EVALUATION OF HEPATOPROTECTIVE POTENTIALS OF METHANOL EXTRACT OF TEPHROSIA VILLOSA AGAINST THIOACETAMIDE INDUCED LIVER TOXICITY IN ALBINO RATS

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INTRODUCTION

The liver disorder has become one of the common health problems worldwide due to exposure human life to various drugs, alcohol, toxins, and hepatitis viral infections [1]. Liver is vital organ of biliary system required to maintain important homeostasis of the body due its various responsibilities. The liver has got its own importance in the physiological system such as metabolism of ingested substances such as carbohydrates, lipids, proteins, blood coagulation, detoxification process, and immunomodulation are the primary functions of the liver [1]. The liver injury is associated with distortion of these metabolic functions [2] and results into disturbance in homeostasis of the body. However, till now, there is no truly satisfactory liver protective drug in the modern system of medicine which is effective and safe. Hence, natural remedies from medicinal plants are considered to be effective and safe alternative drugs for the treatment of hepatotoxicity and a number of medicinal plants in Ayurveda, the Indian system of medicine, are recommended for the treatment of liver disorders [3].

About 600 commercial preparations with claimed liver protecting activity are available all over the world. About 100 Indian medicinal plants belonging to 40 families are used for herbal formulation [4]. The Tephrosia villosa is native to India and it is medicinally important and used in traditional system for the treatment of liver ailments [5]. The T. villosa is commonly known as Shankhpushpi and used in Ayurvedic system of medicine as memory enhancer, neuroprotective [6], and treatment many ailments. The leaves of this plants contains alkaloids, flavonoids, tannins and phenols [7] and scientifically proved for its antidiabetic [9] antilucre [6], antianxiety [10], antioxidant [11], and many other pharmacological activities. The phytoconstituents of plant leaves are capable of reducing liver toxicity due to their antioxidant properties, but the plant has not been scientifically investigated for evaluation of hepatoprotective activity [11]. In view of this, the present study was undertaken to investigate the hepatoprotective activity of methanol extracts of T. villosa (TVME) leaves against thioacetamide (TAA) induced liver damage in rats.

METHODS

Chemicals

All the chemicals and reagents used in the present study were of analytical grade. The hepatotoxin TAA was procured from Sigma-Aldrich Chemical Pvt. Ltd., Bangalore, and standard drug silymarin was obtained from the Himalaya Drug Company, Bangalore (Nice Chemicals Pvt. Ltd., Bangalore), and Estimation Kits for aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), serum bilirubins, sodium, potassium, and glutathione peroxidase (GPX) were obtained from SPAN diagnostics.

Preparation of plant extract

The plant leaves of T. villosa Linn were collected in Sri Venkateswara University, Tirupati, Andhra Pradesh and authenticated by Dr. Madhava Chetty, Department of Botany, Sri Venkateswara University, Tirupati, Andhra Pradesh and authenticated by Dr. Madhava Chetty. The plant leaves were shade dried and powdered; the coarse powder was subjected to successive extraction with petroleum ether and methanol (70%). Then, marc was subjected to extraction using chloroform water as solvent [12].
Preliminary phytochemical studies
The methanol extract of *T. villosa* was subjected to preliminary phytochemical investigation as per the procedure described by Khandelwal. Dragon Droff’s reagent was used to detect presence of alkaloids. Neutral ferric chloride was used to detect phenolic compounds that appear in the form of blue spots. Folin–Ciocalteu test and Fiegel test was used to detect flavonoids and glycosides, respectively [13,14].

Animals
Healthy Adult Wistar rats weighing 180–200 was purchased from the Venkateswara Enterprises, Bangalore. The animals were housed in well ventilated cage and animals had 12 h day and night schedule with temperature between 28±2°C. The animals were housed in large spacious hygienic cages during the course of the experimental period. The animals were allowed free access to standard laboratory pellets and drinking water *ad libitum*. The study protocol was approved by an Institutional Animal Ethics Committee, IJAHSN (Ref.no.IJAHSM/IAEC/2014/03) with the permission from committee for the purpose of control and supervision of experiments on animals, Ministry of Social Justice and Empowerment, Government of India.

Determination of acute oral toxicity
Acute oral toxicity of methanol (TVME) extracts of *T. villosa* was done according to the OECD guidelines No. 423. The overnight fasted mice were divided into four groups, each group consisting of three female animals. The methanol extract (TVME) of *T. villosa* was given in various doses (5, 50, 300, and 2000 mg/kg b.w.) by gastric incubation with a syringe. After administration of the extract, the animal were observed continuously for the first 2 h and at 24 h to detect changes in behavioral responses and also for tremors, convulsion, salivation, diarrhea, lethargy, sleep, coma and also were monitored up to 14 days for the toxic symptoms and mortality [15].

Evaluation hepatoprotective activity
TAA induced hepatotoxicity in rat’s model [16-18] as used for evaluation of hepatoprotective activity for the plant extracts. The experimental design was as follows:

**Figure 1: Histopathology of liver sample from normal group**

**Figure 2: Histopathology of liver sample from toxic group**

**Figure 3: Histopathology of liver sample from standard group**

**Figure 4: Histopathology of liver sample from TVME (200mg/kg)**

**Figure 5: Histopathology of liver sample from TVME (400 mg/kg)**
RESULTS
Phytochemical investigation
The methanol extract of *T. villosa* Linn. was subjected to different preliminary chemical tests to determine the chemical constituents present in the extracts. The results of study suggested that, methanol extracts consist of alkaloids, flavonoids, tannins, and phenolic compounds.

Acute oral toxicity
The results of acute oral toxicity study suggested that the extract of *T. villosa* that is TVME were safe up to 2000 mg/kg. As per the above study, dose fixation was done and hence low dose was decided as 200 mg/kg and high dose was decided 400 mg/kg for the above extract.

Evaluation of hepatoprotective activity
Administration of plant extract has shown variations in various biochemical parameters in animals induced liver damage by TAA as follows:

Effect of TVME on serum enzymes
The serum enzymes ALT, AST, and ALP were significantly (p<0.001) elevated toxic control group due to administration of TAA compare to animals of normal group as a result liver damage while TVME (200 mg/kg and 400 mg/kg) and standard drug silymarin significantly (p<0.001) reduced concentration serum enzymes in therapeutic animals. The effect of methanol extract was comparable standard drug and it was dose (Table 1).

Effect of TVME on direct bilirubin, total bilirubin and total bilirubin
The administration of TAA induced hepatic injury serum direct bilirubin and total bilirubin were significantly increased in toxic control animals as compared to normal group of animals while there was significant (p<0.001) reduction of direct bilirubin and total bilirubin was observed in animals treated with standard drug silymarin and TVME (200 mg/kg and 400 mg/kg) compared to toxic alone animals. The results were equivalent to normal the effect of methanol extract was dose dependent (Table 1).

In toxic control group animals administered with TAA, significant reduction of serum total protein and albumin was observed due to liver damage compared to normal animals but administration of silymarin and TVME (200 mg/kg and 400 mg/kg) caused dose dependent significant (p<0.001) rise in total protein and albumin therapeutic group compared to toxic animals and the results (Table 2).

Effect of TVME on serum ions
TAA induced liver damage may cause ascites and hence there was significant reduction serum ionic concentration was observed in toxic control animals when compared to animals of normal group but serum ionic concentrations were significantly (p<0.001) increased in animals of therapeutic groups treated with silymarin and TVME (200 mg/kg and 400 mg/kg) when compared to toxic animals. The effect of extract was dose dependent and comparable to standard (Table 2).

Table 1: Effect of methanol extracts of *Tephrosia villosa* on serum enzymes and bilirubin against thioacetamide induced hepatotoxicity in rats

| Treatment | Serum parameters | ALP (IU/ml) | Direct bilirubin (mg/dl) | Total bilirubin (mg/dl) |
|-----------|------------------|-------------|--------------------------|------------------------|
| Normal control | ALT (IU/ml) | 62.35±2.342 | 129.8±2.467 | 78.53±2.170 | 0.0263±0.001026 | 0.3453±0.01345 |
| Toxic control | AST (IU/ml) | 151.9±1.302 | 237.6±5.781 | 207.1±6.816 | 0.312±0.007989 | 0.8943±0.01963 |
| Standard (silymarin) | Standard (TAA) | 63.00±3.543 | 129.0±3.233 | 85.32±3.299 | 0.0752±0.005977 | 0.3738±0.01341 |
| TVME 100 mg/kg | TVME 200 mg/kg | 320.1±16.0 | 213.5±3.300 | 192.9±3.327 | 0.256±0.01430 | 0.821±0.01133 |
| TVME 400 mg/kg | Normal control | 129.1±2.770 | 194.6±4.216 | 130.0±1.201 | 0.1289±0.003469 | 0.6673±0.002521 |

Values are mean±SEM, n=6 symbols represent statistical significance. **p<0.05, ***p<0.01, ****p<0.001 versus diabetic control. +p<0.05, ++p<0.01, +++p<0.001 normal control versus positive control. ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, ALP: Alkaline phosphatase

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Effect of TVME on liver weight
Significant increase in weights of rat’s liver was observed which may be due to damage induction by administration of TAA in toxic control animals as compared to normal animals. In animals treated with reference standard silymarin and TVME (200 mg/kg and 400 mg/kg), there was significant (p<0.001) reduction liver weight compared to toxic animals (Table 3).

Effect of TVME on clotting time
The prothrombin time was prolonged due to deficiency of clotting factors toxic animals compared to normal group as a result of TAA induced liver injury. The dose dependent significant (p<0.001) reduction in clotting time was observed animals treated with standard silymarin while TVME (200 mg/kg and 400 mg/kg) (Table 3).

Effect of TVME on GPX
The serum antioxidant enzyme GPX level was significantly (p<0.001) raised by the administration of reference standard silymarin TVME (200 mg/kg and 400 mg/kg) in therapeutic groups compared to animals of normal toxic group (Table 2).

Liver antioxidant enzymes
There was found to be significant (p<0.001) reduction concentration of liver antioxidant enzymes GPX, CAP, GSD, GRD, and LOP in toxic control animals treated with TAA alone compared to normal animals. While animals of therapeutic groups treated with silymarin and TVME (200 mg/kg and 400 mg/kg), have exhibited significant (p<0.001) rise in liver antioxidant enzyme compare to toxic animals.

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Histopathological evaluation
The administration of TAA was caused the complete loss of the normal architecture of livers in positive control animals with the appearance of vacuolization, fatty degenerations, and coagulative necrosis of liver cells were found to be severe in the centrilobular region. The hepatotoxic metabolite TAA produced excessive formation and deposition of fibrous tissue and results in development of scars. The nodular transformation of rat liver treated with TVME 100 mg/kg has shown, large septa of fibrous tissue flowing together which penetrates into the parenchyma cells were found. However, sections of liver samples belongs to therapeutic groups treated with high doses of methanol extract showed almost normal lobular pattern with tiny and a mild degree of fatty degenerations, necrosis and infiltration of lymphocytes which more or less comparable to the standard drug silymarin treated groups.

DISCUSSION
The fungidal drug TAA get converted into potent hepatotoxins sulfine and sulfene metabolites after biotransformation in liver by cytochrome P450 systems which and produce centrilobular necrosis of hepatic cells. Administration of a single large dose of TAA 100 mg/kg is followed by degenerative changes in liver cells of rats leads to centrilobular necrosis. The pre-necrotic changes include loss of glycogen and acidophilic degeneration of cells in the central zone. The liver damage is always followed by disturbances in the several function of live such as metabolism of nutrients, storage functions, synthetic function, and detoxification process.[20-22].

The disturbance in the metabolism of carbohydrates, fats and proteins is main consequence of liver toxicity which leads to fatty change or fatty characterized by the deposition of fat in liver. Hence, the total weight of liver increases due to the deposition of fat and triglycerides in drug induced hepatic damage[22]. In the present study, weight of rat livers from toxic group was significantly increased due to TAA induced hepatic damage. However, administration of methanol extract and silymarin could able to normalize weight of livers in therapeutic groups indicates their liver protective properties.

Storage of various serum enzymes like ALT, AST and ALP is one of the important functions of liver. ALT and AST transaminases that are involved in transamination reactions of various amino acids while ALP is isoenzyme synthesized mainly by liver and has important role in dephosphorylation of biomolecules. These enzymes are leaked into blood in hepatotoxicity due to liver parenchymal damage and hence their concentrations in serum found to be elevated[23,24]. A mother very

Table 2: Effect of methanol extracts of *Tephrosia pumila*, *Tephrosia villosa*, and *Tephrosia calophylla* on serum albumin, proteins and ions against thioacetamide induced hepatotoxicity in rats

| Treatment | Serum parameters | Albumin (mg/dl) | Total protein (mg/dl) | Sodium (mE/L) | Potassium (mE/L) | Chlorides (mE/L) |
|-----------|------------------|----------------|----------------------|---------------|-----------------|-----------------|
| Normal control | 4.69±0.0735 | 5.367±0.1175 | 138.2±0.8504 | 5.065±0.1428 | 77.43±1.125 |
| Toxic control | 2.39±0.1241 | 2.757±0.09793 | 77.05±2.078 | 2.237±0.07632 | 137.0±0.9558 |
| Standard (silymarin) | 4.538±0.1914 | 5.230±0.04712 | 142.0±4.538 | 5.007±0.1126 | 80.35±1.431 |
| TVME 200 mg/kg | 2.407±0.1103 | 3.293±0.08184 | 81.05±0.4004 | 2.423±0.05308 | 132.0±2.598 |
| TVME 400 mg/kg | 3.615±0.05252 | 3.857±0.1712 | 103.5±1.630 | 3.513±0.05308 | 111.1±1.971 |
| TVME 400 mg/kg | 4.877±0.07233 | 4.853±0.03547 | 129.0±0.5275 | 4.922±0.1027 | 81.10±4.155 |

Table 3: Effect of methanol extracts of *Tephrosia pumila*, *Tephrosia villosa*, and *Tephrosia calophylla* on liver antioxidant enzymes and lipid peroxidase against thioacetamide induced hepatotoxicity in rats

| Treatment | Liver enzymes | GPX (mg/G) | CAP (mg/G) | SOD (mg/G) | GST (mg/G) | GRD (mg/G) | LOP (mg/G) |
|-----------|---------------|----------|-----------|-----------|-----------|-----------|-----------|
| Normal control | 8.95±0.2643 | 58.18±0.215 | 9.35±0.2101 | 7.092±0.4456 | 4.045±0.3952 | 7.490±0.1897 |
| Toxic control | 4.757***±0.2648 | 30.70***±1.445 | 5.467***±1.411 | 3.438***±0.2230 | 2.233***±0.4244 | 17.06***±0.4039 |
| Standard (silymarin) | 8.132***±0.3254 | 50.17***±1.118 | 8.661***±1.334 | 6.568***±0.4929 | 3.852***±0.2376 | 10.11***±0.2847 |
| TVME 200 mg/kg | 5.997***±0.2139 | 44.71***±0.495 | 7.119***±1.632 | 5.215***±0.4532 | 3.690***±0.3902 | 13.00***±0.2515 |
| TVME 400 mg/kg | 8.212***±0.2416 | 50.15***±2.292 | 8.965***±1.184 | 6.833***±0.2671 | 4.130***±0.4354 | 10.18***±0.4769 |

Values are mean±S.E.M, n=6 symbols represent statistical significance. *p<0.05, **p<0.01, ***p<0.001 versus diabetic control, p<0.05, p<0.01*, **p<0.01 normal control versus positive control,GPX: Glutathione peroxidase, CAP: Catalase peroxidase, GST: Glutathione S-transferase, GRD: Glutathione reductase, LOP: Lipid peroxidation
| Treatment            | Liver weight(g) | Clotting time(s) |
|----------------------|-----------------|------------------|
| Normal control       | 5.96±0.7054     | 191.2±7.087      |
| Toxic control        | 8.39±3.09691    | 506.5±15.73      |
| Standard(silymarin)  | 6.01±3.09691    | 191.3±3.0573     |
| TVME 100 mg/kg       | 8.00±3.1190     | 466.8±6.101      |
| TVME 200 mg/kg       | 7.51±0.05224    | 354.5±9.468      |
| TVME 400 mg/kg       | 6.38±0.1512     | 23.2±11.77       |

Values are mean±S.E.M, n=6 symbols represent statistical significance. *p<0.05, **p<0.01, ***p<0.001 normal control versus diabetic control. ns = p>0.05, n.s. = p>0.05, ++p<0.01, +++p<0.001 normal control versus positive control

The results obtained from estimation of biochemical parameters suggesting that methanol extract of *T. villosa* leaves posses significant hepatoprotective property in TAA induced liver toxicity in rats model.

**CONCLUSION**

The results obtained from estimation of biochemical parameters suggesting that methanol extract of *T. villosa* leaves posses significant hepatoprotective property in TAA induced liver toxicity in rats model.

The alcohol induced liver toxicity leads to reduction in total protein is observed due decreased albumin synthesis due to cirrhosis. In present study in toxic animals treated with TAA, the significant reduction of serum albumin and total protein was observed while total protein and serum albumin level was increased by methanol extract treated animals indicated its ability to reverse the hepatic damage caused by TAA.

The liver produces all the clotting factors associated with blood clotting mechanism, and it has main role in regulating normal prothrombin time or clotting time. In liver disorders synthesis of clotting factors will be affected and hence clotting time is prolonged [25]. In our esteemed study, animals induced with liver damage by TAA administration have shown prolonged clotting time. However, animals treated with silymarin and methanol extract have shown significant decrease in clotting time compared to positive toxic animals indicating that methanol extract can reverse complications of hepatotoxicity.

The GPX is antioxidant enzyme synthesized by liver involved in the neutralization of free radicals. In the present study, the administration of silymarin and methanol extract significantly increased the amount of GPX in the therapeutic groups compared to toxic animals. This shows the potential of the methanol extract to increase the concentration of GPX and protects the liver cells against TAA induce free radical mediated effects.

The ability of the living system to counteract free radical mediated damages is natural antioxidant mechanism in which GPX, CAP, GST, GRD and lipid peroxidase are produced in the affected organ/tissue. In the present study there was significant increase in the synthesis of liver antioxidant enzymes found in animals treated with TVME indicating its potential to protect the liver cells against TAA induced free radical damage. The drug induced hepatotoxicity is mainly due to oxidative stress and free radicals mediated damage [22]. Hence, free radical scavenging and antioxidant mechanisms are more important to reverse or prevent drug induced liver toxicity. The extracts of *T. villosa* could reduce the most of the complications of TAA induced hepatotoxicity and also significantly increased liver antioxidant enzymes such as GPX, CAP, GST, GRD and lipid peroxidase which may be the possible mechanism of action of extract. Further, studies are required to correlate the hepatoprotective potentials of the extract with increased glutathione concentrations and also to isolate and evaluate hepatoprotective principle from the methanol extract [26].

Hence in conclusion, the possible mechanism of beneficial liver protecting property of our extract due to its potent antioxidant activity. The histopathological studies supported the results of biochemical tests, showing less damage in the cytoarchitecture of the liver.

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**AUTHORS CONTRIBUTIONS**

All the authors have equal contribution in the present research work.

**CONFLICT OF INTEREST**

We hereby declare that there is no conflict of interests.

**ATHORS FUNDING**

The present research work is not funded by any other external agency.

**Table 4:** Effect of methanol extracts of *Tephrosia pumila*, *Tephrosia villosa*, and *Tephrosia calophylla* on liver weights and prothrombin time against Thioacetamide induced hepatotoxicity in rats

| Treatment          | Liver weight(g) | Clotting time(s) |
|--------------------|-----------------|------------------|
| Normal control     | 5.96±0.7054     | 191.2±7.087      |
| Toxic control      | 8.39±3.09691    | 506.5±15.73      |
| Standard(silymarin)| 6.01±3.09691    | 191.3±3.0573     |
| TVME 100 mg/kg     | 8.00±3.1190     | 466.8±6.101      |
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Important role of liver is detoxification of bilirubin which is breakdown product of hem iron component of hemoglobin. The bilirubin uptake by liver parenchyma cells from the blood and conjugates with gluconic acid in presence of enzyme glucuronyl transferase. Later conjugated bilirubin gets excreted through bile. In liver, toxicity of total bilirubin and direct bilirubin concentration are increased in serum due to reduced ability of liver parenchymal cells [25].

In our study, TAA administration caused elevated concentrations of ALT, AST, ALP, direct bilirubin and total bilirubin animals of toxic control which may be due to reduced function of liver due to toxicity. Treatment with silymarin and methanol extract significantly reduced serum concentrations of enzymes ALT, AST, and ALP indicating the enhanced storage function and also reduced bilirubin levels in blood shows the increased detoxification in therapeutic animals compared to toxic group which could be due to possible protection given by methanol extract.

Serum total protein, also called as total protein or plasma total protein is synthesized by the liver and is an important biochemical test for assessing liver function. The albumin and globulin that are produced in drug induced liver toxicity. The ability of living system to counteract free radical mediated damages is natural antioxidant mechanism in which GPX, CAP, GST, GRD and lipid peroxidase are produced in the affected organ/tissue. In the present study, there was significant increase in the synthesis of liver antioxidant enzymes found in animals treated with TVME indicating its potential to protect the liver cells against TAA induced free radical damage. The drug induced hepatotoxicity is mainly due to oxidative stress and free radicals mediated damage [22]. Hence, free radical scavenging and antioxidant mechanisms are more important to reverse or prevent drug induced liver toxicity. The extracts of *T. villosa* could reduce the most of the complications of TAA induced hepatotoxicity and also significantly increased liver antioxidant enzymes such as GPX, CAP, GST, GRD, and lipid peroxidase which may be the possible mechanism of action of extract. Further, studies are required to correlate the hepatoprotective potentials of the extract with increased glutathione concentrations and also to isolate and evaluate hepatoprotective principle from the methanol extract [26].

Hence in conclusion, the possible mechanism of beneficial liver protecting property of our extract due to its potent antioxidant activity. The histopathological studies supported the results of biochemical tests, showing less damage in the cytoarchitecture of the liver.

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