Hepatoprotective Potential of Novel Siddha Formulation *Santha Santhrodhaya Mathirai* against Paracetamol and Methotrexate Induced Hepatotoxicity in Chang liver Cell line Model

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Abstract

Liver is the most important metabolic organ primarily concerned with the biochemical activities in the human body. Hepatocytes (liver parenchymal cells) perform essential metabolic functions. Exposure to environmental pollutants such as toxicants and chemicals often results in liver injury and damage. Liver disorders can result from a wide variety of insults, including infections, drugs, toxins, ischemia, and autoimmune disorders. Hepatic dysfunction is primary causes of morbidity and mortality worldwide. India subcontinent is well known for its usage of traditional preparations and medicines from siddha origin that is often considered to be protective and curative in nature against liver disorders. Siddha has several indigenous preparations which comprises of herbs, minerals and polyherbs which has been used for the treatment of liver injury since several centuries. One such siddha formulation is *Santha Santhrothaya Mathirai* (SSM) which majorly comprises of borax, mercurous chloride, turmeric and lemon juice. As there is increasing evidence that conventional allopathic drugs used for liver rejuvenation has their own side effects hence it is the need of the hour of exploring an alternate complimentary traditional formulation from siddha origin which has both preventive and rejuvenation potential. The main aim of the present investigation is to evaluate the hepatoprotective nature of the formulation SSM against Paracetamol and methotrexate induced hepatotoxicity in chang liver cell line model. Chang liver cells, a human hepatoma cells were now a days been widely used to evaluate the hepatoprotective nature of the drugs and chemicals. The results obtained from the present investigation has revealed that treatment with paracetamol has significantly up-regulated cytochrome P 450 - CYP gene further it was observed that treatment with paracetamol down regulated the UGT- uridine glucuronosyl transferase and ALB-Albumin gene in the chang liver cells. Treatment with SSM at the dose of 10, 100 and 1000 ng/ml dose dependently ameliorated these alterations by up regulating the UGT, ALB and down regulating the CYP gene. Similar results were observed in immunoblotting analysis. Treatment with methotrexate increases the expression of pro-apoptotic BAX gene and decreases the anti-apoptotic BCL-2 , DHFR-Dihydrofolate reductase gene expression. Incubation with SSM at the dose of 10, 100 and 1000 ng/ml dose significantly reverse these changes which was further evident by the results of Immunoblotting assay. It was concluded from the data's obtained from the present investigation that the drug SSM possess promising hepatoprotective activity against paracetamol and methotrexate induced insult in chang liver cell line and hence this formulation may be considered as first drug of choice for the clinical management and treatment of liver disease in humans.

Key Words: Liver, Siddha *, Santha Santhrothaya Mathirai,*, Hepatoprotective, Chang liver cell line ,Paracetamol, Methotrexate, Gene expression.

1. INTRODUCTION

The liver is an important metabolic organ that plays a vital role in the maintenance of homeostasis. The liver is majorly responsible for multiple metabolic functions and physiological processes such as detoxification, bile synthesis, energy production, biotransformation of lipid, proteins and carbohydrates. Toxicology is a branch of science that deals with study of the adverse effects of chemical or physical agents on living organisms. A toxicologist is trained to examine and communicate the nature of those effects on human, animal, and environmental health [1-2]. Toxicological research examines the cellular, biochemical, and molecular mechanisms of action as well as functional effects such as physiological, neurobehavioral and immunological, and assesses the probability of their occurrence. Toxic agents are categorized in a variety of ways, depending on the interests and needs of the classifier. For example, toxic agents are discussed in terms of their target organs (liver, kidney, hematopoietic system, etc), use (pesticide, solvent, food additive, etc), source (animal and plant toxins), and effects (cancer, mutation, liver injury, etc) [3].

Due to non-availability of satisfactory and adequate number of synthetic drugs for prevention and treatment of liver disorders leads to further damage to the liver. Therefore, there is an emergency need of effective drugs from natural origin for the prevention and treatment of liver diseases. Recently there is a greater global interest in non-synthetic, natural drugs derived from Indian system of traditional medicine due to better tolerance and minimum adverse drug reactions. Siddha system of traditional

medicine is one of the oldest practice of medicine known to mankind. It works bend the principles of prevention, curative and rejuvenation. Siddhars the earlier physicians has made several formularies describing the methods on purification, detoxification and formulations derived from herbs, minerals, metals and from natural ores. One such novel siddha formulation is *Santha Santhrothaya Mathirai* (SSM) which majorly comprises of borax, mercurous chloride, turmeric and lemon juice. Curcumin, a chief constituent of turmeric has been proven to have clinically effective and therapeutically beneficial by which its antioxidant properties play an important role in the management of chronic inflammation diseases. Researchers have reported that curcumin has a therapeutic potential as antifungal, antiviral, antioxidant, anti-inflammatory, and further it reveals several pharmacological action in biological system [4].

Extract of lemon traditionally used for the treatment of liver disorders, rheumatism, fever and febrile diseases, further it is used as refresher, a dietary supplement, antidysenteric, antispasmodic for gastralgia, and in colds and fever. The fruit juice is used to treat diabetes and high levels of cholesterol. *Citrus limon*. contains a number of biologically active components such as citric acid, ascorbic acid, minerals, coumarins, limonoids, and flavonoids. Ascorbic acid (vitamin C) is an antioxidant reported to prevent reactive oxygen species (ROS)-mediated microsomal lipid peroxidation and protein degradation in both *in vivo* and *in vitro* [5].The present research study aimed to investigate the hepatoprotective nature of the siddha formulation *Santha Santhrothaya Mathirai* against Paracetamol and methotrexate induced hepatotoxicity in chang liver cell line model.

2. MATERIALS AND METHODS

2.1 Collection of raw materials

The raw drug Venkaaram (Borax) and Pooram (Mercurous chloride) were procured from reputed raw drug store at Erode. Pepper and betel leaves which are used in the purification of pooram, turmeric and lemon which are used in the preparation of SSM were procured from Erode, Tamilnadu,India.

2.2 Authentication of raw materials

The herbal ingredients of SSM such as turmeric and lemon were authenticated to confirm the identity of herbal species using organo-leptic characters by Botanist, National Institute of Siddha and pharmacognostical study of herbal ingredients were done in Plant Anatomy Research Centre, West Tambaram, Chennai. The mineral ingredients of this study drug were authenticated at the Dept. of Geology, Anna University campus, Guindy.Chennai, Tamil Nadu, India.

2.3 Purification of ingredients of Santha Santhrothaya Mathirai (SSM)

2.3.1 Purification of Pooram (Mercurous chloride)

30 g of black pepper was coarsely powdered and added with 30 g of sliced betel leaves in a black stone mortar and made into karkam (Pasty consistency) by trituration with water. The karkam was mixed with 3.6 L of water in a mud pot. 120 g of Raw Pooram (Sample P) was knotted in

a cotton cloth and soaked in the water by hanging in the above pot as Thula Iyanthiram. The mud pot was heated using firewood under low flame until the water was reduced to 900ml. The knotted Pooram was taken out and the cloth over the Pooram was removed and washed with water and dried under sunlight[4]. This purified form of Pooram was coded as Sample P1 and observed for loss of weight. The same process was repeated for the purification of another two samples of raw Pooram and the obtained purified Poorams were coded as Sample P2 and P3. The raw sample P and purified Pooram samples P1, P2 & P3 were analyzed.

2.3.2 Purification of Vengaram (Borax)

Vengaram –180 g was coarsely powdered using the black stone mortar and roasted over the mud plate until water evaporated out. The fried Vengaram became puffed[4]. Loss of weight was calculated. The purified form of Vengaram was coded as sample V1 and observed for loss of weight. The same process was repeated for the purification of another two samples of raw vengaram and the obtained purified Vengaram were coded as sample V2 and V3.

2.3.3 Preparation of SanthaSanthrothayaMathirai [6]

The purified Pooram, Vengaram and Kappumanjal were powdered separately and mixed uniformly in a kalvam (Black stone Mortar). To this mixture, lemon juice was added constantly and triturated for 12 hours and made into pill rolling pasty consistency. It is then made into pepper sized pills (60mg) and dried in shade.

Three batches of this preparation (SSM-A, SSM-B, SSM-C) were made in a similar manner as per Siddha literature for standardization purpose. The trial drugs were prepared at a GMP certified manufacturing company, Erode.The weight and size of SSM Pills were determined by using analytical balance and vernier caliper respectively.

2.4. Cell culture and maintenance [7]

Chang liver cells, a human hepatoma cells were obtained from National Centre for Cell Science (NCCS), Pune, India. Cells were maintained in Minimum Essential Media (MEM) supplemented with 10% Fetal Bovine Serum (FBS), with 100units/ml penicillin and 100µg/ml streptomycin. Cells were cultured in $75cm^2$ culture flask and incubated at humidified atmosphere with 5% CO_2 at 37°C.

2.5.Paracetamol induced hepatotoxicity in chang liver cells [8]

Chang liver cells were seeded in 6 well plates at a density of $1X10^5$ cells/well and allowed to grow for a period of 24 h. Test drug was administered at a concentration of 10 ng, 100 ng and 100 µg / ml. One hour following test drug exposure, paracetamol dissolved in syringe water (100µg/ml) was added to each well except the control and incubated for a period of 24 h. Cells were then trypsinised for measuring gene and protein expressions of albumin (ALB), uridine glucuronosyl transferase (UGT), and cytochrome P450 (CYP).

2.6.Methotrexate (MTX) induced hepatotoxicity in chang liver cells [9-10]

Chang liver cells were seeded in 6 well plates at a density of $1X10^5$ cells/well and allowed to grow for a period of 24

h. Test drug was administered at a concentration of 10, 100 and 1000 ng/ml. One hour following test drug exposure, methotrexate dissolved in DMSO (100mM) was added to each well except the control and incubated for a period of 24 h. Cells were then trypsinised for measuring gene and protein expressions of DHFR, BAX and BCL2.

2.7.Reverse transcriptase - Polymerase Chain Reaction (RT-PCR) [11]

Total RNA was extracted using TRIzol Reagent (Sigma, USA). After homogenizing the cells with TRIzol reagent, the tubes was incubated for 10 min and centrifuged at 1000 rpm for 5 min. 200 µl of chloroform was added to the supernatant, allowed to incubate for 5min at room temperature and centrifuged at 12000 rcf for 20min. Then 500 µl of isopropyl alcohol was added to the supernatant to precipitate the total RNA and centrifuged at 12000 rcf for 15min following the incubation period of 10 min. The supernatant was decanted carefully; the pellet was washed thrice with 75% ethanol, centrifuged at 12000 rcf for 15 min. The pellet was air dried and re-suspended in 20 µl of RNase free water and stored in -80°C until use. RT-PCR was carried using PCR master cycler gradient (Eppendorf, Germany) and semi-quantified using Bio1D software in gel documentation (Vilber Loumart, France).

Primer sequence used were as follows,

Primer	Forward Primer	Reverse Primer
GAPDH	5'-CGACAGTCAGCCGCATCTT-3'	5'-CCAATACGACCAAATCCGTTG-3'
ALB	5'-CTTGAATGTGCTGATGACAGG-3'	5'-GCAAGTCAGCAGGCATCTCAT-3'
UGT	5'-CCTCTTTCCTATGTCCCCAATGA-3'	5'-CCTTAGTCTCCATGCGCTTTGC-3'
CYP	5'-GTGATGCCCTGGCTGCAG-3'	5'-AATCGAGCTGGATCAAAGTTC-3'
DHFR	5'-CTGTCATGGTTGGTTCGC-3'	5'-AAGCTTTTGGTATTTCCA-3'
BAX	5'-TTTTGCTTCAGGGTTTCATC-3'	5'-GACACTCGCTCAGCTTCTTG-3'
BCL2	5'-ATGTGTGTGGAGAGCGTCAACC-3'	5'-TGAGCAGAGTCTTCAGAGACAGCC-3'

2.8.Immunoblotting [12]

Cells were homogenised in 0.1 M ice cold Tris hydrochloric acid (pH 7.4) and centrifuged at 3500 rpm for 10 min. Protein concentrations of the supernatant were measured using Bradford reagent (Bradford, 1976). Aliquots containing 10 µg total proteins per sample were mixed with sample loading dye (β-mercaptoethanol and bromophenol blue) and electrophoresed on a 12% sodium deodecyl sulphate polyacrylamide gel (Hoefer, USA). Separated proteins were transferred to polyvinylidene difluoride membranes, which were blocked overnight in 3% bovine serum albumin in Tris-buffered saline. Membranes were then washed thrice for 5 min each in Tris buffered saline and probed with primary antibodies. After three 5 min washes in Tris base saline (TBS), membranes were probed with secondary antibodies for 1 h then washed again in TBS and incubated in BCIP/NBT substrate for alkaline phosphatase kit for 10min. Bands obtained were visualised and semi-quantified using Bio1D software in gel documentation (Vilber Loumart, France). The following primary antibodies were used: mouse anti ALB (1:100; Santa cruz biotechnology, USA), rabbit anti UGT (1:200; Santa cruz biotechnology, USA), mouse anti CYP (1:200; Santa cruz biotechnology, USA), mouse anti BAX (1:200; Santa cruz biotechnology, USA), rabbit anti BCL2 (1:200; Santa cruz biotechnology, USA) and mouse anti DHFR (1:100; Santa cruz biotechnology, USA). Secondary antibodies used were as follows: Goat anti-rabbit IgG (for UGT and BCL2; Santa cruz biotechnology, USA) and goat anti-mouse IgG (for ALB, CYP, BAX and DHFR; Santa cruz biotechnology, USA) at 1:1000 dilution.

2.9.Data analysis

Data were expressed in mean±SEM. Mean difference between the groups were analyzed by one way anova followed by turkey's multiple comparison test using graph pad prism 5.0. $p < 0.05$ was considered as statistically significant [#, ## - indicates $p < 0.05$ and 0.01, respectively vs group I; *, ** - indicates $p < 0.05$ and 0.01, respectively vs group II)

3. RESULTS

3.1 Effect of SSM on CYP, UGT and ALB gene Expressions on Paracetamol induced Chang liver cell toxicity

Paracetamol exposed chang liver cells showed significant ($p < 0.01$) up-regulation of CYP expression and down-regulation of UGT and ALB gene expressions when compared to normal cells. Treatment with siddha formulation SSM at 10, 100 and 1000 ng/ml significantly and dose dependently ameliorated these alterations and reverse the scenario by up regulating the UGT, ALB and down regulating the CYP gene. The results were represented in figure 1 and 2 A-C.

3.2 Effect of SSM on Immunoblotting analysis of CYP, UGT and ALB genes

Treatment with paracetamol shown significant ($p < 0.01$) up-regulation of CYP and down-regulation of UGT and ALB protein expressions in chang liver cells when compared to normal cells. Test drug exposure at 10, 100 and 1000 ng/ml dose dependently ameliorated these changes. The results were represented in figure 3 and 4 A-C.

3.3 Effect of SSM on BAX, BCL2 and DHRF gene Expressions on Methotrexate induced Chang liver cell toxicity

Anti-apoptotic BCL-2 gene and Pro-apoptotic BAX gene plays a crucial role in liver cell degeneration and apoptosis. The results of the present study has shown that significant ($p < 0.01$) increase in BAX and significant ($p < 0.01$) decrease in BCL2 and DHFR gene expressions were observed in methotrexate induced chang liver cells when compared to normal cells. Treatment with test drug significantly decreased BAX and increased BCL2 and

DHFR gene expressions. The results were represented in figure 5 and 6 A-C.

3.4 Effect of SSM on Immunoblotting analysis of BAX, BCL2 and DHRF genes.

A significant (p<0.01) increase in BAX and significant (p<0.01) decrease in BCL2 and DHFR protein expressions were observed in methotrexate induced chang liver cells in comparison to normal cells. Test drug exposure significantly and dose dependently ameliorated these changes. The results were represented in figure 7 and 8 A-C.
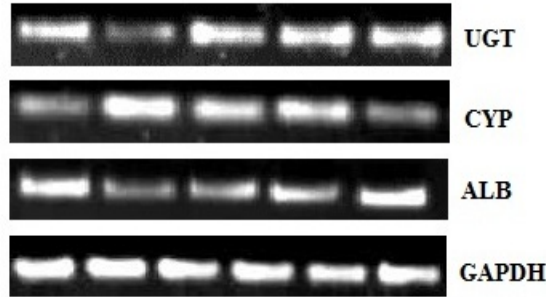
Figure 1: Quantitative representation of mRNA expression of CYP, UGT and ALB gene Expressions
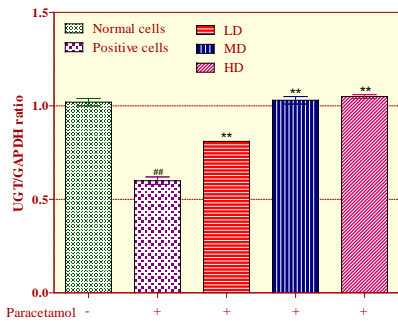
Figure 2 A: UGT/GADPH Ratio   **Figure 2 B: CYP/GADPH Ratio**   **Figure 2 C: ALB/GADPH Ratio**
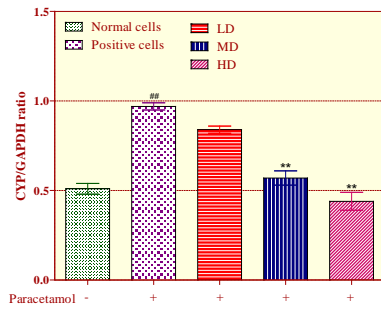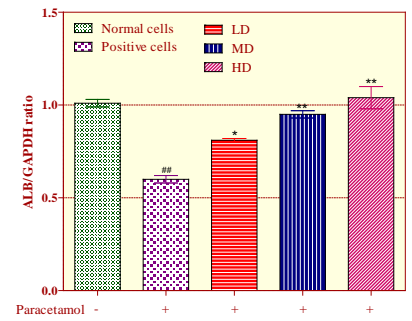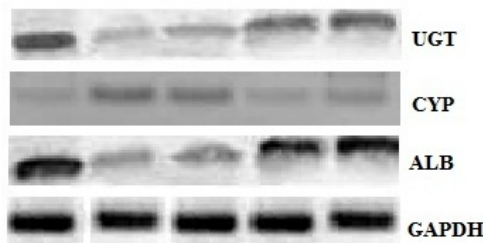
Figure 3: Immunoblotting Analysis on expression of CYP, UGT and ALB gene Expressions
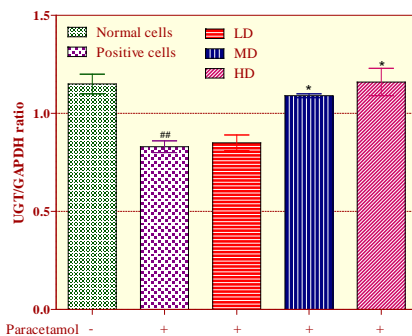
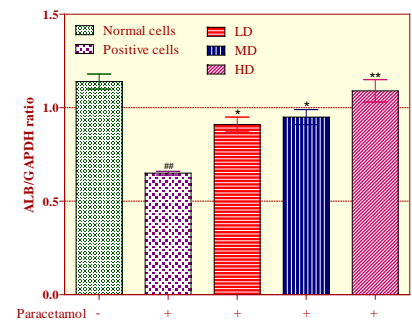**Figure 4 A: UGT/GADPH Ratio**   **Figure 4 B: CYP/GADPH Ratio**   **Figure 4 C: ALB/GADPH Ratio**
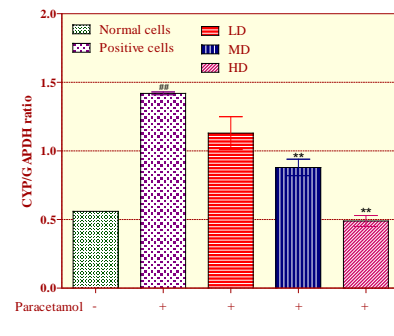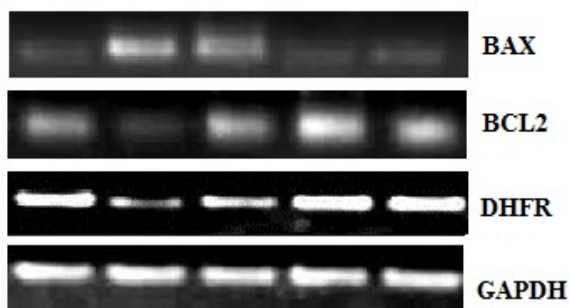
Figure 5 : Quantitative representation of mRNA expression of BAX, BCL2 and DHRF gene Expressions
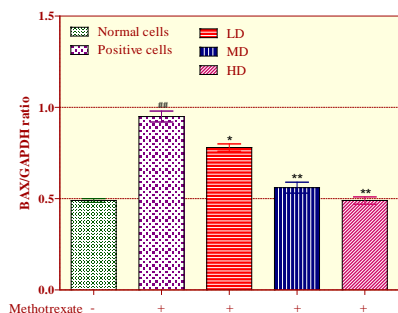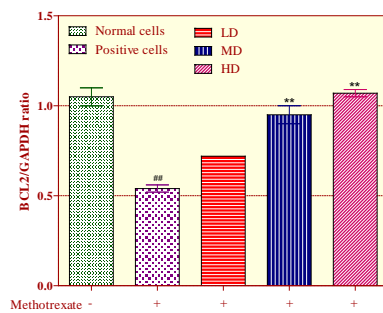

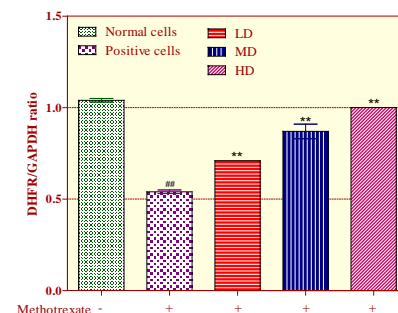**Figure 6 A: BAX/GADPH Ratio** **Figure 6 B: BCL2/GADPH Ratio** **Figure 6 C: DHFR/GADPH Ratio**
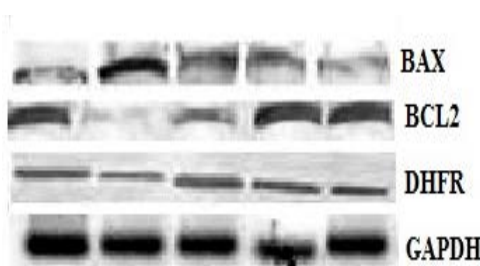

Figure 7: Immunoblotting Analysis on expression of BAX, BCL2 and DHRF gene Expressions
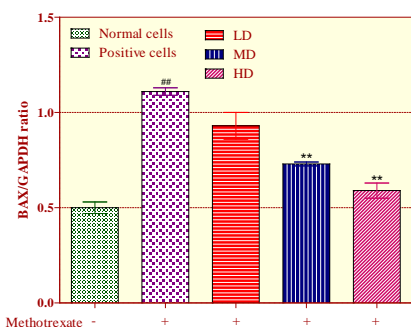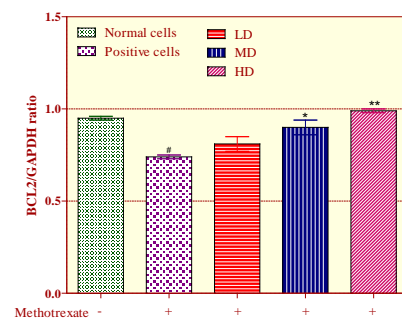

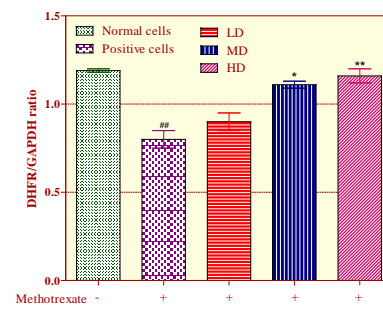**Figure 8 A: BAX/GADPH Ratio** **Figure 8 B: BCL2/GADPH Ratio** **Figure 8 C: DHFR/GADPH Ratio**

4. DISCUSSION

Liver being a major metabolic organ critically involved in the biochemical processing of chemicals and toxins. Present drug of choice such as steroids, vaccines and antivirals available for clinical management of liver disorder offers tremendous deleterious side effects [13-14]. Due to existing lacuna on the need of the therapy towards liver disorders, there diverse shift of research focus towards alternative complimentary therapy and drugs from traditional system of Indian medicines. Since

several years siddha medicines are considered to be effective and safe for the treatment of liver disorders.

Paracetamol is primarily metabolized by the enzyme uridine diphosphate glucuronosyl transferase (UGT) and excreted, but with an increasing dose rate; these pathways become saturated and a greater proportion is oxidised by the microsomal cytochrome P-450 system (Amar and Schiff, 2007). N-Acetyl-P-benzoquinone Imine (NAPQI) is the product of this pathway which is thought to be responsible for the subsequent hepatic damage. NAPQI is a highly reactive electrophile and is detoxified in liver by

either reduction to the parent compound, acetaminophen, or conjugation at the metaposition with glutathione, in which both reactions consume GSH [15]. Depletion of GSH, under conditions of continuous intracellular oxidative stress, leads to oxidation and damage of lipids, proteins, and DNA by the reactive oxygen species [16].

Liver cell degeneration is predicted by consistent change in the enzyme gene expression.To evaluate liver injury, gene markers (UGT, ALB, CYP) expression levels are measured. In this present investigation the hepatotoxicity due to acetaminophen was confirmed by increased expression of CYP expression and decrease in UGT and ALB gene expressions. Increase CYP expression often due to remarkable degeneration in liver cells, Hence increases CYP expression is an indicator of chang liver cell damage. Paracetamol exposed chang liver cells showed significant (p<0.01) up-regulation of CYP expression and down-regulation of UGT and ALB gene expressions when compared to normal cells. Treatment with siddha formulation SSM at 10, 100 and 1000 ng/ml significantly and dose dependently ameliorated these alterations and reverse the scenario by up regulating the UGT, ALB and down regulating the CYP gene.

Methotrexate can induce a variety of histologic changes including steatosis, stellate (Ito) cell hypertrophy, anisonucleosis (nuclei of varying sizes), and hepatic fibrosis. Hepatic folate stores are depleted by MTX at the doses used in arthritis. MTX competitively inhibits dihydrofolate reductase (DHFR), an enzyme that participates in the tetrahydrofolate synthesis thereby inhibiting the synthesis of DNA, RNA, thymidylates, and proteins. The affinity of MTR for DHFR is about one thousand-fold that of folate. Methotrexate acts specifically during DNA and RNA synthesis, and thus it is cytotoxic during the S-phase of the cell cycle [17].

Apoptosis involves a series of biochemical events leading to characteristic changes in cell morphology and death. These changes include blebbing, loss of membrane symmetry and attachment, cell shrinkage, nuclear fragmentation, chromatin condensation and chromosomal DNA fragmentation [18]. *Bcl-2* gene family is the major regulator of cell apoptosis. Various homogenous or heterogeneous dimers can be formed within this gene family, enhancing or inhibiting apoptosis. For example, *Bcl-2* (B-cell lymphoma-2) gene expression inhibits apoptosis, but Bax (Bcl-2 associated-x) promotes it [19].

Anti-apoptotic BCL-2 gene and Pro-apoptotic BAX gene plays a crucial role in liver cell degeneration and apoptosis. The results of the present study has shown that significant (p<0.01) increase in BAX and significant (p<0.01) decrease in BCL2 and DHFR gene expressions were observed in methotrexate induced chang liver cells when compared to normal cells. Treatment with test drug significantly decreased BAX and increased BCL2 and DHFR gene expressions.

5. CONCLUSION

In recent times there is a tremendous diverging interest focused in particular towards the Indian medicines as there is no proper therapy available in the current allopathic system of medicine. As an evident of this the data's obtained from the present investigation that the siddha formulation SSM offers greater hepatoprotective activity against paracetamol and methotrexate induced hepatotoxicity in chang liver cell lines. This study provides evidence based data on exploring the siddha formulation SSM for being a good drug of choice for better clinical management of liver disorders.

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