Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

E-ISSN: 2320-7078
P-ISSN: 2349-6800
JEZS 2020; 8(1): 505-510

Received: 08-11-2019
Accepted: 12-12-2019

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Ameliorative effect of hydro-ethanolic extract of *Tinospora cordifolia* against methotrexate induced toxicity in mice

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Abstract
Present study conducted on ameliorative effect of *Tinospora Cordifolia* against Methotrexate induced toxicity in mice on the thirty-two either sex mice having 4-5 weeks of age with approximately 20-30 gm body weight or divided into four equal groups. During experiment, mice showed dullness, depression, lethargy and loss of decreased spontaneous activity and body weight like clinical sign and symptoms. Significant decreased in body weight, body weight gain, feed consumption and significantly increased in relative organ weight of liver were recorded in methotrexate treated group. Hematological finding revealed significant decreased ($P \leq 0.05$) in Hb, PCV, TEC, TLC and lymphocyte count were also observed in methotrexate treated group. The mild to moderate intensive lesions were observed in clinical sign, organ weight and hematological findings in *Tinospora cordifolia* treated group. It was revealed that supplementation of hydro-ethanolic extract of *Tinospora cordifolia* through oral route may reduce the severity of methotrexate toxicity in mice.

Keywords: General parameter, methotrexate, *Tinospora cordifolia*, mice

Introduction
For the last 40 years, chemotherapy has a key role in cancer management in human and animal patients. In human medicine, more than 50 anticancerous agents are present However; very few of them are used in Veterinary medicine as cancer is diagnosed at a very late stage 4. In India, cancer cases or its incidence estimated to grow by 25 per cent by 2020 in humans (Indian Council of Medical Research). Cancer is the uncontrolled, abnormal growth of cells or tissues in the body which proliferates continuously without control. In cancer treatment, chemotherapy is one of the most widely used therapy. Chemotherapeutic agents have a cytotoxic effect on cancer cells as well as normal cells of the body. Methotrexate (MTX) is primarily used in the veterinary field to treat the animal patients that may be suffering from certain neoplastic diseases like lymphoma and osteosarcoma [6]. MTX is also used to treat the other susceptible solid tumors, psoriasis cases and certain inflammatory disease [5]. MTX is used to treat rheumatoid arthritis, autoimmune diseases, mammary tumor, Cancer of invasive urinary bladder cancer and used in veterinary oncology [25]. Chemically MTX is (2*S*)-2-[[4-[(2, 4-diaminopteridin-6-yl) methyl-methylamino] benzoyl] amino] pentanedioic acid having chemical formula $C_{20}H_{22}N_8O_5$. It is a derivative of aminopterin and interferes with the metabolism of folic acid. MTX is specific for the S phase cell cycle and inhibits the enzyme, dihydrofolate reductase. The use of MTX is decreasing due to its side effects like hepatotoxicity [35], severe side-effects on the hematopoietic system [36] and liver enzymes. MTX is primarily cleared by the kidney and its concentration is high in the kidney, gall bladder, spleen and liver [2]. MTX seems to be hepatotoxic, nephrotoxic, toxic to the respiratory and reproductive system. In ancient India, *Tinospora cordifolia* is non controversial and widely used shrub in folk and ayurvedic systems of medicine.

Tinospora cordifolia is an herbaceous vine of the family Menispermaceae which is an indigenous to the tropical areas of India, Myanmar and Sri Lanka [33]. The plant is commonly known as heartleaf moonseed in English; Giloe, Gulancha in Bengali and giloya, amrita in Hindi. Giloya which is a mythological term that refers to the heavenly elixir that saved celestial beings from old age and kept them eternally young. In phytochemistry, *Tinospora cordifolia* has different active phytoconstituents classes. The phytoconstituents responsible for the biological activities like anti-inflammatory, anti-arthritic, anti-osteoporotic activity,

anti-allergic, anti-hyperglycemic, anti-pyretic, antioxidant, diuretic and cardioprotective activity [32]. The purpose of the present study is to induce MTX toxicity in mice and to evaluate possible ameliorative effect of *Tinospora cordifolia* on general performance of the body.

Materials and Methods

Approval from IAEC

Experiment was carried out as per the national guidelines of the Committee for Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment, forest and climate changes, Government of India. Before start of the experiment the protocol of the experiment was approved from the Institutional Animal Ethical Committee (IAEC) of Post Graduate Institute of Veterinary and Animal Science, Akola.

Chemicals

The test item Methotrexate tablets (Folitrax® 2.5 Batch No. AT-181117 Ipca Laboratories Ltd.) having 2.5mg active drug. The shrub *Tinospora cordifolia* (Plate 3.1) was obtained from the campus of Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola. Stem were Separated and prepared hydroethenolic extraxt in Department of Veterinary Pharmacology and Toxicology, PGIVAS, Akola. The standard whitish brown colour pellet feed was supplied from M/s. Nutrivet Life Science (Laboratory Animal Diets) Sihagad road, Pune with the Batch no: 010718 for experiment purpose.

Experimental design

Thirty-two, either sex mice of 4-5 weeks old of 20-30 gm was procured from the recognized CPCSEA (The Committee for the purpose of control and supervision of experiment on animals) approved animal breeding unit. These mice were then divided into four equal groups comprising eight mice each. After acclimatization, from starting day of experiment A, B, C and D were given respective oral treatment up to end of 4 weeks. The A group served as control, B group was treated with methotrexate @1.75 mg/kg body weight biweekly orally, whereas hydroethenolic extract of *Tinospora cordifolia* @ 200 mg/kg body weight once daily orally given in group C and D group was given methotrexate @ 1.75 mg/kg body weight biweekly combined with hydroethenolic extract *Tinospora cordifolia* @ 200 mg/kg body weight once daily given by oral route for 28 days.

General performance

During experimental period of 4 weeks mice of each group were kept under close observations for clinical symptoms and mortality. Average weekly body weights (gm), Average weekly body weight gain (gm) and Average weekly feed consumption (gm) were recorded for each group of mice during experimental period.

Relative organ weight (g/100 g B.WT.)

At the end of 4th week, after gross pathological observations liver, kidney and spleen were separated out and weighed on digital weighing balance. Calculated relative organ weight by using following formula:

$$\text{Organ weight factor} = \frac{\text{Organ weight}}{\text{Whole body weight}} \times 100$$

Hematological observations

For hematological examination, blood sample were collection from eight mice from each group at the end of experiment (4th week). Blood samples were collected at the end of experiment from all the mice from retro orbital or cardiac puncture. Hematological parameter studied included Hemoglobin (Hb, g/dL), Packed cell volume (PCV,%), Total erythrocyte count (TEC, x 106 /cumm), Mean corpuscular volume (MCV), Mean corpuscular hemoglobin (MCH), Mean corpuscular hemoglobin concentration (MCHC), Total leucocytes count (TLC, x 103 cumm) described and Differential leucocytes count (DLC) were estimated as per the standard methods described as under.

Statistical analysis

The obtained data during present investigations was analyzed by applying Completely Randomized Design (CRD) as described by Snedecor and Cochran (1989).

Results and Discussion

Clinical symptoms and Mortality

All the mice from A, C and D experimental group did not reveal any clinical sign and symptoms throughout the experimental period except B group. Nausea, inactiveness, nasal bleeding, loss of hairs, abnormal bowel clearance, intestinal mucositis and increased urine excretion like clinical signs were noted in second week while diarrhoea was present between 3rd to 4th week of experiment in the MTX treated (B) group. The few mice were exhibited signs of dullness, depression, lethargy, loss of body weight, decreased spontaneous activity till the end of experimental period. Similar observation was recorded by Lobo and Balthasar [15], Karri and Vanithakumari [12] and David *et al.* [7] in rats. The lesion of intestinal mucositis was observed by Tran *et al.* [31] in mice. There was no mortality recorded in any of the treatment groups from A to D during the entire period of the experiment. Similar findings of no mortality were reported by Patel [24], Mohamed and Nor-Eldin [19] in rats and Kim *et al.* [14] in mice. In the present study, dullness, lethargy and diarrhoea like clinical signs were observed in MTX treated group. Due to corrosive and irritating action of MTX on the gastrointestinal mucosa causes gastrointestinal toxicity results in decreased in feed consumption.

Relative organ weight (g/100 g B.WT.)

In B group the relative weight of liver was significantly increased when compared with A, C and D groups (Table 1). In group D, the relative weight of liver was significantly decreased as compared with B group. The statistical data revealed restoration in levels of relative weight of liver towards normal level might be due to the effect of *Tinospora cordifolia* plant. Similar findings of significantly increased in the relative weight of liver in MTX treated group was reported by Iqbala *et al.* [9] and Moghadam *et al.* [18], Cao *et al.* [4] in rats, Kalantari *et al.* [11] and Mukherjee *et al.* [20] in mice. However, similar results of significantly reduced in relative weight of liver from group treated with *Tinospora cordifolia* along with MTX was also reported by Kavitha *et al.* [13] in rats. The relative organ weight of kidney and spleen differ non-significantly at end of 4th week.

Average weekly feed consumption

The average weekly feed consumption revealed numerically

decreased in methotrexate treated group as compared with control and C and D group which might be due to toxic effect of methotrexate on intestine (Table 2). The numerically increased feed consumption was observed in group C followed by A and D group. Similar findings of numerically decreased in average weekly feed consumption in MTX treated group B was also reported by Patel [24] and Deepak [8] and Iqbala *et al.* [9] in rats. Whereas, non significant difference were recorded by Saka and Aouacheri [28]. Similar results of improvement in feed consumption in *Tinospora cordifolia* plant was recorded by Bala and Gupta [3], Deepak [8] and Nageswari *et al.* [21] Sprague Drawly rats and Wankhede [34] in cockerels against toxicities.

Average weekly body weights (g) and Average weekly body weight gain (g)

The non significant differences were recorded in average weekly body weight and average body weight gain at 1st and 2nd week of experiment. However, average weekly body weight and average body weight gain at 3rd and 4th week of experiment showed significant (P≤0.05) decrease in body weight in B group mice treated with methotrexate @1.75 mg/kg body weight biweekly orally (Table 3, 4). These changes might be due to loss of appetite with decreased in feed consumption leads to reduction in the body weight possibly owing to MTX toxicity [7].Significantly improvement in average weekly body weight and average weekly body weight gain in D group mice when compared to B group which indicated the ameliorative effect of *Tinospora cordifolia* against MTX toxicity indicates restoration of body weight towards normal control group.

Similar results of significant decreased in average weekly body weight in B group was also reported by Tran *et al.* [31], Karri and Vanithakumari [12], Kim *et al.* [14] in mice and Jurcovicova *et al.* [10], Perianayagasamy *et al.* [26], Patel [24], Iqbala *et al.* [9], David *et al.* [7], Deepak [8] and Cao *et al.* [4] in rats. Whereas, significantly increased in average weekly body weight in *Tinospora cordifolia* along with various toxicities was also reported by in Mamta and Jakhar [16] in broiler against colibacillosis, Ambasta *et al.* [1] in mice against arsenic and Padmaa *et al.* [23] against Cadmium, Bala and Gupta [3] against sodium nitrite, Deepak [8] against MTX and Nageswari *et al.* [21] in rats against zidovudine.

Hematological Parameters

At the end of 4th week significantly (P≤0.05) decrease in Hb, PCV and TEC value was observed in methotrexate treated B group but progressive improvement in D group birds receiving *Tinospora cordifolia* with methotrexate (Table 5). This reduced in Hb and PCV value in MTX treated group might be due to interference to bone marrow because MTX having cytotoxic effects on erythropoiesis and haemopoietic system as evident from the state of anemia. The reduced in TEC count might be due to MTX causes premature death of RBCs due to the oxidative injury and ulcers in the digestive tract resulting in gradual blood loss and ultimately leads to decrease in hemoglobin value. MTX drug can also causes decreased in feed intake leading to hypoproteinemia indicates to lower hemoglobin values were observed in the present study.

Decreased in Hb and PCV value in methotrexate treated B group is in accordance with Ohbayashi *et al.* [22] in mice, Patel [24] and Deepak [8] in rats and reversed in level near to normal level is in accordance with Deepak [8], Bala and Gupta [3] in rats, Sharma and Pandey [30] in mice. They recorded beneficial effect *Tinospora cordifolia* against various toxicity suggested the hematinic property of the *Tinospora cordifolia* plant.

Similar findings of decreased in TEC is in accordance with Ohbayashi *et al.* [22] in mice and Patel [24], Mhatre and Marar [17], Saka and Aouacheri [28] and Deepak [8] in rats. However, ameliorative effect of *Tinospora cordifolia* against various toxicity was also reported by Sharma and Pandey [30] in mice. This might be due *Tinospora cordifolia* act as blood purifier and stimulates liver and spleen which remove defective and damage RBC's from peripheral blood circulation. It stimulates hemopoiesis in the bone marrow leads to increase in Hb level.

At the end of 4th week, MCV and MCH value were significantly increased in methotrexate treated group when compared group D. This findings is in accordance with Patel [24] in rats and Similar results of normalized levels due to the effect of *Tinospora cordifolia* against ziram were observed by Wankhede [34] in cockerels, Sharma and Pandey [30] against lead toxicity. The mean (± SE) values of TLC (10^3/cu mm) in B group were significantly decreased ($p< 0.05$) as compared to those of A, D and C groups of animals (Table 6). The mice of C group found significantly higher as compared to B and D group revealed beneficial effect of *Tinospora cordifolia*. These finding were similar with the Sharma and Pandey [30] as they recorded the beneficial role of *Tinospora cordifolia* in mice and Wankhede [34] in cockerels. In the group D, values was a significantly higher ($p< 0.05$) when compared to B group revealed restoring effect of *Tinospora cordifolia* over the MTX toxicity due to immunomodulatory property of the plant. Similar finding of significant decreased in mean (± SE) values of TLC (10^3/cu mm) in MTX treated group was reported by Ohbayashi *et al.* [22] and Kim *et al.* [14] in mice, Patel [24], Mhatre and Marar [17], Saka and Aouacheri [28], Deepak [8] in rats. Whereas, ameliorative effect of *Tinospora cordifolia* against various toxicity was also observed by Shankhala [29] in mice against acephate toxicty, Bala and Gupta [3] against sodium nitrtite, Saha and Ghosh [28], Deepak [8] against MTX in rat and Sharma and Pandey [30] in mice against lead. This is might be due to immunostimulatory role of *Tinospora cordifolia.*

In group B observed significantly increased in lymphocyte count and decreased in neutrophil count as compared to control group. This is might be due oxidative stress which was plays important role in MTX induced small intestinal damage, reactive oxygen species causes the accumulation of leukocytes in the tissues thus leads to aggravate tissue injury indirectly through activated neutrophils. However restorative effect was obsereved in group D.

Similar results of a significant increase in the mean values of lymphocytes and decreased in neutrophil count in MTX treated group was reported by Patel [24] and Saka and Aouacheri [28] in rats. Significant improvement in the leucocytes count due to *Tinospora cordifolia* was reported by Wankhede [34] in cockerels against ziram induced toxicity, Sharma and Pandey [30] and Shankhala [29] in mice by giving stem and leaves extract of *Tinospora cordifolia* @ 400 mg/kg body weight orally once daily for 30 days to showed beneficial effects against lead toxicity and non significant difference was observed in MCHC, eosinophil, basophil, monocyte count in methotrexate treated group as compared to control group.

Table 1: Average organ weight (g) in control and different treatment groups at 4th week of experiment

Groups	Relative liver Weight	Relative kidney weight	Relative spleen weight
T0	4.32±0.00^{c}	1.70±0.02	0.40±0.01
T1	4.77±0.00^{a}	1.70±0.02	0.38±0.01
T2	4.31±0.01^{d}	1.68±0.01	0.39±0.01
T3	4.53±0.00^{b}	1.70±0.02	0.40±0.01
Significance/ NS	**	NS	NS
CD (0.05)	0.013	-	-

Mean values with common alphabet as superscript do not differ significantly NS= Non Significant

Table 2: Average weekly feed consumption (g) in different treatment groups during experimental period from 1st to 4th week

Group	1st week	2nd week	3rd week	4th week	Total feed consumption
T0	23.16	24.59	25.98	28.95	25.67
T1	22.42	23.21	21.29	18.21	21.28
T2	23.75	25.29	26.82	28.35	26.05
T3	22.54	23.84	23.46	21.23	22.77

NS= Non Significant

Table 3: Average weekly body weights (g) per mice in different groups during experimental period from 1st to 4th week

Group	0 day weight	1st week	2nd week	3rd week	4th week	Pooled mean
T0	25.96 ±0.27	28.04 ±0.17	30.70 ±0.23	33.34 ±0.14^{ab}	37.01 ±0.14^{a}	33.79±0.08^{a}
T1	26.02 ±0.25	27.98 ±0.25	30.55 ±0.18	31.71 ±0.22^{c}	32.53 ±0.32^{c}	32.60±0.11^{c}
T2	25.68 ±0.30	27.90 ±0.30	30.61 ±0.24	33.39 ±0.21^{a}	37.44 ±0.44^{a}	33.93±0.19^{a}
T3	25.68 ±0.23	27.77 ±0.23	30.40 ±0.25	32.66 ±0.28^{b}	34.77 ±0.24^{a}	33.16± 0.15^{b}
Level of significance	-	-	-	**	**	**
CD(0.05)	NS	NS	NS	0.522	0.399	0.402

Mean values with common alphabet as superscript do not differ significantly NS= Non Significant

Table 4: Average weekly body weight gain (g) per mice in different groups during experimental period from 1st to 4th week

Group	1st week	2nd week	3rd week	4th week	Pooled mean
T0	2.08±0.24	2.66±0.30	2.64±0.32^{a}	3.67±0.19^{a}	2.76±0.07^{ab}
T1	1.96±0.35	2.57±0.21	1.17±0.27^{b}	0.82±0.16^{c}	2.30±0.06^{c}
T2	2.22±0.19	2.71±0.26	2.78±0.32^{a}	4.05±0.55^{a}	2.94±0.09^{a}
T3	2.09±0.20	2.63±0.16	2.26±0.39^{a}	2.11±0.26^{b}	2.65±0.09^{b}
Level of significance	-	-	**	**	**
CD (0.05)	NS	NS	0.941	0.957	0.229

Mean values with common alphabet as superscript do not differ significantly NS= Non Significant

Table 5: Haematological values related to erythrocytes in different groups at the end of 4th week of experiment

Groups	Hb (g/dL)	PCV (%)	TEC (10^{6}/cumm)	MCV (fL)	MCH (Pg)	MCHC (%)
T0	11.48± 0.24^{b}	33.20± 1.07^{b}	7.46±0.30^{b}	44.72± 2.15^{bc}	15.47±0.67^{bc}	34.61±0.50
T1	9.01±0.44^{c}	27.00±1.32^{c}	4.81±0.21^{c}	56.46±0.96^{a}	18.83±0.26^{a}	33.38±0.41
T2	14.10±0.22^{a}	42.17±0.79^{a}	9.84±0.35^{a}	42.95±0.79^{c}	14.40±0.32^{c}	33.61±0.18
T3	10.90 ±0.30^{b}	32.50±0.96^{b}	6.86±0.16^{b}	47.36±0.66^{b}	15.88±0.10^{b}	33.45±0.43
Significance/ NS	**	**	**	**	**	NS
CD (0.05)	0.924	2.979	0.734	3.358	1.065	-

Mean values with common alphabet as superscript do not differ significantly NS= Non Significant

Table 6: Hematological values related to leucocytes in different groups at the end of 4th week of experiment

Groups	TLC (10^{3}/ cumm)	Lymphocyte (%)	Neutrophil (%)	Monocyte (%)	Eosinophil (%)	Basophil (%)
T0	9.64 ±0.23^{a}	64.40±0.51^{c}	31.20±0.86^{b}	1.80 ±0.20	1.60±0.24	0.60±0.24
T1	7.04±0.29^{c}	69.00±0.58^{a}	28.00 ±0.58^{c}	1.33±0.21	1.17±0.31	0.50 ±0.22
T2	9.78±0.21^{a}	63.00 ±0.37^{c}	33.00±0.58^{a}	1.83±0.17	1.67±0.21	0.50±0.22
T3	7.94 ±0.26^{b}	66.00 ±0.58^{b}	30.00±0.58^{b}	1.67±0.21	1.50±0.34	0.83±0.31
Significance/ NS	**	**	**	NS	NS	NS
CD (0.05)	0.743	1.459	1.826	-	-	-

Mean values with common alphabet as superscript do not differ significantly NS= Non Significant

Conclusion

From this study it can be concluded that exposure of methotrexate for 28days produced reduction in average weekly body weight, average weekly body weight gain and weekly feed consumption which was restored towards normal due to *Tinospora cordifolia* treatment in group treated with *Tinospora cordifolia* along with methotrexate. In comparisons with haematological parameter, methotrexate causes reduced in Hb, PCV, TEC, TLC and neutrophil count and increased in MCV, MCH and lymphocyte count was also ameliorated due *Tinospora cordifolia* treatment. For this reason, it can be proposed the using of routine consultation of *Tinospora cordifolia* extract in patient using long term MTX cure.

Acknowledgements
The authors are highly hearty thankful to Associate Dean, Post Graduate Institute of Veterinary and Animal Science, Akola – 444104 (Maharashtra) India for their valuable help and technical support for this research work.
American journal of undergraduate research

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