Pharmacognosy Magazine [Phcog Mag.]
Vol 5, Issue 20, Oct-Dec, 2009 Page 372-380
(An Official Publication of Pharmacognosy Network Worldwide)
Received: Feb 12, 2009
Modified: Jun 3, 2009
Accepted: Jun 3, 2009

PHCOG MAG.: Research Article

Effect of Subacute Exposure of *Wrightia tinctoria* Bark Extract on Hematological, Biochemical and Antioxidant Enzyme Parameters of Rat.

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ABSTRACT

Wrightia tinctoria (Roxb.) R.Br. extensively used in the Indian system of medicine, is a small deciduous tree of the family Apocynaceae The plant is very useful as stomachic, antidysenteric, carminative, astringent, aphrodisiac and diuretic, used in the treatment of abdominal pain, skin diseases and bilious affections. This plant is reported to have fungicidal, antinociceptive, wound healing, immunomodulatory and antiulcer activity. The major phytoconstituents are triacontanol, tryptanthrin, β-amyrin, ursolic acid, oleanolic acid, β-sitosterol, cycloartenone, cycloeucalenol, β-sitosterol, lupeol, wrightial, 14α-methylzymosterol desmosterol and clerosterol. A number of poly herbal formulations containing *W. tinctoria* is available in market for psoriasis, diarrhoea and dysentery, dandruff and for rejuvenation of joint function.

The present study was undertaken to investigate the effect of sub-acute administration of *W. tinctoria* bark extract on some haematological, biochemical, histological and antioxidant enzyme status of rat liver and kidney following 21 and 45 days treatment. The animals were observed for gross physiological and behavioural responses, food and water intake and body weight changes. Free radical scavenging activity and histopathology was done on liver and kidney samples. *W. tinctoria* showed significant hemopoiesis with increase in body weight signifying anabolic effect. It significantly reduced serum SGOT level and increased glucose levels. *W. tinctoria* caused increased SOD activity of liver along with catalase of both liver and kidney and decreased liver peroxidase ($P<0.001$). These features indicate that *W. tinctoria* upto 1000 mg/kg daily dose is safe and has potential to be consumed for long time in management of various diseases.

KEYWORDS: *Wrightia tinctoria* Bark, Ethanolic extract, Triterpene, Sub-acute, Biochemical parameters, Serum marker.

INTRODUCTION

During the last decade an explosion in the consumption of herbal remedies has been witnessed in all over the world. China and India now lead the world in the sales of herbal remedies including dietary supplements. Phytomedicines containing many chemical constituents have complex pharmacological effects on the body. Apart from determining efficacy, safety of herbal products should also be assessed, as not all herbal medicines are harmless. Herbals are traditionally considered as harmless since they belong to natural sources and consumers assume that natural means safe. In this context the incidence of 1991 and 1992 at Brussels, Belgium, can be taken in to account in which 30 women treated with a Chinese herbal sliming preparation died from renal failure caused by the presence

of aristocholic acid (1). Policy makers, health professionals and general public all over the world are wrestling with question about the safety, quality, availability, preservation, standardization and further development of this health care system.

A commonly heard argument in favour of herbal medicines is that these products have a longstanding history of traditional use, resulting in considerable experience and knowledge about their wanted and unwanted effects. However not all adverse reactions occur immediately after starting the therapy. The importance of delayed reactions was recently underlined by a retrospective studies covering clinical safety trials. An example is the occurrence of muscular weakness due to hypokalemia in long term users of herbal anthranoid laxatives (2). Although preliminary assessment of efficacy can be obtained through the results of in vitro testing and experiments on animals but the effect of long term consumption should also be explored. It is very much needed to review continually and assess the safety of botanicals, with an emphasis on surveillance of the use of these products to identify unknown hazards or risk associated with their continuous use.

In general herbal medicines are prescribed for relatively long term use (2 weeks to 3 months), and during that period some other medicines are occasionally co-administered. Moreover there is a possibility that the patient will self-medicate and sometimes herbal medicines are prescribed with synthetic medicines also. It is therefore necessary and also highly desirable to access the kind of effects of herbals upon long term uses on body weight, relative organ weight, haematological parameters, serum enzyme parameters, antioxidant enzyme status and histopathology of liver and kidney.

Wrightia tinctoria (Roxb.) R.Br. is a small deciduous tree of the family Apocynaceae distributed in Central India, Burma and Timor. This plant is extensively used in the Indian system of medicine. Fresh leaves are pungent and are chewed for relief from toothache. Bark and seeds are antidysenteric, carminative, astringent, aphrodisiac and diuretic, used in flatulence, stomach pain and bilious affections. The plant is very useful as stomachic, in the treatment of abdominal pain, skin diseases, as antidiarrhoeal and antihaemorrhagic (3, 4). Oil emulsion of *W. tinctoria* pods is used to treat psoriasis and also have fungicidal activity against *Pityrosporum ovale* recovered from dandruff (5, 6). Ethyl acetate, acetone and methanol extracts of *W. tinctoria* bark showed antinociceptive activity in mice (7) and wound healing effect in rats (8). *Wrightia tinctoria* bark alcoholic extract showed immunomodulatory activity, protection against experimentally induced acute gastric ulcers, moderate analgesic and anti-inflammatory as well as potent diuretic activity (9–11).

Triacontanol and tryptanthrin have been isolated from *W. tinctoria* leaves (12). The mature powdered pods of *W. tinctoria* showed co-occurrence of β-amyrin, ursolic acid and oleanolic acid along with β-sitosterol (13). Methanol extract of the immature seedpods contains cycloartenone, β-amyrin, cycloeucalenol and β-sitosterol and wrightial, a new terpene (14). A new sterol 14α-methylzymosterol in addition to four rare plant sterols, desmosterol, clerosterol, 24-methylene-25-methylcholesterol and 24-dehydropollinastanol have been isolated from *W. tinctoria* seeds (15). The stem bark of *W. tinctoria* contains β-amyrin, lupeol, β-sitosterol and a new triterpenoid (16). The present study was performed to assess effect of long term administration of *W. tinctoria* bark alcoholic extract once a day continuous treatment for 21 and 45 days on growth, haematology and biochemistry of experimental animals.

MATERIALS AND METHODS

Collection and extraction of plant material

W. tinctoria bark was collected from Hoshangabad district of Madhya Pradesh, India in September 2005. The plant was identified with the help of available literature and authenticated by Dr. A. P. Shrivastava, Principal, P. K. S. Govt. Ayurveda College and Institute, Bhopal, India. A voucher specimen was deposited in the herbarium of department (Herbarium No. Bigoniya 1084).

The barks were made free of adhering woods air dried under shade and milled into coarse powder. 70% ethanolic extract of bark powder was prepared by cold maceration. The macerated mixture was filtered through muslin cloth and evaporated under 40°C up to one third of initial volume, remaining solvent was completely evaporated at 40°C using a rotary vacuum evaporator (Superfit, India). The brownish residue (yield 19.145 % w/w) designated as hydro-alcoholic extract was employed for the experimental studies. Crude plant extract was subjected to qualitative phytochemical investigation for alkaloid, steroidal saponin, reducing sugar, tannin and flavonoid (17, 18). Extract give negative test for glycoside, fixed oil, gum and resin.

Test animals

Laboratory bred Wistar albino rats of both sexes (150 – 200 g) maintained under standard laboratory conditions at 22 ± 2°C, relative humidity 50 ± 15% and photoperiod (12-hrs dark and light), were used for the experiment. Commercial pellet diet (Hindustan Lever, India) and water were provided ad libitum. In order to avoid diurnal variation all the experiments were carried out at same time

of the day i.e. between 10 a.m. to 5 p.m. Approval was obtained from Institutional Animal Ethical Committee (approved body of Committee for the Purpose of Control and Supervision of Experiments on Animals, Chennai, India) of Dr. H. S. Gour University, Sagar, before carrying out the experiments and care provided to the animal was as per the 'WHO guidelines for the care and use of animals in scientific research'.

Determination of LD_{50}

Dried crude extract was freshly suspended in 2% (w/v) carboxy methyl cellulose (CMC) prepared in distilled water. LD_{50} of the extract was determined following the Organizations for Economic Co-operation & Development guidelines (acute toxicity class method: OECD guideline No. 423 and revised Up & Down method: OECD guideline No. 425) were followed for the testing of extract. A Limit test was performed to categorize the toxicity class of the compound and then Main test was performed to estimate the exact LD_{50}. The animals (nulliparous and non-pregnant female Wistar albino rats) were fasted overnight with free access to water, weighed before dosing and test substance administered. All the animals survived at Limit test on 2000 mg/kg and subsequently on 5000 mg/kg dose, as this is the upper limit of testing further dosing are not needed. The *W. tinctoria* bark extract is practically a nontoxic class of compound as it is non-toxic upto 5000 mg/kg dose. A dose of 300, 500 and 1000 mg/kg was selected for pharmacological studies (19).

Study protocol

Animals were divided into four groups of 16 rats each, group 1 served as control received 2% CMS (0.5 ml/100gm) only, group II, III and IV were treated with *W. tinctoria* extract 300, 500 and 1000 mg/kg, p.o dose respectively on daily basis for 45 days. Eight rats from each group were sacrificed on 21st day and remaining eight rats on 45th day two hours after last dose administration to assess all the parameters.

The animals were observed for physiological and behavioural responses, mortality, food and water intake, and body weight changes. Body weight of the animals were noted before and after extract treatment for 21 and 45 days and percent increase in body weight was calculated. The animals were sacrificed by cervical dislocation and blood collected by cardiac puncture in clean dry heparinized tubes were used for estimation of haematological parameters. Another aliquot of blood was allowed to coagulate for 30 min in room temperature and centrifuged at 3000 rpm for 5 mins. The supernatant serum was separated and used for estimation of marker enzyme. The animals were quickly dissected to remove liver, kidney, spleen and heart, washed with cold saline solution, pressed between filter paper pads and weighed. Relative organ weight (weight of organ/100 gm of body weight) was calculated and recorded. A part of liver and kidney was preserved in cold saline for estimation of free radical scavenging activity and reminder in Aqua Bouine's fluid for histopathology.

Haematological parameters

Estimation of hemoglobin content (Sahli's Hemoglobinometer), total WBC and RBC count (Neubauer hemocytometer; Feinoptik, Germany) was done using standard technique and differential WBC count (Neutrophil, Eosinophil, Basophil, lymphocyte and monocyte) was done by Leishman's staining method (20).

Estimation of blood marker enzymes

Serum alkaline phosphatase (21), total protein content (22), total cholesterol (23), serum triglycerides (24) and high density lipoprotein (25) were determined spectrometrically (Shimadzu UV-1700; pharmaspec) using the commercially available standard kit (Span Diagnostics Ltd., India). Serum low density lipoprotein (26) content was calculated from known concentration of total cholesterol, triglyceride and HDL cholesterol. Glucose content of serum was estimated following the method of Trinder (27) using a standard kit of Transasia Bio-medicals Ltd., Daman. Serum glutamate pyruvate transaminase and serum glutamate oxaloacetate transaminase (28) was estimated using kit obtained from Stangen Immuno Diagnostics, Hyderabad.

Estimation of free radical scavenging ability of liver and kidney

Lipid peroxides was estimated as thiobarbituric acid reactive substance malondialdehyde (MDA) at 532 nm following the method of Ohkawa et al. (29). The level of lipid peroxidases was expressed as nM of MDA/ mg of liver protein. Peroxidase is a class of important enzymes such as NAD-peroxidase, NADP- peroxidase and glutathione peroxidase involved in scavenging of hydrogen peroxide. Liver homogenate 0.5 ml (10% in 0.1 M KCl) was added to 1 ml of potassium iodide and sodium acetate solution each then the absorbance was noted at 532 nm. To the above solution 20 µl of hydrogen peroxide solution was added and the change in absorbance was again noted after 5 min. One unit of peroxidase activity is defined as change in absorbance per min and expressed in terms of units/ min/mg of protein.

Catalase was determined as per the method of Aebi (30). Decomposition of H_2O_2 by catalase is directly

proportional to decrease in absorbance at 340 nm. The results were expressed as unit of H_2O_2 decomposed/min/mg of protein, using 71 as molar extinction coefficient of H_2O_2. Superoxide dismutase was estimated as per the method of Misra and Fridovich (31). One enzymatic unit of SOD is the amount of protein in the form of enzyme present in 100 µl of sample required to inhibit the reduction of 24 micromolar nitro-blue tetrazolium (NBT) by 50% and is expressed as unit/mg of protein. All these estimations were done on Shimadzu (UV-1700; pharmaspec) spectrophotometer.

Histological study of liver and kidney

Permanent tissue slides of liver and kidney sections were prepared and stained (Hematoxylin & Eosin) based on method of Nanji et al. (32).

Statistical analysis

All data are presented as means ± SEM. Experimental data was analysed using one-way ANOVA followed by Student's t-test to compare the difference between the control and treated values. P value <0.05 were considered significant. Graph Pad Prism Version 3.02 was used for statistical calculations.

RESULTS

Prolonged administration of *W. tinctoria* extract for 21 and 45 days showed no significant change in relative organ weight except at 1000 mg/kg dose where spleen weight had significantly increased (P<0.05) after 45 days. *W. tinctoria* extract (1000 mg/kg dose) after 21 and 45 days treatment showed 33.33 and 55.55% increase in body

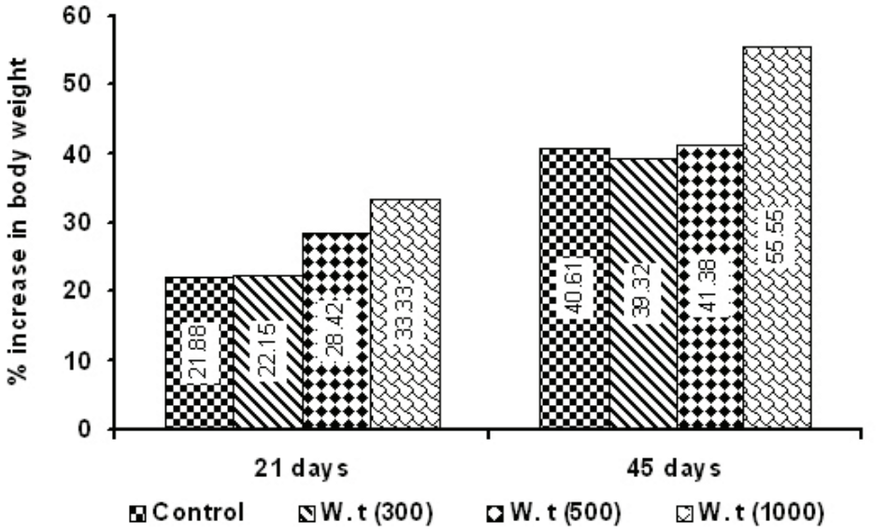


Figure 1: Effect of daily oral administration of *W. tinctoria* bark extracts up to 45 days on percentage body weight increase of rats. W.t – W. tinctoria.

Table 1: Effect of daily oral administration of *W. tinctoria* extract up to 6 weeks on relative body and organ weight of rats

Day	Treatment (mg/kg, p.o)	Relative organ weight (gm/100 gm of body weight) M ± SEM				% increase in body weight
		Liver	Kidney	Heart	Spleen	
21	Vehicle control	3.034 ± 0.156^{ns}	0.803 ± 0.047	0.379 ± 0.026	0.234 ± 0.044	21.88
	W. t (300)	3.126 ± 0.495^{ns}	0.804 ± 0.072^{ns}	0.377 ± 0.080^{ns}	0.301 ± 0.025^{ns}	22.15
	W. t(500)	3.471 ± 0.320^{ns}	0.844 ± 0.035^{ns}	0.385 ± 0.063^{ns}	0.331 ± 0.014^{ns}	28.42
	W. t (1000)	3.915 ± 0.282^{ns}	0.877 ± 0.090^{ns}	0.420 ± 0.055^{ns}	0.382 ± 0.054^{ns}	33.33
45	Vehicle control	3.305 ± 0.678	0.926 ± 0.034	0.384 ± 0.059	0.286 ± 0.055	40.61
	W. t (300)	3.816 ± 0.220^{ns}	0.805 ± 0.022^{ns}	0.396 ± 0.027^{ns}	0.307 ± 0.020^{ns}	39.32
	W. t (500)	4.173 ± 0.631^{ns}	0.925 ± 0.027^{ns}	0.447 ± 0.081^{ns}	0.377 ± 0.024^{ns}	41.38
	W. t (1000)	4.514 ± 0.387^{ns}	0.984 ± 0.071^{ns}	0.479 ± 0.036^{ns}	0.450 ± 0.028*	55.55

n = 6,

*P <0.05 and

ns = not significant when compared to respective control group. W.t – *W. tinctoria* extract.

Table 2: Effect of daily oral administration of *W. tinctoria* extract upto 6 weeks on peripheral blood parameters of rats

Day	Treatment (mg/kg, p.o)	Hb (gm/dl) M ± SEM	RBC (10^6/mm^3) M ± SEM	WBC (10^3/mm^3) M ± SEM	Differential leukocyte count in % (M ± SEM)				
					N	L	M	E	B
21	Vehicle control	11.70 ± 0.50	4.80 ± 0.13	6.02 ± 0.82	52.45 ± 1.27	45.28 ± 1.94	1.30 ± 0.12	0.77 ± 0.17	0.20 ± 0.11
	W. t (300)	13.40 ± 0.84^{ns}	5.91 ± 0.41^{ns}	6.42 ± 0.23^{ns}	56.22 ± 1.45^{ns}	41.94 ± 1.62^{ns}	1.50 ± 0.10^{ns}	0.40 ± 0.11^{ns}	0.06 ± 0.01^{ns}
	W. t (500)	15.20 ± 0.16***	6.42 ± 0.24*	6.54 ± 0.13^{ns}	62.50 ± 1.31***	35.62 ± 1.69**	1.40 ± 0.22^{ns}	0.40 ± 0.13^{ns}	0.08 ± 0.02^{ns}
	W. t (1000)	15.80 ± 0.12***	7.05 ± 0.42***	6.72 ± 0.42^{ns}	65.73 ± 1.57***	32.65 ± 1.58***	1.02 ± 0.14^{ns}	0.40 ± 0.12^{ns}	0.20 ± 0.13^{ns}
45	Vehicle control	12.20 ± 0.97	5.31 ± 0.25	7.20 ± 0.53	53.14 ± 1.51	44.17 ± 1.59^{ns}	1.02 ± 0.17	1.20 ± 0.15	0.47 ± 0.14
	W. t (300)	15.00 ± 0.47*	6.53 ± 0.22*	7.32 ± 0.12^{ns}	60.27 ± 1.72^{ns}	37.24 ± 1.70*	1.40 ± 0.18^{ns}	1.09 ± 0.25^{ns}	0.40 ± 0.12^{ns}
	W. t (500)	15.50 ± 0.41**	7.24 ± 0.11***	7.25 ± 0.21^{ns}	64.63 ± 1.47**	34.02 ± 1.49**	1.01 ± 0.12^{ns}	0.30 ± 0.11**	0.04 ± 0.01*
	W. t (1000)	16.20 ± 0.52**	7.87 ± 0.41***	7.26 ± 0.60^{ns}	70.14 ± 2.40***	27.84 ± 1.63***	1.00 ± 0.11^{ns}	0.94 ± 0.14^{ns}	0.08 ± 0.03*

n = 6,
*P<0.05,
**P<0.01,
***P<0.001 and
ns = not significant when compared to respective control group. W.t – *W. tinctoria* extract, N – Neutrophil, L – Lymphocyte, M – Monocyte, E – Eosinophil, B – Basophil.

Table 3: Effect of daily oral administration of *W. tinctoria* extract upto 6 weeks on serum biochemical parameters of rat

Day	Treat-ment (mg/kg, p.o)	SGOT (IU/L) M ± SEM	SGPT (IU/L) M ± SEM	ALP (U/l) M ± SEM	Cholesterol (mg/dl) M ± SEM	Triglyceride (mg/dl) M ± SEM	Protein (gm/dl) M ± SEM	HDL (mg/dl) M ± SEM	LDL (mg/dl) M ± SEM	Glucose (gm/dl) M ± SEM
21	Vehicle control	44.72 ± 2.66	47.21 ± 3.15	65.74 ± 4.34	91.70 ± 3.22	63.50 ± 3.70	7.60 ± 0.11	42.67 ± 2.86	36.33 ± 2.11	109.72 ± 5.30
	W. t (300)	41.23 ± 2.69^{ns}	89.15 ± 4.46***	62.28 ± 4.21^{ns}	132.95 ± 5.25***	67.58 ± 3.10^{ns}	8.72 ± 0.41^{ns}	57.16 ± 3.76*	62.27 ± 3.14***	149.23 ± 4.28***
	W. t (500)	37.06 ± 2.03^{ns}	92.60 ± 4.64***	62.52 ± 4.02^{ns}	125.56 ± 5.60***	68.27 ± 3.20^{ns}	8.84 ± 0.45^{ns}	52.24 ± 3.22^{ns}	59.67 ± 2.82***	147.37 ± 5.03***
	W. t (1000)	30.72 ± 2.45**	99.65 ± 4.11***	60.55 ± 4.33^{ns}	147.36 ± 5.12***	82.31 ± 3.33**	8.63 ± 0.23^{ns}	46.19 ± 2.12^{ns}	84.71 ± 3.56***	125.56 ± 4.95^{ns}
45	Vehicle control	45.55 ± 3.71	46.19 ± 3.22	68.24 ± 3.84	104.72 ± 5.17	65.17 ± 3.07	7.82 ± 0.40	45.40 ± 3.10	46.29 ± 2.12	112.63 ± 4.68
	W. t (300)	32.60 ± 2.30**	55.43 ± 3.52^{ns}	72.52 ± 4.13^{ns}	115.60 ± 4.08^{ns}	69.79 ± 3.53^{ns}	8.50 ± 0.91^{ns}	52.23 ± 2.74^{ns}	49.41 ± 2.55^{ns}	121.14 ± 4.09^{ns}
	W. t (500)	14.13 ± 1.21***	57.51 ± 3.12^{ns}	70.87 ± 3.70^{ns}	104.55 ± 4.71^{ns}	72.26 ± 3.11^{ns}	8.25 ± 0.74^{ns}	50.35 ± 2.30^{ns}	39.75 ± 2.05^{ns}	104.55 ± 4.22^{ns}
	W. t (1000)	12.97 ± 1.25***	57.08 ± 3.23^{ns}	73.33 ± 4.27^{ns}	88.84 ± 4.35^{ns}	100.15 ± 4.65***	8.94 ± 0.83^{ns}	43.96 ± 2.21^{ns}	24.85 ± 2.12***	78.84 ± 3.54***

n = 6,
*P<0.05,
**P<0.01,
***P<0.001 and
ns = not significant when compared to respective control group. W.t – *W. tinctoria* extract, SGOT – Serum glutamate oxaloacetate transaminase, SGPT – Serum glutamate pyruvate transaminase, ALP – Serum alkaline phosphatase, HDL – High density lipoprotein, LDL – Low density lipoprotein.

Table 4: Effect of daily oral administration of *W. tinctoria* extract upto 6 weeks on liver and kidney antioxidant enzyme parameters of rat

Day	Treatment (mg/kg, p.o)	Lipid peroxidase (nmol/mg of protein) M ± SEM		Peroxidase (unit/min/mg of protein) M ± SEM		Catalase (unit/min/mg of protein) M ± SEM		SOD (unit/mg of protein) M ± SEM	
		Liver	Kidney	Liver	Kidney	Liver	Kidney	Liver	Kidney
21	Vehicle control	28.65 ± 1.28	6.78 ± 0.50	2.68 ± 0.26	1.17 ± 0.13	3.31 ± 0.37	5.36 ± 0.46	1.86 ± 0.12	1.71 ± 0.23
	W. t (300)	24.33 ± 1.68^{ns}	9.27 ± 0.78^{ns}	0.44 ± 0.12***	1.47 ± 0.15^{ns}	4.76 ± 0.25^{ns}	4.97 ± 0.32^{ns}	5.36 ± 0.32***	1.93 ± 0.14^{ns}
	W. t (500)	18.62 ± 1.24***	9.87 ± 0.82*	0.12 ± 0.01***	1.54 ± 0.24^{ns}	5.17 ± 0.27^{ns}	4.36 ± 0.15^{ns}	7.62 ± 0.33***	3.10 ± 0.22^{ns}
	W. t (1000)	10.55 ± 0.91***	10.52 ± 0.95*	0.07 ± 0.04***	0.43 ± 0.12*	5.90 ± 0.86**	5.29 ± 0.19^{ns}	8.23 ± 0.65***	4.63 ± 0.32***
45	Vehicle control	29.94 ± 1.22	7.44 ± 0.34	2.71 ± 0.25	1.30 ± 0.25	3.16 ± 0.47	4.17 ± 0.48	1.76 ± 0.23	4.31 ± 0.29
	W. t (300)	15.34 ± 1.04***	7.32 ± 0.68^{ns}	0.93 ± 0.28***	0.75 ± 0.13***	4.67 ± 0.27^{ns}	3.15 ± 0.27^{ns}	4.03 ± 0.47*	4.16 ± 0.44^{ns}
	W. t (500)	11.37 ± 0.80***	7.15 ± 0.48^{ns}	0.15 ± 0.03***	0.11 ± 0.00***	5.04 ± 0.44*	4.47 ± 0.35^{ns}	4.59 ± 0.47**	4.52 ± 0.23^{ns}
	W. t (1000)	5.76 ± 0.61***	6.82 ± 0.24^{ns}	0.34 ± 0.02***	0.05 ± 0.01***	5.65 ± 0.57**	6.09 ± 0.32*	4.89 ± 0.59***	4.64 ± 0.45^{ns}

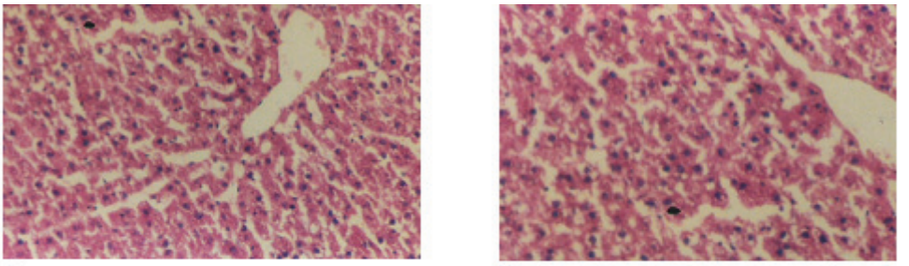
n = 6,
*P<0.05,
**P<0.01,
***P<0.001 and
ns = not significant when compared to respective control group. W.t – *W. tinctoria* extract, SOD – Superoxide dismutase.

weight respectively compared to 21.88 and 40.61% of control as compiled in Figure 1 and Table 1.

W. tinctoria extract treatment at 500 and 1000 mg/kg dose caused significant (P<0.05 to 0.001) increase in total RBC count and Haemoglobin content both on 21 and 45 days of treatment as depicted in Table 2. Extract also increased neutrophil and lymphocyte percentage which was highly significant (P<0.01 to 0.001) at 500 and 1000 mg/kg dose.

W. tinctoria extract treatment caused highly significant (P<0.01) decrease in serum SGOT at 1000 mg/kg dose on 21 days treatment but after 45 days treatment it showed highly to extremely significant (*P*<0.01 to 0.001) decrease in all the tested doses. Extract treatment showed extremely significant (*P*<0.001) rise in SGPT and serum cholesterol at all the tested doses on 21 days but this was normalized after 45 days. *W. tinctoria* extract treatment did not show significant change in serum ALP, HDL and protein content. Serum triglyceride concentrations were significantly (P<0.01 and 0.001) increased in 1000 mg/kg dose both at 21 and 45 days treatment. *W. tinctoria* extract treatment extreme significantly (P<0.001) increased serum LDL at all the tested doses on 21 days and this was normalized after 45 days but in 1000 mg/kg dose extract showed extremely significant (P<0.001) decrease. *W. tinctoria* extract initially at 21 days treatment showed extremely significant (P<0.001) increase in serum glucose at 300 and 500 mg/kg dose but the increase was insignificant at 1000 mg/kg dose, on the other hand after 45 days treatment significantly (P<0.001) decreased serum glucose (Table 3).

W. tinctoria extract treatment caused extremely significant (P<0.001) decrease in liver lipid peroxidase activity both at 21 as well as 45 days treatment. Kidney lipid peroxidase was increased initially after 21 days treatment and after 45 days treatment it was normalized. Decrease in liver peroxidase activity was extremely significant (P<0.001) at all the tested doses both on 21 and 45 days treatment. Decrease in kidney peroxidase activity was extremely significant (P<0.001) at 45 days



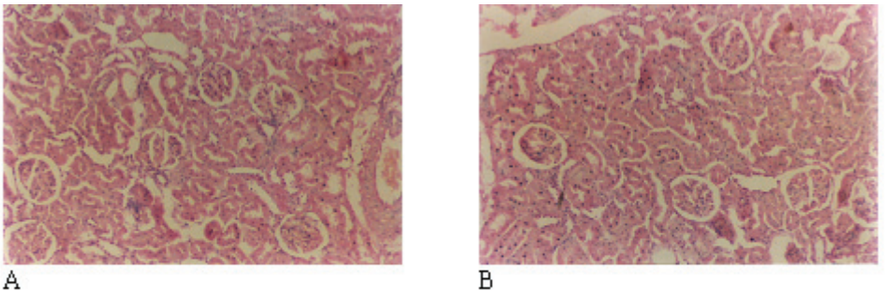
A

Photomicrograph of liver tissue of W. tinctoria 500 mg/kg treated rat

B

Photomicrograph of liver tissue of W. tinctoria 1000 mg/kg treated rat

Figure 2: Effect of daily oral administration of *W. tinctoria* bark extracts up to 45 days on liver histology of rats (10×).



A

Photomicrograph of rat kidney tissue treated with W. tinctoria extract 500 mg/kg

B

Photomicrograph of rat kidney tissue treated with W. tinctoria extract 1000 mg/kg

Figure 3: Effect of daily oral administration of *W. tinctoria* bark extracts up to 45 days on kidney histology of rats (10×).

treatment on all the tested doses. Liver catalase activity showed a highly significant (P<0.01) increase in 1000 mg/kg dose both at 21 and 45 days treatment. Kidney catalase activity was increased significantly (P<0.05) on 45 days treatment at 1000 mg/kg dose only. Liver SOD activity also showed a very significant (P<0.05 to 0.001) increase at all the tested doses both on 21 and 45 days treatment. It showed extremely significant (P<0.001) rise in kidney SOD only at 1000 mg/kg dose after 21 days treatment (Table 4).

Histopathological studies of the liver of the treated rats showed normal hepatocyte exhibiting minimal fatty changes along with mild portal inflammation. Photomicrographs of kidney had normal architecture with structural and functional integrity of nephrons (Figures 2 and 3).

DISCUSSION AND CONCLUSION

Organ body weight ratios are normally investigated to determine whether the drug treatment has any effect on size and weight of the vital organs comparative to total body weight. The measurement of the activities of enzymes in tissues and body fluids plays a significant and well-known role in investigation and diagnosis of diseases (33). Tissue enzyme assay can also indicate cellular damage long before structural damage of tissues can be picked up by conventional histological techniques. Such measurement can also give an insight to the site of cellular tissue damage as a result of assault by sub-acute or chronic use of plant extract. Alkaline phosphatase is a 'marker' enzyme for the plasma membrane and endoplasmic reticulum functionality (34).

No acute mortality was observed even at 45 days treatment of *W. tinctoria* extract. All animals were found to be normal during and at the end of observation period. In the subacute toxicity study, no death occurred during the treatment periods either in the control or in the treated groups. General behaviour or other physical activities of the animals were normal. The control and drug treated groups showed normal body weight gain during the treatment period, which was quiet higher for the extract treated groups. *W. tinctoria* extract showed non-significant effect on the relative liver and kidney weight on 45 days treatment implicating the defensive mechanism of the animal has not been overcome and the drug has not accumulated sufficient enough to manifest any significant change. However the significant increase in spleen weight after 45 days administration of *W. tinctoria* may be attributed to its immunostimulant properties (12). The extract did not have any adverse effect on the normal growth of the animals. On the contrary, a significant increase in the body weight gain, hemoglobin, RBC count and increase in glucose levels were observed in the treated animals suggesting an anabolic effect of the preparation.

It was observed that RBC count had undergone a sharp increase in the *W. tinctoria* alcoholic extract treated group than the control group during the whole experimental period of 45 days. Hemoglobin concentration in the blood had also followed a similar trend. The effect of extract on total RBC and hemoglobin concentration appeared to be dose dependent. Some plants like Withania somnifera has been reported to produce anabolic effect, enhancing the synthesis of certain modulator proteins in rat liver and increasing the body weight in humans (35). Qualitative phytochemical investigation of extract revealed the presence of alkaloid, steroid, tannins and flavonoids. It is well known that glucocorticoids increase the hemoglobin content and RBC count and also affect circulating white blood cells. Administration of glucocorticoids leads to an increase in the number of polymorphonuclear leucocyte in the blood because of increased rate of entrance into the blood stream from the bone marrow and diminished rate of removal from circulation. Moreover the number of eosinophils, monocytes and basophils of the blood decreases after the administration of glucocorticoids. The thymus derived lymphocytes (T-cells) are also known to decrease proportionally more than those derived from the bone marrow (B-cells), indicating that subpopulations of lymphocytes are differentially affected by the steroids (36).

When liver cell plasma membrane is damaged, a variety of enzymes normally located in the cytosol are released into the blood stream. SGPT is a cytosolic enzyme primarily present in the liver. Serum levels of SGPT mostly increases due to damage of the tissue producing acute hepatic necrosis, such as viral hepatitis and acute cholestasis. Alcoholic liver damage and cirrhosis also can associate with mild to moderate elevation of transaminase (37). The prolonged and repeated administration of *W. tinctoria* for 45 days has not adversely affect functional parameter of rat. The extract significantly reduced serum SGOT level without effecting SGPT and ALP level, which signifies its hepatoprotective effect. On a contrary 21 days treatment of *W. tinctoria* affects to significant increase in SGPT level which was again normalized after continuous 45 days treatment. Increment in the enzyme activity following the 21 days administration of extract may be attributed to enhancement of membrane component and stimulation of the enzyme molecule in the extracellular fluids. This is needed for the synthesis of two major membrane phospholipids, phosphatidylethanolamine and phosphatidylcholine, respectively with the attendant

consequence of affecting membrane fluidity and increasing the permeability of the epithelial cells (38).

Extract induced increase in cholesterol and triglyceride concentration may be due to lipolytic effect and mobilization of fat from adipose tissue. This increase in lipid profile along with increased body weight, hemopoiesis and glucose suggest anabolic effect of the plant, which can also be associated with cardiovascular risk upon prolonged use.

Currently purified SOD is therapeutically used in the treatment of oxidative and inflammatory diseases. SOD's are reported widely in plant sources and superoxide scavenging effect of fresh juice and methanolic extract of *Emilia sonchifolia* leaves are reported (39). Alcoholic extract of *Hypericum perforatum* also showed reasonable superoxide anion scavenging activity (40). *W. tinctoria* increased SOD activity of liver along with catalase of both liver and kidney. Highest activity of catalase observed coincides very well with the highest activity of SOD, indicating that the H_2O_2 formed by SOD was effectively removed by catalase. Plant catalases are reported to be very sensitive to environmental conditions and have a rapid turnover rate. The high catalase activity of *W. tinctoria* extract was associated with a low activity of peroxidase indicating the role of catalase in peroxide removal. An inverse relationship was observed between lipid peroxidase and catalase (41). The protective effect of *W. tinctoria* extract on concomitant increase in catalase level observed in the present study is attributed to the inbuilt antioxidant properties.

The global changing scenario is showing a tendency towards use of nontoxic plant products having good traditional medicinal background. *W. tinctoria* is easily available plant which grows in large quantity in the dry hilly areas of North and Central India. This plant can be used as a cheap source of active therapeutics. This plants has potent antimicrobial properties against dermatophytic microbes, which is used extensively in superficial skin ailments like eczema, psoriasis, keratosis and dandruff. A number of poly herbal formulations containing *W. tinctoria* as one of the active phyto-pharmaceutical is available in market for psoriasis, diarrhoea and dysentery, dandruff and for rejuvenation of joint function, having reach in overseas markets also (42–45). This plant has proved effectiveness against commonly occurring ailments and can be used for long duration for alleviation of peptic ulcer, cut and wounds, immunosuppression, anxiety and depression, arthritis and pain relief by the poor and under privileged people of central India region.

The heamopoietic, growth promoting and antioxidant activity of the *W. tinctoria* extract may be due to presence of steroids and flavonoids. These features indicate that *W. tinctoria* extract had the safety potential to be consumed for a very long time in traditional medical practice in the management of various diseases. It is worth to isolate and characterize the active compound(s) of *W. tinctoria* with a view of development of new potent drugs. This particular experimental study substantiate safety following subchronic treatment of the extract as it was well tolerated with no overt sign of clinical toxicity (in terms of salivation, diarrhoea, yellowing of hair, loss of hair, postural abnormalities, behavioural changes, decrease in food and water intake and body weight), non-nephrotoxic (in terms of relative kidney weight) and non-heamatotoxic (in terms of Hb, total RBC, WBC and differential WBC count). This plant can be used as anabolic, growth stimulant and haematinic. However pronged consumption of *W. tinctoria* in high dose can be implicated with increased risk of cardiovascular diseases, liver damage and hyperglycemia. More studies are required to assess its safety on chronic exposure focusing on bone forming elements, extensive tissue enzyme level and teratogenic effects before the extracts can be recommended for long term treatment of diseases.

ACKNOWLEDGEMENTS

The authors are grateful to All India Council of Technical Education, New Delhi for the financial assistance by awarding National Doctoral Fellowship to one of the authors (Dr. P. Bigoniya, grant no.FD/NDFS/2003-04). We express our sincerest gratitude to the authority of Dr. H. S. Gour University, Sagar for providing sufficient laboratory facilities during this experiment.

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