



E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2018; 7(3): 384-390
Received: 01-03-2018
Accepted: 05-04-2018

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Exploration of hepatoprotective potential of methanolic extract of *Tridax procumbens* against isoniazid-rifampicin induced toxicity in albino rats

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Abstract

Objective: This study explored antioxidant and hepatoprotective effect of methanolic extract of *T. procumbens* L., *in-vitro* using Chang (human hepatoma) cell line and isoniazid-rifampicin induced toxicity in male Wistar rat, Silymarin is used as a standard drug.

Methods: 2, 2 diphenyl- 1- pycryl hydrazyl (DDPH) free radical scavenging assay was used to calculate antioxidant activity. The hepatoprotective properties of MTPL, MTPS and MTPF (methanolic extracts of *T. procumbens* Leaves, Stem, flower) and hepatotoxicity of isoniazid-rifampicin *in vitro* on Chang liver cells as percentage cell viability was calculated by MTT assay. The animals were divided into 6 groups (6 rats in each), treated for 14 days. Group-1 (control), normal saline (1ml/Kg) P.O. Group-2 (Toxic), INH+RMP (50 & 100mg/kg) i. p. after day 14th. Group-3 (positive) Silymarin-suspension 200mg/Kg p. o. and toxin same as group-2. Group-4, 5 & 6 received MTPL, MTPS and MTPF (500mg/Kg) p. o. and toxin similar as group 3. After treatment, blood was collected for biochemical estimation and liver was dissected for histological examination.

Results: In DPPH assay, MTPL and MTPS demonstrated strong antioxidant property ($34.52 \pm 0.67 \mu\text{g/mL}$ and $33.14 \pm 0.76 \mu\text{g/mL}$); INH+RMP has significant effects on cell viability, MTPL, MTPS and MTPF were not cytotoxic. Significant elevation of serum liver enzyme were observed ($P < 0.001$) along with considerable decrease of SOD, CAT levels in toxic group. Treatment groups compared with standard drug Silymarin prohibited rise in liver enzymes level while increased SOD, CAT and TP ($P > 0.05$) level. Histopathological profile was helpful to support biochemical parameters.

Conclusion: INH+RMP induce liver injury. MTPL recorded more hepatoprotective potential as compared to MTPS and MTPF.

Keywords: Isoniazid, rifampicin, catalase, hepatocytes, MTPL, MTPS, MTPF.

Introduction

Liver is the principal organ which plays a vital role in metabolism. Almost 75% of hepatic blood comes directly from gastrointestinal tract. Hence, several biochemical reactions result in the production of highly reactive free radicals which attack the membranes and turn into lipid per-oxidation, alter permeability of membrane and cause liver toxicity [1-2]. Managing liver diseases is still cumbersome to the modern society. Though, natural products have been used as therapeutic agents to manage liver injury. While intrinsic antioxidants like SOD, CAT and GSH reduce the tissue damage. Thus, herbal drugs may facilitate in protecting organs whereas the conventional drugs do not have appropriate results in liver protection. Generally, herbal medicines accelerate the curative property because of their antioxidant property with efficacy and lesser side effects. Several medicinal plants have been used traditionally in the management of liver diseases since prehistoric time [3].

T. procumbens (Asteraceae) one of the most versatile medicinal plants, though native to tropical America, but found throughout the world is used in Ayurvedic medicine for treatment of various ailments traditionally. It is used as anti-inflammatory, wound healer, immunomodulatory, antimicrobial, bradycardiac etc. Numerous constituents like luteolin, quercetin, β -sitosterol, dexamethason, esculetin, fumeric acids were isolated from the *T. procumbens* [4-7]. Till date there is no study about evaluation of hepatoprotective activity of leaves stem and flower of *T. procumbens* against isoniazid-rifampicin induced toxicity. Therefore, this study was carried out to evaluate the hepatoprotective potential of extract of *T. procumbens* by *in vitro* and *in vivo* methods.

Materials and Methods

Chemicals

The solvents used were of analytical grade purchased from Merck, India Ltd. DMEM (Dulbecco's modified essential eagle medium), FBS (fetal bovine serum) were taken from Gibco, India. Methyl blue tetrazolium bromide (MTT) dye, HEPES, trypsin-EDTA, antibiotic/ antimycotic (Ab/Am), phosphate buffer saline (PBS) were from Sigma Aldrich, USA. DPPH was procured from Sigma-Aldrich, Bangalore, India. Sodium chloride (NaCl), Sodium bicarbonate (NaHCO₃), dimethyl sulphoxide (DMSO), Polyoxyethylene 20 sorbitan monooleate (Tween 80) and isopropanol were purchased from Merck, India Ltd. Alanine transaminases (ALT), aspartate transaminases (AST), alkaline phosphatase (ALP), bilirubin, total protein, albumin and creatinin were assayed by using standard kits. All drugs (Isoniazid-Rifampicin), and reagents used were purchased from Himedia and Sigma-Aldrich USA.

Collection and extraction of plant material

The fresh plants of *T. procumbens* were collected in the month of April-May 2015 from the field of CSIR-CIMAP, Lucknow, Uttar Pradesh, India. Identification and authentication was done by Dr. Muhammad Arif (Assistant professor), and referral voucher specimen IU/PHAR/HRB/15/23 has been deposited in the Department of Pharmacognosy and Phytochemistry, Integral University. The authenticated plant materials were washed firstly with tap water then distilled water. Leaves, stem and flowers were separated from the plant, dried completely under shade and pulverized into coarse powder. To make methanolic extract, 2000gm of coarse powder of leaves, stem and flower were subjected to methanol at room temperature overnight and filtered. The filtrate was dried under vacuum evaporator (Buchi- V200, Switzerland) and the same process repeated thrice. Dried extract was weighed; percent yield was calculated and stored at 4° C for further use. The yield was calculated by using the formula:

$$\% \text{ Extract yield} = \frac{\text{weight of dried extract}}{\text{weight of dry plant material}} \times 100$$

Extract	% yield (w/w)
MTPL	9.5
MTPS	8.6
MTPF	7.1

Determination of free radical reducing power using DPPH assay

The DPPH assay was performed by earlier method with minor amendment [8]. DPPH is of dark violet color, forms free radical in methanol and gets bleached by antioxidant activity. 1000µg of MTPL, MTPS and MTPF was dissolved in 1 mL DMSO to obtain stock solution (1mg/mL), from which different dilutions were prepared. Various concentrations (2µg/mL, 10µg/mL, 50µg/mL and 100µg/mL) of each extract were mixed with 0.1M Tris- HCl buffer (pH 7.4) along with 500µM DPPH and allowed to incubate for 30 min in the dark at room temperature. The decrease in ultraviolet absorbance (Ab) was recorded at 517nm against control. The experiment was performed in triplicate. Ascorbic acid (100µg/mL) was used as a positive control. The percent scavenging potential was calculated by using formula:

$$\% \text{ Inhibition} = \frac{\text{Ab of control} - \text{Ab of test sample}}{\text{Ab of control}} \times 100$$

Cell Culture

The Chang cell line (normal hepatocytes) were used for screening of hepatoprotective potential of the methanolic extract of *T. procumbens*, obtained from NCCS (National Centre for Cell Sciences), Pune, India. Chang cell line were grown in DMEM (Dulbecco's modified eagles medium) supplemented with 10% Fetal Bovine Serum (FBS) and 1% Antibiotic/Antimycotic, in a humidified atmosphere of 5% CO₂ at 37° C.

Cell viability by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay

MTT assay was performed by Mossman (1983) method [9]. MTT assay is based on hypothesis that dead cells are unable to reduce tetrazolium. The principle involved in reduction of tetrazolium salt into blue colored formazan product by mitochondrial dehydrogenase. For the assay, cells were seeded in 96 well plates 0.1mL of cell suspension was added to each well (approximately 2X10³cells/well). Treatment of the compound (MTPL, MTPS, MTPF and INH+RMP) in different conc. was given for 24 hours. Then, MTT dye (500µg/ml) was added to the media in each well and plate was further incubated at 37°C in 5% CO₂ for 4 hours. After flicked off media, 100µl DMSO was added to dissolve the formazan crystal. The absorbance was recorded at 570nm through Thermo Fisher Scientific Multiskan™ Go Microplate spectrophotometer and data acquisition was done by SkanIt Software which was provided with Multiskan Go plate reader. MTT assay was performed in duplicate with each experiment having triplicate values. IC₅₀ values were calculated from Table Curve 2D Windows Version 4.07 (SPSS Inc., Chicago, IL, USA).

The percentage growth inhibition was calculated using the following formula:

$$\% \text{ Growth Inhibition} = 100 - \frac{\text{Mean OD of Control group}}{\text{Mean OD of individual test group}} \times 100$$

Animals

Male Wistar albino rats weighing about 120-150gm acquired from CSIR-CDRI, Lucknow were used in experiment. The protocol was approved by the Institute's Animal Ethics Committee (IU/IAEC/16/25), Integral University, Lucknow and the experimental protocols were conducted according to the method of Organization for Economic Cooperation and Development following guidelines No. 423 [10].

The animals were kept in the animal house of Pharmacy, Integral University, in well ventilated poly acrylated cages, at an ambient temperature of 22° C (± 3° C) and 50-60% relative humidity with 12hr:12hr dark-light cycle. Animals were allowed pellet diet and distilled water ad -libitum.

Experimental design

Before starting the experiment the rats were acclimatized for 3-5 days to the environment. The treatment groups received the methanolic extract of leaves stem and flower of *T. procumbens*, compared with silymarin from 'milk thistle' (*Silybum marianum*) 200mg/kg p. o. The animals were randomly divided into 6 groups (6 rats in each), and treatment was given for 14 days, the divided groups were as follows: group1 (control)- received 1 ml of normal saline/day; group2 (negative control)- received 1 ml/day saline for the first

14 days then, after 14 days rats received acute dose of INH+RMP (50mg/kg/ & 100mg/kg b. w.) i. p. (in normal saline) for 5-6 hrs; group3 (positive control), received oral silymarin suspension (200mg/kg/day b. w.) and received toxin as in group2; group 4, 5 and 6 (treatment) received acute oral dose of MTPL, MTPS and MTPF (methanolic extract of *T. procumbens* leaves, stem and flower), 500mg/kg/day b. w. (oral suspension in tween 80) and received toxin as in group III. On day 15th before collecting the blood, centrifuge tubes were labeled and blood samples were collected from orbital plexus. The animals were sacrificed under ether anesthesia; organs were dissected out and weighed. The blood samples were left to clot for 30 min at room temperature, centrifuged for 10 min at 3000 rpm to find the sera and preserved at -20°C, which was used for the biochemical assay. Organ index was calculated according to the formula:

$$(\text{Organ weight/rat body weight}) \times 100\%$$

Histopathology

For histopathological study, animal's liver tissue of each group was fixed in 10% formalin. Liver tissues of each lobe were obtained and embedded in paraffin wax. Thin sections of 4-5µm thickness were made by using standard microtome and liver tissues were routinely stained with hematoxylin and eosin and histological study was done under light microscope [11].

Biochemical analysis

AST, ALT, and ALP activities were expressed in IU/L and Total protein, albumin, bilirubin and creatinin (mg/L) concentration was estimated by using standard kits and absorbance was recorded by spectrophotometer. Homogenizing the liver tissue, 10% of homogenate was prepared, by adding phosphate buffer saline (pH 7.4). Hemoglobin was calculated by Darbkin's reagent. WBCs, RBCs were counted in upright microscope using haemocytometer.

Estimation of SOD activity

The method is based on the ability of enzyme to inhibit the autoxidation of pyrogallol in presence of EDTA in the pH range 7.9-10.6, with increasing pH the rate of autoxidation increases. The reaction is inhibited to 99% by superoxide dismutase at pH 7.9, signifying an almost total dependence on the involvement of the superoxide anion radical. The solution first becomes yellow-brown with a spectrum showing a peak between 400 and 425 nm. After few min the color changes to green and finally a yellow color appears [12-13]. For the estimation of SOD, 5µL of sample (liver homogenate 10%) and 280 µL of phosphate-EDTA buffer solution was taken to 96 well plate and 10 µL of pyrogallol was added just before the kinetic study, then it was examined at 420 nm using spectrophotometer for 4 min at interval of 30 sec. The unit for enzyme activity was calculated to be 50% inhibition of pyrogallol autoxidation; performed along with control (without adding enzyme).

$$\text{SOD (U)} = (\text{Change in control OD} - \text{Change in sample OD}) / (\text{Change in control OD} \times 0.05) \times 6.$$

Estimation of CAT activity

The CAT activity was measured according to the method of Beer & Seizer (1952) with some modification. 100µL of supernatant was added, containing 1.9 mL of 0.05 M

phosphate buffer of pH 7.0. The enzyme catalyses the decomposition of hydrogen peroxide (H₂O₂) to water and oxygen. In order to estimate the catalase activity, 2.5 µL samples (liver homogenate) and 300 µL of 5×10⁻³M H₂O₂ solution was added and examined at 240 nm for 4 min at interval of 30 sec. [14, 12]. CAT activity was expressed as U/mg of protein by using the formula:

$$\text{Catalase} = \text{Change in OD} \times 1000 / 43.1 \times \text{mg of protein per mL of reaction of mixture.}$$

Statistical Analysis:

In biochemical estimation, each assay was performed in triplicate and average values and standard deviation (±SD) were calculated by Microsoft office excel 2007. The data was analyzed by one-way analysis of variance (ANOVA), followed by Dunnett's multiple-comparison procedure to all groups against control using Prism software (Graph pad version 5.3; San Diego, CA, USA).

Results:

DPPH free radical scavenging assay

The DPPH free radical scavenging of MTPL, MTPS and MTPF was estimated (Fig.1). Ascorbic acid was used as standard which scavenged DPPH radicals with IC₅₀ of 25.55µg/mL whereas the IC₅₀ of extracts are 32.21±0.83µg/mL, 34.52±1.2µg/mL and 33.14±.92µg/mL correspondingly.

Cytotoxic effect of MTPL, MTPS, MTPF and INH+RMP on Chang Cells:

The cytotoxic effects of MTPL, MTPS, MTPF and INH+RMP was observed on Chang cells for 24hr as shown in (Fig.2). The leaves, stem and flower extracts of the *T. procumbens* was not found toxic as the decrease in cell viability was not 60% more as compared with untreated cells. Whereas in INH+RMP (100µg/mL) drug treated cells, there was significant decrease in cell viability as compared with control cells. Hence it was found that the extract is safe to be used to evaluate hepatoprotective effect while anti tubercular drug to be used as toxin.

Body weight and relative organ weight

As compared to the weights before the treatment in group and after the treatment; there was no significant decrease in body weight (Table1) and organ weight (Fig3-4).

Isoniazid- rifampicin induced toxicity

The results of biochemical parameters showed that elevation of serum activities such as ALT, AST, ALP and bilirubin was significant ($P < 0.001$) whereas there was decrease in the levels of serum TP and Alb. It indicates, INH+RMP induced group caused liver injury. Whereas the pre-treatment with MTPL, MTPS and MTPF significantly decreased the elevation of ALT, AST and ALP level ($P > 0.005$) and increase of TP, Alb. There was no significant difference in serum activity when compared with standard Silymarin group and control group. (Table 2-4)

SOD and catalase activity

Superoxide dismutase activity was significantly decreased in INH+RMP, MTPS and MTPF treated group as compared with control ($P < 0.001$) while in pre-treated with SYL (silymarin) and MTPL stopped reduction of SOD significantly ($P > 0.05$).

The catalase was decreased in INH+RMP, MTPS and MTF treated groups ($P < 0.001$) and pre-treated with SYL and MTPL prevented the reduction in elevation of the catalase enzyme compared with control ($P > 0.05$) (Table 5).

Histopathological studies

Histopathological study of the liver section of the INH+RMP treated group demonstrated an intense centrilobular necrosis and cytoplasmic vacuolization as an evidence of liver injury. The rats with pre-treated standard SYL, MTPL, MTPS and MTPF showed a good symptom of protection against the toxin as it marked the formation of hepatic cord and absence of necrosis (Fig 5).

Discussion

In the present study, Chang cell line was used to assess the hepatoprotective effect of MTPL, MTPS, MTPF and INH+RMP in combination. The leaves, stem and flower extracts of the *T. procumbens* were not considered as toxic because the decrease in cell viability was not more than 60% compared with untreated cells. Whereas in INH+RMP treated cells, there was a significant decrease in cell viability compared with control cells. The concentration of INH+RMP for > 60% cytotoxicity was approximately 100 $\mu\text{g/mL}$. The MTT salt was taken up by the cells and reduced into formazan through mitochondrial reaction which does not pass through the plasma membrane as it accumulates within the cell. The ability of cells for reduction of MTT provides an indication of mitochondrial reliability and interpreted as an inference of viability cell count [15]. We can say that MTPL, MTPS and MTF extracts have ability to provide strength to mitochondria of cell by which they were able to reduce MTT.

On the other hand, no significant difference in body weight was observed between the groups before and after the treatment; there was minor decrease in body weight of group V and VI (Fig.3-4).

Amino transferases are essential group of enzymes involved in carbohydrate and protein metabolism. Hence, there is a visible connection between the intermediates of the citric acid and amino acids. The liver is the most important place for bilirubin detoxification and excretion, albumin synthesis and regulation of plasma lipids and lipoproteins. As a result, ALT, AST and ALP are distinguished as diagnostic markers of liver injury [16]. Whereas, the albumin, TP and bilirubin levels can be used as indicators of liver function.

According to previous literature, INH itself does not cause liver damage completely [17, 18] because the role of INH induced hepatotoxicity is not clear.

Paolo Preziosi (2007) study shows, INH is metabolized in the liver by acetylation and hydrolysis which produces acetylated metabolites hydrazine. Another study shows, INH itself is an inducer of CYP2E1 [19-20]. Literature suggests acetylated metabolites hydrazine; considered to be hepatotoxins has a

direct affect on CYP2E1 induction and hepatotoxicity production [21-23]. Moreover, Sarich *et al.* supported that hydrazine plays a role in liver injury in INH treated rats [24-25]. One of the studies also show that the rate of liver injury increases when INH and RMP were given in combination. In the hepatotoxic effects of INH and RMP several mechanism are involved [26]. Generally credited to the development of highly reactive oxygen species (ROS) initiated the lipid peroxidation, resulting the destruction and damage to the liver cell membrane [27]. The effect of RMP on INH is considered to be involved by cytochrome P450 synergistically [28]. So, the results of this study suggested that in INH+RMP induced (group II) a significant difference in biochemical indicators such as ALT, AST and ALP, statistically as compared to control which indicates hepatic damage.

The results of pre-treated rats with Silymarin 200mg/kg. b.w. (group III) and MTPL, 500 mg/kg b. w. (group IV) showed effective protection against the INH+RMP, as the elevated level of ALT, AST and ALP were significantly reduced ($P < 0.01$). Whereas TP and albumin remained fix to their normal value. However, the MTPS and MTPF, 500mg/kg/b. w. (group IV, V) did not produce any significant change in ALT, AST and ALP level compared with control group. MTPL treated group showed insignificant difference for total bilirubin, and direct bilirubin while significant change in toxicant group. (Table2).

Antioxidant enzymes SOD and CAT suggested a defense against tissue damage. Oxygen is converted to hydrogen peroxide by SOD which in turn metabolized to non-toxic products through CAT [29-30].

Conclusion:

The present study demonstrated that the Methanolic extract of *T. procumbens* may well play an imperative role in medicine as preventive and curative drug for hepatoprotection due to presence of a number of chemical constituent which is useful in managing free radicals scavenging and stimulating antioxidant enzymes activities against the drug-induced hepatotoxicity. More studies are needed to isolate the active constituent and assess the correct mode of action.

Acknowledgement

The authors are very thankful to Department of Biochemistry King George's Medical University, Department of Chemistry and Pharmacy, Integral University Lucknow (IU/R&D/2018-MCN000266). The study is not financially supported by any funding agency. We are also expressing the heartiest thanks to Dr. Santosh Kumar Srivastava (Emeritus Scientist) and Dr. Arvind Singh Negi (Principal Scientist) CSIR-CIMAP, Lucknow for their valuable suggestions in providing facilities.

Conflict of interest

Nothing to declare.

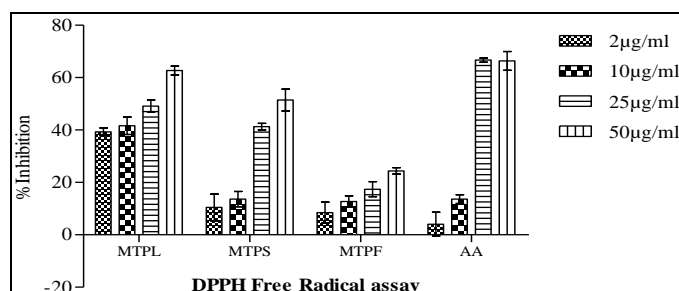


Fig 1: Concentration-dependent DPPH (free radical scavenging) activity of MTPL, MTPS and MTPF (methanolic extract of leaves, stem and flower of *T. procumbens*) in three independent testing of replicates at each concentration and values are mean of \pm SD.

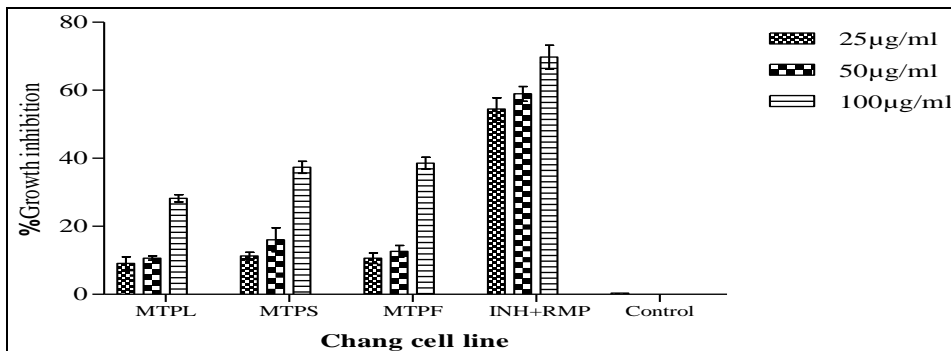


Fig 2: % Growth inhibition of MTPL, MTPS, MTPF and INH+RMP on chang (normal hepatoma) cell line by MTT assay

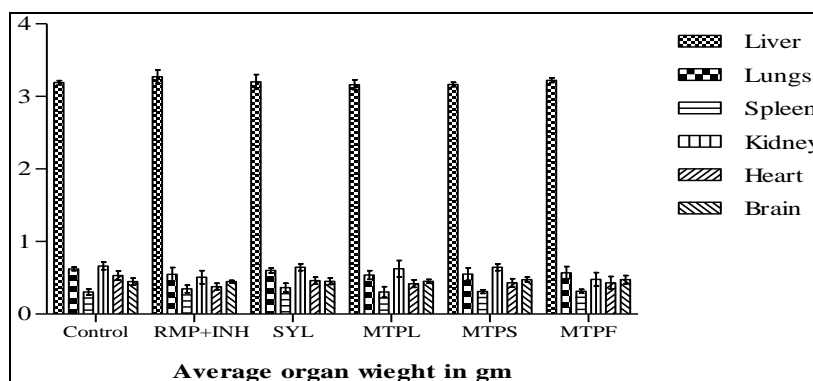


Fig 3: Methanolic extract of leaves stem and flower treated groups of *T. procumbens* compared with normal control group showed no change in average organ weight.

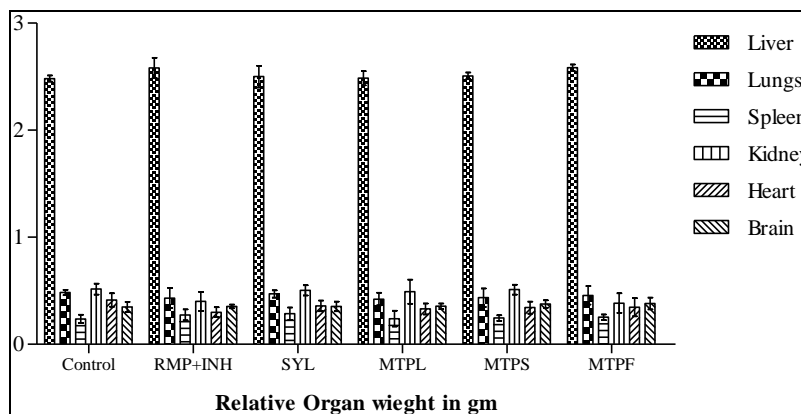


Fig 4: No change in Relative organ weight of methanolic extract of leaves, stem and flower of *T. procumbens* compared with Control

Table 1: Effect of methanolic extract of *T. procumbens* on male Wistar albino rats' average body weight before and after treatment.

Group	Initial body weight (g)	Final body weight(g)
Control	128.25±0.96	128.5±0.58
RMP+INH	126.75±0.50	126.5±0.58
SYL	127.5±0.57	128±0.83
MTPL	126.75±0.95	127.25±0.50
MTPS	127±0.82	126.25±0.50
MTPF	125.75±0.96	124.75±0.96

Table 2: Effect of methanolic extract of leaves stem flower of *T. procumbens* on serum aspartate amino transferase (AST), alanine amino transferase (ALT), alkaline phosphatase (ALP) in different groups.

Group	AST(U/L)	ALT(U/L)	ALP(U/L)
Control	45.25±1.08	45.11±2.29	71.52±2.79
RMP+INH	62.42±1.40	62.42±2.76	104.31±6.53
SYL	45.18±1.60	44.45±3.75	71.36±2.34
MTPL	45.69±1.04	45.18± 2.27	71.75±4.67
MTPS	46.05±1.39	45.47±1.35	75.40±2.26
MTPF	46.42±2.95	45.76± 3.38	74.56±2.06

Table 3: Effect of methanolic extract of leaves stem flower of *T. procumbens* on total protein (TP), albumin (Alb), creatinin (Creat), bilirubin total (Bili T.) and bilirubin direct (Bili. di).

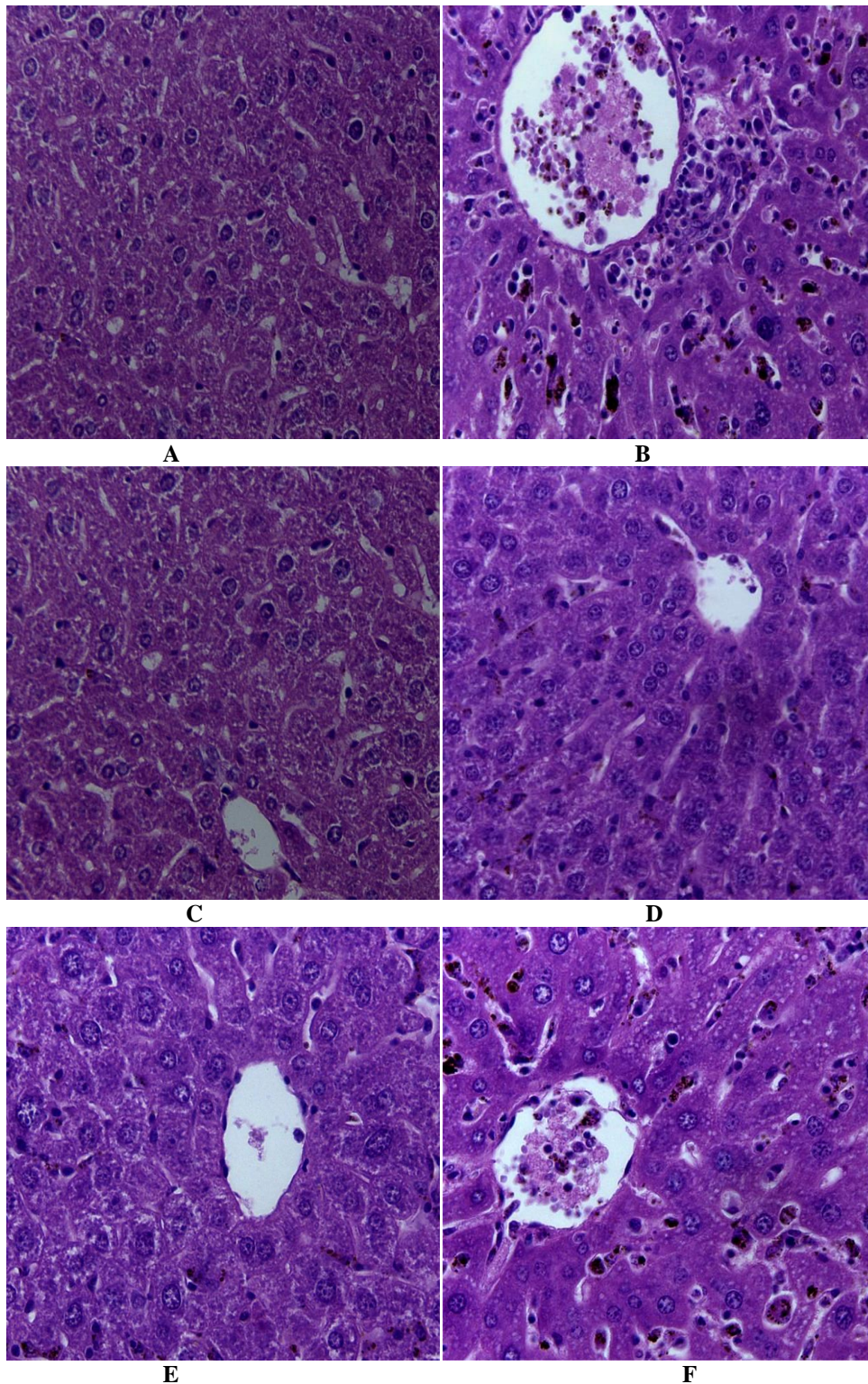
Group	TP(mg/L)	Alb(mg/L)	Creat(mg/L)	Bili di (mg/L)	Bili T.(mg/L)
Control	43.58±1.06	2.34±0.17	0.95±0.06	0.54±0.07	0.17±0.01
RMP+INH	36.77±0.63	1.48±0.23	1.18±0.07	1.15±0.09	0.41±0.03
SYL	43.04±0.50	2.79±0.12	0.94±0.07	0.54±0.09	0.16±0.03
MTPL	42.60±1.38	2.34±0.29	0.96±0.08	0.56±0.08	0.17±0.01
MTPS	38.82±0.82	2.30±0.23	0.99±0.05	0.62±0.10	0.18±0.03
MTPF	38.15±1.3	2.17±0.29	0.99±0.05	0.61±0.07	0.19±0.02

Table 4: Effect of methanolic extract of leaves stem flower of *T. procumbens* on hematology in all groups.

Group	RBCs (million/mm ³)	WBCs (Thousand/mm ³)	Hb (g/dL)
Control	7.44±0.45	6.30±0.58	18.08±0.21
RMP+INH	5.09±0.27	4.70±0.57	11.96±0.35
SYL	7.61±0.55	6.79±0.49	18.38±0.23
MTPL	7.56±0.50	6.63±0.86	18.15±0.22
MTPS	7.24±0.54	5.93±0.72	15.02±0.38
MTPF	6.81±0.42	6.10±0.47	14.92±0.63

Table 5: Effect of methanolic extract of leaves stem flower of *T. procumbens* on liver antioxidant enzyme in all groups.

Group	SOD (U/mg prot)	CAT(U/mg prot)
Control	162.32 ± 3.12	30.07± 1.23
RMP+INH	73.19 ±2.34	17.19± 1.29
SYL	160.39±3.11	29.84± 2.11
MTPL	159.71±3.16	30.04± 1.19
MTPS	103.88±2.14	25.27± 2.21
MTPF	96.49±2.12	24.38± 1.15

**Fig 5:** Histology of the Livers under light microscope. A. Control (group I) ; B. INH+RMP (group II) confirmed membrane disintegration with extreme vacuolation along with cytoplasmic rarefaction. ; C. Silymarin (group III) modified the vacuole and checked the membrane disintegration no evidence of necrotic areas. ; D. MTPL (group IV) adjusts the vacuolation and preventing the degradation of cell membrane. E. MTPS (group V) increases the vacuole with cytoplasmic disintegration; F. MTF (group6) showed vacuolation with increasing necrotic area.

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