/12-hour light/dark cycle). All the rats were acclimatized for one week prior to initiation of experiment. Standard feed and water were provided *ad libitum* and bedding material (wheat straw) was changed on alternate days. All procedures employed in this study were approved by the institutional animal ethical committee (IAEC).

Collection of plant materials and extraction

Leaves of *Morus alba* (Fig. 1) was procured from Vallabhnagar village, CVAS campus Navania and was authenticated by Department of Horticulture, Rajasthan College of Agriculture, MPUAT, Udaipur (Raj).

(a) **(b)**

Fig. 1(a): Leaves of *Morusalba* **(b)** dried leaves of *Morus alba*

Morus alba leaves were air-dried in shed at room temperature (26°C) for 2 weeks and then grinded to a uniform powder. Methanolic extracts were prepared by soaking 480g of the dried powdered leaves in 1 litre of 70% methanol in sterilized measuring cylinder wrapped with aluminium foil at room temperature for one week in dark place. The extracts were filtered first through muslin cloth and they were concentrated by using a rotary vacuum evaporator with the hot water bath set at 40°C (Khan *et al*., 2013).

The yield % of *Morus alba* leaves extract was 5.84%.

Yield (%) of extracts was calculated as below:

Yield % = (weight of dried extract × 100) / (weight of original sample)

Experimental design

The dose of fipronil was selected on the basis of its oral LD_{50}. The oral LD_{50} of fipronil technical grade in rats was reported to be 97mg/kg b.w. (California Environmental

Protection Agency, 2001). The dose of fipronil for the 28 days subacute toxicity study was 10 mg/kg b.w. ($1/10^{th}$ of LD_{50}). *Morus alba* leaves extract was given at the dose rate of 500mg/kg b.w. based on previous literature (Govinda and Asdaq, 2011; Sabsung *et al.,* 2004; Giribabu *et al.,* 2014).

Clinically healthy rats were divided into four groups (six rats per group) and were treated with fipronil and/or *Morus alba* leaves extract. Corn oil was used as vehicle for fipronil and *Morus alba* leaves extract was dissolved in normal saline. The different rats group and their respective doses are mentioned in the Table 1. Each dose was adjusted according to their body weight. Rats in the group III were pre-treated with *Morus alba* leaves extract 2 hour before administering dose of fipronil (i.e. 10 mg/kg). The whole experimental procedures were followed as per OECD guideline number 407 of 2008 (OECD, 2008). The experimental schedule is mentioned in Table 1.

Table 1: Experimental schedule

Groups	Treatment	No. of rats	Dose (Oral) (mg/kg b.w.)	Period of exposure
I	Control (corn oil)	6	10 ml/kg	28
II	Fipronil toxicity	6	10 mg/kg	28
III	Fipronil toxicity + extract of *Morus alba*	6	10 mg/kg + 500 mg/kg	28
IV	Extract of *Morus alba*	6	500 mg/kg	28

Immunotoxicity assay

Total leucocytes count (TLC), differential leucocytes counts (DLC), serum total protein, albumin, globulin and A : G ratio were estimated using haematology auto analyzer (Mindray BC-2800 vet Mahwah-USA).

Evaluation of humoral immune response by serum antibody titer/haemagglutination (HA) titer

Sheep blood was collected and mixed with Alsever's solution in proportion of 1:1. Sheep blood (in Alsever's solution, about 4 ml) was washed thrice in Dulbecco's phosphate buffer saline (DPBS), at 1500 rpm/ 10 min. From final RBC pack, 1:10 and 1:100 dilutions were prepared for the purpose of counting RBCs. Final volume of RBCs was adjusted 5×10^9 cells/ml. For evaluation of HA titre, rats from various group were immunized by i.p. injection of SRBCs (0.5×10^9 cells/rat, 100µl/rat) in saline seven days before the completion of treatment period (Shukla *et al.,* 2009). At the end of experiment (day 29 for fipronil treatment), sera was prepared from peripheral blood sample of each group of immunized rats and decomplemented by heating at 56°C for 30 minutes. The microtitre HA technique was employed to determine the serum antibody titer.

Evaluation of cell mediated immune response by delayed type hypersensitivity (DTH) response

On day 18^{th} of the exposure period rats were sensitized by s/c injection of 50 µl SRBCS suspended in Freund's complete adjuvant (FCA) on their back by using l ml plastic tuberculin syringe fitted with 26 G needle. SRBCs were separated from blood as mentioned above. To prepare SRBC-FCA emulsion, equal volume of SRBC and FCA (2.5 ml of each, sufficient to be injected for rats) was taken. After 10 days (i.e. on day 28), sensitized rats were challenged by injecting (1.5×10^9 cell/ml) 100 µl of SRBCs in right hind foot pad with the help of 26 G needle mounted on tuberculin syringe. Before injecting SRBCs in foot pad rats were lightly anaesthetized and skin thickness was recorded. Swelling in the right hind foot pad was measured by digital vernier calliper, 24, 48 and 72 hours after challenge. Histopathology of foot pad was also performed to study cellular changes.

Statistical analysis

Statistical differences between respective means for various parameters were evaluated by using SPSS software. Comparison among the treatment groups were made by using one way ANOVA with Duncan multiple comparisons as post hoc test. All p values ($p<0.05$) were considered to be statistically significant.

RESULTS AND DISCUSSION

Effect of extract of *Morus alba* on TLC and DLC against toxicity induced by sub-acute exposure of fipronil in rats are presented in Table 2. TLC and lymphocyte levels were found to be significantly ($p<0.05$) decreased in fipronil treated groups while significantly ($p<0.05$) higher

Table 2: Effect of extract of *Morus alba* on TLC and DLC against toxicity induced by sub-acute exposure of fipronil in rats

Groups	TLC($\times10^3$µl)	Lymphocyte (%)	Monocyte (%)	Neutrophil (%)	Basophils (%)	Eosinophil (%)
I	8.37±0.33^a	76.11±0.11^a	2.09±0.02^c	19.91±0.07^c	0.22±0.005^b	1.61±0.09^b
II	5.56±0.06^c	65.44±0.58^c	5.29±0.14^a	26.80±0.52^a	0.28±0.003^a	2.03±0.02^a
III	6.91±0.15^b	72.72±0.43^b	3.26±0.11^b	22.09±0.35^b	0.24±0.010^b	1.54±0.02^b
IV	8.34±0.23^a	76.16±0.13^a	2.08±0.02^c	19.86±0.09^c	0.22±0.005^b	1.60±0.02^b

Values are (Mean±SE; n=6) bearing different superscripts within a column do not differ significantly ($p<0.05$). One-way ANOVA followed by Duncan new multiple range test post-hoc comparison was performed for the studied parameters.

percentage values for monocytes, neutrophils, basophils and eosinophils as compared to control group were found. Significant restoration of TLC and percentage values for monocytes, neutrophils, basophils and eosinophils in group III was observed as compared to group II. Groups IV observed non significant alteration in TLC and DLC count and were comparable to control.

The immune suppressive nature of fipronil is further confirmed by significant alteration in TLC and DLC values. In the present investigation, leukopenia and lymphocytopenia was observed while monocyte, neutrophil, basophil, eosinophil count was increased in fipronil treated group. These results were in agreement with the results of Basir *et al.* (2011) who reported administration of lambda-cyhalothrin to rabbits caused significant decrease in white blood cell and lymphocyte count, while neutrophils, monocytes and eosinophils were increased. In broiler chicks, leukopenia was recorded after cypermethrin administration (Sharaf *et al.,* 2009). Anand *et al.* (2016) also reported decrease in TLC and lymphocyte count in acetamiprid treated group which reflected its immunosuppressant effect.

Effect of extract of *Morus alba* on total protein, albumin, globulin & A : G ratio against toxicity induced by sub-acute exposure of fipronil in rats are presented in Table 3. There was significant ($p<0.05$) decrease in total protein, albumin & globulin in fipronil treated groups as compared to control. In group III the values were significantly increased in comparison to group II. Non significant alteration in total protein, albumin and globulin were observed in group IV in comparison to control. A: G ratio was lower in fipronil toxicity group in comparison to control the values were towards restoration in group III. The values in group IV were comparable to control.

In the present study, serum proteins (total protein, albumin, and globulin) were significantly decreased in fipronil treated group. These results indicated immunosuppressive effect of fipronil. These results were in agreement with the findings of Doltade (2012) who also observed that daily oral administration of acetamiprid caused significant decrease in total protein and albumin levels in male rats. Decrease in the level of serum proteins could be due to the increase in protein metabolism and decrease in protein synthesis.

Table 3: Effect of extract of *Morus alba* on total protein, albumin, globulin & A : G ratio against toxicity induced by sub-acute exposure of fipronil in rats

Groups	Total Protein (g/dl)	Serum Albumin (g/dl)	Serum globulin (g/dl)	A : G
I	7.12±0.11^a	4.63±0.05^a	2.48±0.07^a	1.86±0.05^a
II	3.37±0.06^d	2.13±0.04^d	1.23±0.02^d	1.73±0.03^a
III	4.83±0.03^c	3.07±0.06^c	1.77±0.04^c	1.74±0.07^a
IV	7.17±0.08^a	4.60±0.06^a	2.32±0.08^a	1.85±0.08^a

Values are (Mean±SE; n=6) bearing different superscripts within a column do not differ significantly ($p<0.05$). One-way ANOVA followed by Duncan new multiple range test post-hoc comparison was performed for the studied parameters.

In the present study total protein, albumin, and globulin were restored by fipronil co treatment groups. The findings were in agreement with the result of Gupta *et al.* (2014) who also reported that *Morus alba* significantly restored total protein level in CCl_4 induced toxicity in rats.

Effect of administration of fipronil alone and in combination with extract of *Morus alba* in rats on haemagglutination titer against sheep RBCs in terms of log 2/0.05 ml are presented in Table 4. In fipronil treated group HA titer was significantly ($p<0.05$) decreased as compared to control.

In group III there was significant increase in HA titer as compared to fipronil treated group and was towards amelioration. Group IV was comparable to control.

Table 4: Effect of extract of *Morus alba* on Serum antibody titer/Haemagglutination (HA) titer against toxicity induced by sub-acute exposure of fipronil in rats

Groups	Log 2 Antibody titer
I	7.10±0.24[a]
II	3.23±0.30[c]
III	5.21±0.23[b]
IV	7.03±0.20[a]

Values are (Mean±SE; n=6) bearing different superscripts within a column do not differ significantly ($p<0.05$). One-way ANOVA followed by Duncan new multiple range test post-hoc comparison was performed for the studied parameters.

The present investigation further confirmed immunotoxic effect of fipronil by decrease in the haemagglutination titer against SRBCs antigen as compared to control. The results were in agreement with the findings of Badgujar *et al.* (2013) who reported subacute exposure to imidacloprid in Balb/C mice reduced anti-SRBC antibody titer.

T-helper (TH) cells are involved in the production of B-cell responses leading to generation of antibodies against T-dependent antigens such as SRBC. In the present findings suppression of HA titer with fipronil treatment could be attributed to the impairment of TH cell activity. HA titer demonstrated that fipronil induce severe impairment in T and B cell activity where their ability to respond to SRBC antigen was remarkably decreased.

The plant extracts co administered with fipronil produces protective effect by restoring all classical indices related to immune system which are discussed above. In the present investigation, leaves extract of *Morus alba* significantly restored haemagglutination titer as compared to fipronil treated group. Similarly, Venkatachalam *et al.* (2009) observed that aqueous leaves extract of *Morus alba* increased DTH response but no significant changes was observed in haemagglutination titer against SRBC antigen at dose of 200 mg/kg b.w. and 400 mg/kg b.w. in rats. They stated that aqueous leaves extract of *Morus alba* stimulate cell mediated immune response but not humoral immune response. Bharani *et al.* (2010) similarly elucidated that methanolic leaves extract of *Morus alba* increased the levels of serum immunoglobulins and circulating antibody titre in indirect haemagglutination test, phagocytic index in carbon clearance assay in rats and they stated that *Morus alba* increased both humoral and cell mediated immunity.

Immunosuppressive effect of fipronil was further assessed by measurement of DTH response as footpad thickness in rats. DTH response is one of the best and well validate test for assessment of cell mediated immune response. In the present study DTH response was decreased after 24, 48 and 72 hour as compared to control. Similar results were reported by Kumar *et al.* (2012) in cypermethrin toxicity in mice and Anand *et al.* (2016) in acetamiprid toxicity in rats.

Delayed type hypersensitivity (DTH) response to SRBCs was characterized by intense local inflammatory reaction with edema, erythema, vesiculation and swelling in control rats. In fipronil treated group only mild inflammatory reaction with mild edema and erythema was observed. Group III and IV showed erythema, vesiculation and swelling of foot pad.

Effect of extract of *Morus alba* on delayed type hypersensitivity (DTH) response (foot pad thickness at a given time point) against toxicity induced by sub-acute exposure of fipronil in rats is presented in Table 5.

Table 5: Effect of *Morus alba* on Delayed type hypersensitivity (DTH) response (foot pad thickness in mm) after subacute exposure of fipronil in rats

Groups	24 h later	48 h later	72 h later
I	1.49±0.04[a]	2.04±0.04[a]	1.83±0.03[a]
II	1.02±0.03[c]	1.11±0.02[c]	0.83±0.02[c]
III	1.26±0.03[b]	1.23±0.03[b]	1.19±0.03[b]
IV	1.47±0.02[a]	2.04±0.02[a]	1.81±0.02[a]

Values are (Mean±SE; n=6) bearing different superscripts within a column do not differ significantly ($p<0.05$). One-way ANOVA followed by Duncan new multiple range test post-hoc comparison was performed for the studied parameters.

The foot pad thickness was significantly decreased ($p<0.05$) in fipronil treated group after 24 h, 48 h and 72 h as compared to control. In group III foot pad thickness was significantly increased as compared to group II. In groups IV foot pad thickness was comparable to control. The result was in agreement with the observations of Anand

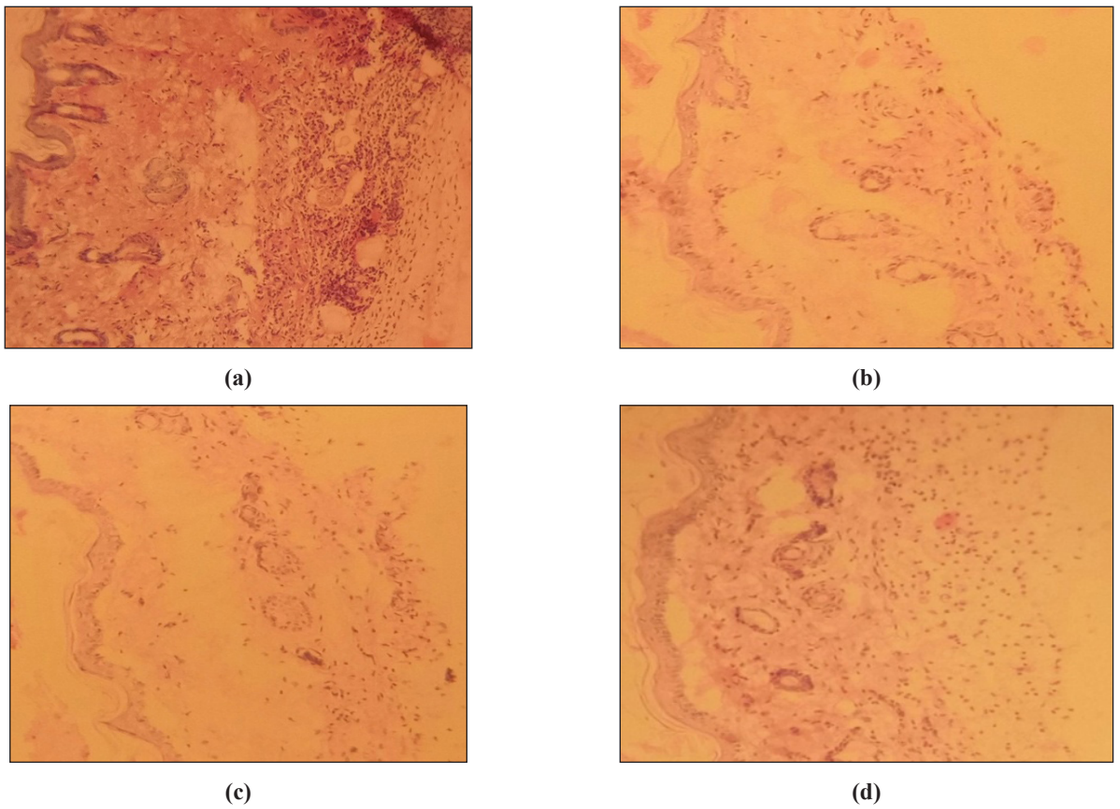

Plate 1: Photomicrograph of right hind foot pad of rats tested for DTH reaction after 72 hours. **(a)** vhicle control- severe infiltration of mononuclear cells (H&E×100). **(b)** fipronil treated group- very few infiltration of mononuclear cells (H&E×100). **(c)** fipronil co treatment *M. alba* extract showed moderate inflammatory response (H&E×100). **(d)** *M. alba* extract exhibit severe infiltration of mononuclear cells (H&E×100)

et al. (2016) where there was significant increase in foot pad skin thickness in Panax treated group as compared to Acetamiprid treatment group alone in the study reflecting the immune restoration of Panax ginseng.

Effect of extract of *Morus alba* on histopathology of footpad against toxicity induced by sub-acute exposure of fipronil in rats is presented in Plate 1. The footpad section of control rats revealed a severe inflammatory reaction characterized by the presence of a large number of mononuclear cells (macrophages and lymphocyte). Footpad section of fipronil treated group exhibited very mild infiltration of mononuclear cells, suggesting decrease in severities of inflammatory reaction. Fipronil co treatment with *M. alba* significantly increased population of inflammatory cells. Inflammatory reaction in *M. alba* group was comparable to control.

Histopathological evaluation of footpad section of rats tested for DTH reaction revealed significant cellular or pathological alterations. Comparative histological evaluation of food pad sections from vehicle control and fipronil treated group confirmed the gross symptoms of DTH reaction and the decreases in paw thickness seen in fipronil treated group. Significant reduction in DTH response to SRBC (T-cell dependent antigen) often is indicative of decrease in cell-mediated immunity. Alterations in the magnitude of DTH reaction, symptomatically and/or at a histologic level, are usually indicative of an impairment of Th1 effector cells (Badgujar *et al.,* 2013). Th1 effector cells (also termed TDTH cells) are responsible for the DTH reaction. Specifically, following interaction with a specific antigen, the Th1 cells produce cytokines that invoke mononuclear cell infiltration, mononuclear cell interaction, and increased vascular permeability in the vicinity of

stimulus. Histopathological findings in the present study indicating reductions in mononuclear cell involvement at the injection site (foot pad) and a generalized lower inflammatory response could be explained by fipronil-induced cytotoxic effect on Th1 cells. Hence, results of important indices of immune system assessed in this study emphatically demonstrated suppression of humoral and cellular immunity after subacute exposure to fipronil.

CONCLUSION

Thus it was concluded that extract of *Morus alba* has the potential to combat immunotoxicity caused by subacute exposure to fipronil in rats.

Conflicts of interest: The authors declare that there is no conflict of interest in publication of this research paper.

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