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Original Article

ANTIOXIDANT ACTIVITY OF TRADITIONAL SIDDHA FORMULATION ON CCL_4 INDUCED LIVER FIBROSIS IN RATS

DIWAKAR MANICKAM[a], KAMATCHI PRIYA RAMAMOORTHY[b], MYTHILY UDHAYA KUMAR[c], B. SANTHOSH KUMAR[d], SAMU SUBRAMANIAM[e*], SHYAMA SUBRAMANIAM[f]

[a,b,c,d]Department of Biochemistry, Regenix Super Speciality Laboratories, Affiliated to University of Madras, Chennai, Tamilnadu, India, [e*]Head of the Department, Department of Biochemistry, Regenix Super Speciality Laboratories, Chennai, Tamil Nadu, India, [f]Consultant, Lab Services, Apollo Hospital, Chennai, Tamil Nadu, India
Email: subramaniam@regenix.in

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ABSTRACT

Objective: The main objective of this study was to evaluate the antioxidant activity of Traditional Siddha Formulation (TSF) on CCl_4 induced liver fibrosis in rats.

Methods: In this study, plant materials were collected, shade dried, mixed in equal proportion and extraction process was done to prepare TSF. Liver injury was induced by intraperitoneal injection of 1 ml/kg body weight of both CCl_4 and olive oil (2:3 v/v) mixture weekly twice for 8 w. The levels of thiobarbituric acid reactive substances (TBARS), lipid peroxides (LPO), protein carbonyl (PC), superoxide dismutase (SOD), catalase (CAT), Glutathione peroxidase (GPx), glutathione reductase (GR), Vitamin C (VIT C), Vitamin E (VIT E), GSH and Total thiols (TTS) were measured in the liver of experimental rats. Histopathological changes in the liver of experimental rats were assessed for each group using hematoxylin and eosin

Results: At the end of the 8-week experimental period, histopathological examination was demonstrated which indicates TSF could attenuate the inflammation and reduced the score of liver fibrosis. The administration of TSF significantly decreased the levels of TBARS (4.11±0.09, $p<0.01$), LPO (53.15±0.79, $p<0.01$), PC (7.31±0.26, $p<0.01$) and significantly increased the levels of antioxidant enzymes such as SOD (7.10±0.16, $p<0.01$), CAT (62.14±1.61, $p<0.01$), GPx (90.79±1.05, $p<0.01$), GR (144.06±1.61, $p<0.01$), GSH (35.24±0.82, $p<0.01$), VIT C (3.29±0.10, $p<0.01$), VIT E (2.42±0.14, $p<0.01$) and TTS (15.15±0.21, $p<0.01$).

Conclusion: TSF inhibits CCl_4 intoxicated hepatic fibrosis in Wistar Albino rats which may be due to the action of synergistically active phytochemicals present in the formulation. Enzymatic and non-enzymatic antioxidant enzyme levels were found to be increased in the treatment group which may be related to the therapeutic effect of TSF.

Keywords: Antioxidant, TSF, Siddha and CCl_4

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INTRODUCTION

Liver fibrosis is a wound healing process which is characterized by the accumulation of large amounts of extracellular matrix which leads to architectural changes of the liver, pathological disturbance, development of intrahepatic shunts and portal hypertension [1]. Viral hepatitis, chronic alcohol abuse, toxic drug exposure and nonalcoholic fatty liver disease are the pathological factors that contribute to the development of liver fibrosis [2]. CCl_4 is a potent hepatotoxic chemical which induces hepatocellular damage and liver fibrosis in animal models [3]. CCl_4 induced liver disease model is similar to human liver diseases, especially hepatic fibrosis [4]. CCl_4 damages liver by the production of reactive oxygen species (ROS) during the process of metabolism and induces lipid peroxidation in the cellular organelles of liver [5]. The liver is a major organ affected by ROS [6].

Siddha system of medicine is one of the oldest systems of medicine practiced in South India which is more popular in Tamil Nadu. The aim of Siddha medicine is to make the body perfect, imperishable and increase longevity. TSF is a polyherbal Siddha formulation containing seven ingredients. The plants used in TSF possess various therapeutic activities which are reported in Siddha literature. Preliminary phytochemical analysis of TSF showed a wide range of therapeutic phytochemicals such as phenolics, flavonoids and terpenoids [7]. TSF possesses strong antioxidant, free radical scavenging activity and it is nontoxic to normal human embryonic kidney cell lines *in vitro* [8]. TSF is a promising anti-fibrotic formulation which improves biochemical markers and reverses the histopathological changes induced by CCl_4 [9]. In scientific literature, *Terminalia chebula* is regarded as the mother of the herb, its seed coat possesses antioxidant [10] and antibacterial activities [11]. *Phyllanthus amarus* has an effective free radical scavenging activity and helps to reduce oxidative stress [12]. *Sphagneticola calendulacea is an* effective hepatoprotective medicinal plant commonly used to treat jaundice [13]. *Curcuma longa* commonly known as turmeric in English and manjal in Tamil used as a medicinal food with effective antioxidant activity [14]. *Cuminum cyminum* a common medicinal seed possesses free radical scavenging and antioxidant activity [15]. *Emblica Officinalis* commonly known as Indian gooseberry has the potential of antimicrobial, antioxidant activity, immunostimulatory and hepatoprotective activity [16]. *Terminalia belerica* is regarded to possess a good anti-diabetic and antioxidant activity [17]. Most of the herbs used in Traditional Siddha formulation have been used in Indian traditional food.

Free radicals are atom or molecule that have an unpaired electron, usually unstable and highly reactive that damage cells and contribute to ageing and diseases [18]. Oxygen free radicals such as superoxide, hydroxyl radicals and peroxide radicals are the few known reactive oxygen species which are generated during the metabolic process of oxygen [19]. ROS has special chemical characteristic can initiate lipid peroxidation, which leads to breaking down of molecules present in the biological membrane which leads to cell death. [20]. Oxidative stress plays a major role in the initiation and progression of various liver diseases [21]. There is scientific evidence which proves that increased intake of antioxidants decreases the incidence of cancer [22]. The aim of the present study was to observe the antioxidant activity of TSF on liver fibrosis induced by CCl_4 in Wistar rats.

MATERIALS AND METHODS

Chemicals

CCl_4 was purchased from Sigma-Aldrich, USA. CCl_4 was diluted with olive oil in the ratio of 2:3 v/v and all other chemicals used were of high purity and analytical grade.

Traditional Siddha formulation preparation

All the botanicals in TSF were authenticated by the Central Siddha Research Institute, Chennai Tamil Nadu and India. Preparation of TSF was described earlier [7].

Animals and experimental design

Institutional Animal Ethical Committee (IAEC) at Saveetha University approved the experimental protocol of this study (SU/BRULAC/RD/ 019/2014). Albino male Wistar rats weighing 180–200 g were used in this study. All rats were housed in polyethene cages under controlled laboratory conditions of temperature 20-22˚C with 12h dark and light cycle and provide with standard rat pellet and water, *ad libitum.* All the animal procedures were performed under the guidelines set by CPCSEA. Animals were randomly divided into four groups (n = 6 rats per group). Group 1 (Normal control) received free access to pure drinking water and normal pellet diet. Group 2 (Model group) received CCl_4 1 ml/kg body weight intra-peritoneal twice weekly for eight weeks as described by Diwakar *et al.* (2017) [9]. Group 3 (Therapeutic model) received CCl_4 as per group 2 followed by TSF orally 400 mg/kg body weight daily from 5th week to end of the experiment. Group 4 (TSF control) received TSF orally 400 mg/kg body weight daily throughout of experiment.

Assessment of antioxidants and lipid peroxidation

The TBARS was measured by the method of Ohkawa *et al.* [23]. Lipid peroxides were estimated by the method of devesagayam and tarachand [24]. Protein carbonyl was measured by the method of Reznick and Packer [25] Catalase was assayed by the method of Sinha [26]. Superoxide dismutase was assayed by the method of Misra and Friedrich [27]. Glutathione peroxidase was assayed by the method of Rotruck *et al.* [28]. Glutathione reductase activity was measured by the method of Maron *et al.*, [29] Vitamin E was estimated by the method of Quaife and Dju [30] Vitamin C was estimated by the method of Omage *et al.* [31]. Reduced glutathione was measured by the method of Linden Maier *et al.* [32].

Assessment of histopathological examination

The liver specimen of the experimental rat was fixed in 10% neutral buffered formalin solution, dehydrated with graded alcohol, embedded in paraffin. A thin section of 5-micrometer thickness was made and stained with hematoxylin and eosin. Histological changes were examined under a light microscope (Labomed).

Statistical analysis

The values are expressed in the mean±SD for the six rats in each group. Statistically significant differences between the groups were calculated using SPSS. Hypothesis testing methods included one-way analysis of variance (ANOVA). The value of $p<0.05$ was considered to be statistically significant.

RESULTS

Macromolecular damage

In experimental rats, CCl_4 induced oxidative stress which was proud to the increased levels of LPO, TBARS, and PC when compared to control group. TSF significantly ($p< 0.05$) reverted to normal levels in the rats of therapeutic and TSF control groups.

Antioxidants

Values of enzymatic antioxidant enzymes such as SOD, CAT, GPx, and GR in the liver of experimental rats were shown in table 2. CCl_4 induced group showed a significant decline of SOD, CAT, GPx, and GR when compared to the normal group. However, rats in the therapeutic group showed a significant increase ($p< 0.05$) in the activities of the antioxidant enzyme to the near normal level as shown in table 2.

Table 1: Effect of TSF on macromolecular damage in liver of experimental animals

Parameter	Control	Model Group	Therapeutic Group	Drug Control
TBARS	3.99±0.11	8.44±0.21^{a*}	4.11±0.09^{b*}	3.94±0.10
LPO	49.88±1.19	69.26±1.17^{a*}	53.15±0.79^{b*}	46.78±0.76
PC	6.76±0.18	16.82±1.25^{a*}	7.31±0.26^{b*}	6.50±0.14

Values are expressed as mean±SD. (n=6). Statistical significance at $p< 0.05$. Comparisons are made with 'a' control group and 'b' induced group. $^{*}p< 0.05$. Units: TBARS: nmoles/100 g tissue, LPO: nmoles of MDA formed/mg protein and protein carbonyl: nmoles of DNPH formed/min/mg protein.

Table 2: Effect of TSF on enzymatic antioxidants in the liver of experimental animals

Parameter	Control	Model group	Therapeutic group	Drug control
SOD	7.80±0.21	4.35±0.22^{a*}	7.10±0.16^{b*}	7.38±0.11
CAT	65.67±1.64	43.52±1.98^{a*}	62.14±1.61^{b*}	63.88±0.62
GPx	101.18±0.80	56.42±1.25^{a*}	90.79±1.05^{b*}	102.26±0.63
GR	158.77±1.37	100.27±1.41^{a*}	144.06±1.61^{b*}	157.68±1.06

Values are expressed as mean±SD. (n=6). Statistical significance at $p < 0.05$Comparisons are made with 'a' control group and 'b' induced group. $^{*}p< 0.05$. Units: SOD activity is expressed as 50% inhibition of epinephrine auto-oxidation, CAT activity is expressed as µmol of H_20_2 decomposed/min/mg protein, GPx activity is expressed as µmol of GSH oxidized/min/mg protein and GR activity is expressed as µmol of NADPH oxidized/min/mg protein.

The levels of non-enzymatic antioxidants such as vitamin C, vitamin E, GSH and total thiols in the liver are depicted in table 3. Similar to enzymatic antioxidant status, the level of Vitamin C, Vitamin E, GSH and total thiols were also significantly decreased in CCl_4 induced group when compared to the normal group. However, treatment with TSF after CCl_4 intoxicated group showed significantly ($p<0.05$) elevated the reduced level of Vitamin C, Vitamin E, GSH and Total thiols to near normal level shown in table 3.

Table 3: Effect of TSF on non-enzymic antioxidants in liver of experimental animals

Parameter	Control	Model group	Therapeutic group	Drug control
GSH	40.24±1.04	17.71±0.48^{a*}	35.24±0.82^{b*}	40.94±0.56
Vitamin C	3.64±0.20	1.51±0.20^{a*}	3.29±0.10^{b*}	3.79±0.12
Vitamin E	2.70±0.15	1.34±0.08^{a*}	2.42±0.14^{b*}	2.81±0.10
Total thiols	5.51±0.08	3.59±0.12^{a*}	15.15±0.21^{b*}	5.68±0.06

Values are expressed as mean±SD. (n=6). Statistical significance at $p< 0.05$. Comparisons are made with 'a' control group and 'b' induced group. $^{*}p< 0.05$. Units: GSH mg/100 g tissue, Vitamin C and E (mg/g wet tissue), TTS µg/mg protein.

Effect of TSF on histopathological examination

Control rats showed the normal histological structure (fig. 1A) whereas CCl_4 induced showed the severe fibrous expansion with septae (fig. 1B). TSF treated rats showed the complete reversal of pathological anomalies to near normal histological appearance when compared to CCl_4 induced group (fig. 1C). TSF treated group showed normal histology (fig. 1D).

DISCUSSION

CCl_4 induced liver damage is attributed to the formation of the metabolic product, i.e. trichloromethyl radical and peroxy trichloromethyl radical generated during biotransformation reaction by the cytochrome p450 system [33]. Both of this highly reactive radical bind covalently to the macromolecules and initiate lipid peroxidation of fatty acids (especially polyunsaturated fatty acid) in the cell membrane, resulting in cell damage [34]. CCl_4 intoxicated rats showed increased lipid peroxidation product such as MDA and TBARS levels indicating hepatocellular damage and failure of the antioxidant defence mechanism [35]. Lipid peroxides induce fibrogenic cytokines and initiate HSC activation resulting in increased collagen synthesis [36]. The present study demonstrates that increased MDA and TBARS levels in CCl_4 intoxicated group correlate with previous studies [37]. Interestingly TSF administration reverted the MDA and TBARS levels to near normal level due to its free radical scavenging activity and polyphenol-rich compound present in the TSF. Protein carbonyl is an effective biomarker for oxidative stress due to its high stability and it increases in CCl_4 induced hepatocellular damage [38]. Dietary antioxidant and polyphenol effectively act against reactive oxygen species thereby reducing protein carbonyl content [39]. TSF treatment effectively decreased the protein carbonyl content to a near normal level which is attributed to the presence of polyphenols, flavonoids, and other phytochemicals that act as an antioxidant by scavenging the free radicals generated by CCl_4.

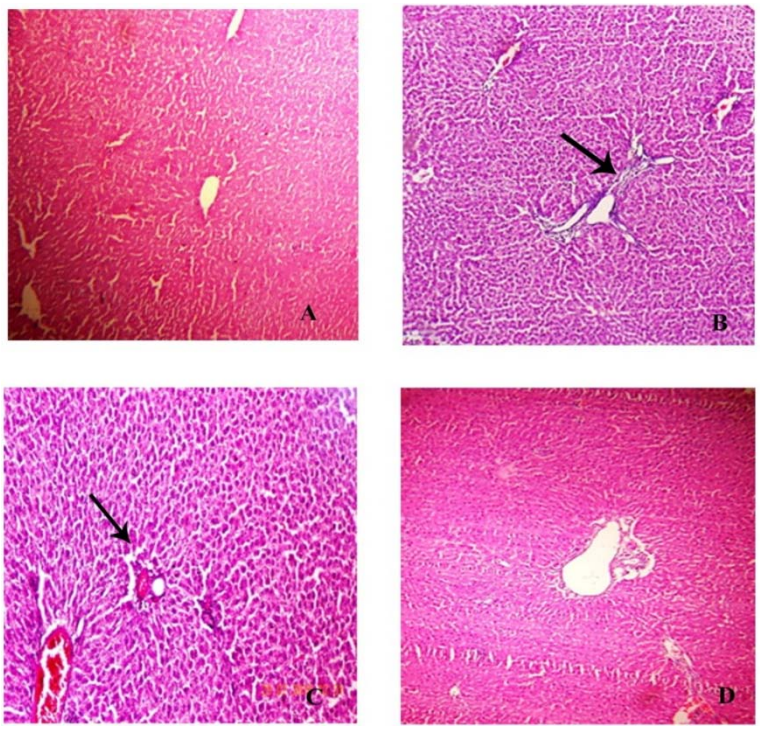

Fig. 1: Microscopic examination of the liver in experimental groups determined by hematoxylin and eosin (original magnification 10x liver section in rats) staining is presented in A–D; (A) Control; (B) CCl_4 induced liver showing fibrous expansion with septae; (C) CCl_4+TSF treated group showing nil fibrosis and very mild inflammation; (D) TSF alone group showing normal histology

The decreased levels of enzymatic antioxidants like SOD, CAT, GPx, and GR was observed in CCl_4 intoxicated group. Antioxidant enzymes protect the biological system from oxidative damage. SOD is a metalloenzyme acts as the first line of defence against superoxide radicals by dismutation, converting into H_2O_2 and O_2 [40]. Catalase and GPx is a very important enzyme defence against dangerous reactive oxidizing molecules converted to H_2O, thereby providing protection to the cells from free radicals and also GPx reduces the lipid hydroperoxide and H_2O_2 to non-toxic product [41].

GR maintain the ratio of GSSH/GSH present in the cell thereby maintaining the oxidative balance of the cell [42]. It has been a demonstration that many plants have abundant natural antioxidants, and are capable of eliminating free radicals and protecting the liver from oxidative stress [43]. Interestingly TSF treatment increased the enzymatic antioxidants like SOD, CAT, GPx, and GR to normal levels mainly, due to phyto ingredients such as polyphenol, flavonoid, tannin, and alkaloids. These antioxidant activities of the TSF are based on free radical scavenging, hydrogen donation and chelating metal ions.

The severe depletion of non-enzymatic antioxidants such as GSH, Vitamin C and Vitamin E was recorded in CCl_4 induced group. GSH plays an important role in the detoxification of xenobiotic compound [44], a decrease of GSH in CCl_4 induced rats were observed in this study. TSF supplementation increased the GSH level significantly, this might be due to a high level of phenolics and flavonoids present in compounds of TSF. Vitamin C and Vitamin E is an effective biological antioxidant showed could act synergistically in the scavenging of various reactive oxygen species, Vitamin C supplementation reduces diseases associated with oxidative stress [45]. Tannin is reported to be responsible for retrieval of Vitamin C activity [46]. The increase in the non-enzymatic antioxidant markers after the TSF treatment might be due to plants in the TSF as such natural source of vitamins and minerals. Total thiols decreased in the CCl_4 intoxicated rats, due to antioxidant and free radical scavenging activity of TSF, the total thiols were resumed to near normal level in TSF treated animals. The present study provides strong evidence that TSF has a therapeutic effect on CCl_4 induced liver fibrosis, reverses the free radical damage from CCl_4 by increasing both the enzymatic and non-enzymatic defence mechanism.

CONCLUSION

The protection by TSF against oxidative stress in CCl_4 induced liver fibrosis might be due to decreased macro molecular damage and stabilization of antioxidant defense mechanism. These findings suggest that TSF can attenuate CCl_4 induced liver fibrosis via synergistically active phytochemicals present in this formulation.

CONFLICTS OF INTERESTS

All authors have none to declare

AUTHORS CONTRIBUTION

Design, experimental part of the work and writing of the manuscript was done by the first author Diwakar Manickam. Standardization of above-mentioned TSF was done by Sri Kamatchi Priya Ramamurthy. Formatting of the manuscript was done by Mythily Udhayakumar and B. Santhosh Kumar. Design of the work was done by Dr. Samu Subramaniam and correction of the manuscript was done by Dr. Shyama Subramaniam

CONFLICT OF INTERESTS

Declared none

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