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EFFECT OF SATWA FROM THREE TINOSPORA SPECIES ON LIPID METABOLISM AND INFLAMMATORY MARKERS IN ACETAMINOPHEN AND ALCOHOL-INDUCED HEPATO-TOXICITY IN RATS

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Keywords:

Acetaminophen, Alcohol, Hepatotoxicity, Guduchi satwa, Silymarin

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ABSTRACT: To investigate the possible protective effects of satwa from three *Tinospora* species against acetaminophen and alcohol-induced hepatotoxicity in rats. Male albino wistar rats were divided into six groups (n=6); healthy control, negative control (Acetaminophen 1000mg/kg b.w./day, p.o. or 30%; alcohol 1ml/100g b.w./day, p.o.), positive control (Silymarin; 100mg/kg b.w./day, p.o.), Treatment 1 (*Tinospora cordifolia* satwa; 200mg/kg b.w/day p.o.), Treatment 2 (*Tinospora sinensis* satwa; 200mg/kg b.w/day, p.o.) and Treatment 3 (*Neem-giloe* satwa; 200mg/kg b.w/day., p.o.). Liver injury in the rats was induced by repeated dosing of acetaminophen or alcohol for 15 days. Expression analysis of fatty acid-binding protein 1 (FABP1), peroxisome proliferator-activated receptor-gamma (PPARγ), sterolregulatory element-binding protein 1 (SREBP1), nuclear factor-kappa β (NF-κβ) and tumor necrosis factor-alpha (TNF-α) genes from liver were assayed by a semi-quantitative polymerase chain reaction. Expressions of FABP1, PPARγ were downregulated while SREBP1, NF-κβ and TNF-α were upregulated in negative control. Treatment with *Neem-giloe* satwa upregulated the expression of FABP1 and down-regulated the expression of NF-κβ, SREBP1, TNF-α as compared to acetaminophen treated rats. Treatment with *T. sinensis* satwa upregulated the expression of FABP1 and PPARγ while the expression of NF-κβ and TNF-α was down-regulated as compared to alcohol-treated rats. These results suggest that the satwa from three *Tinospora* species exhibit different protective effects of the transcription factors and genes (inflammatory and lipid metabolism pathways). The formulation or combination of satwa may boost hepatoprotective actions can potentially be an effective liver tonic in animals.

INTRODUCTION: The liver is the largest gland in the body weighing about 1500g in an adult and accounts for approximately 2.5% of total body weight [1]. The liver is also called as the metabolic "engine-room of the body" [2] and performs more than 500 vital functions of metabolic importance [3].

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Hepatotoxicity is most commonly seen in the form of malfunction or damage to the liver due to an excess number of drugs or xenobiotics [4].

Hepatotoxicants are exogenous substances of clinical relevance, which may include an overdose of certain medicinal drugs (acetaminophen, nimesulide, and antitubercular drugs like isoniazid, rifampicin, *etc*.), industrial chemicals (alcohol, CCl_4, beta galactosamine, thioacetamide) *etc*., which causes liver injury [5]. Hepatotoxicity may result in cytotoxic effects (necrosis, apoptosis), cholestasis, steatosis, fibrosis, cirrhosis, hepatitis,

and liver tumors [6]. Liver diseases are fatal and a leading cause of illness and deaths worldwide [7].

Acetaminophen is over-the-counter analgesic and anti-pyretic medicine [8]. Therapeutic dose of acetaminophen is safe, but its overdose leads to 'Acetaminophen hepatotoxicity', causing liver injury and is one of the most common reasons for poisoning all over the world [9]. Acetaminophen is metabolized into intermediate N-acetyl-p-benzoquinoneimine (NAPQI), which accumulates in the liver, causing depletion of glutathione (an important antioxidant in the liver) resulting in direct damage to liver cells. It has been reported as the most common drug overdose, either accidentally or intentionally, resulting into acute liver failures (ALF) in the United Kingdom (UK, 60-75% of ALF etiology), Europe (2% of ALF etiology in France), Canada and United States (US, approximately 20% of ALF etiology), and Australia [10, 11]. In India, 33.2% of patients were reported with acetaminophen overdose in a four-year clinical observation study on 1024 patients (Median age 23 years, 82.0% female) [10]. The data on acetaminophen self-poisoning in India is highly insufficient as compared to that of Western countries [12].

Alcohol is one of the main causes of end-stage liver disease and a leading cause of morbidity and mortality worldwide [7]. Alcoholic liver disease (ALD) is considered a major health and economic problem worldwide [13]. Alcohol overdose causes an increase in the reduced form of nicotinamide adenine dinucleotide (NADH) resulting in fat accumulation [14], oxidative stress, mitochondrial damage [15], induction of free radicals leading to peroxidation and inflammatory response [16]. Deaths due to alcoholic liver diseases have been increased since the last decade [17] and have become a common reason for cirrhosis in western countries [18]. A WHO study in 2012 reported about 3.3 million deaths worldwide, of which 5.9% were caused by alcohol consumption [19]. About 3.8% of global mortality is accounted for alcohol consumption [20, 21]. In the USA, the second leading cause for liver transplantation in alcoholic cirrhosis [22]. In India, 5% of all deaths are because of liver diseases, for which the most critical culprit is alcohol [22]. The prevalence of alcohol consumption ranges from 7% in Gujarat, to 75% in Arunachal Pradesh [23].

Overdose of acetaminophen or alcohol is known to exert hepatotoxic effects, which are reflected at biochemical, histological, and molecular levels in the form of altered liver function tests, mild to severe alterations in the histological architecture of hepatocytes and modulation in the expression of several genes. Several studies have identified important genes such as nuclear factor-kappa β (NF-κβ), sterol regulatory element-binding protein 1 (SREBP1), fatty acid-binding protein 1 (FABP1), tumor necrosis factor-alpha (TNF-α) involved in acetaminophen and alcohol-induced hepatotoxicity in rodents [24, 25, 26, 27, 28]. Despite considerable progress in modern medicine, the drugs or agents which can stimulate liver function or help regeneration of hepatic cells or offer protection to the liver damage, still have many undesirable side effects [2]. Hence, there is a recent renewal of interest in the search for natural resources like medicinal plants, which have promising potential to offer several herbal medicines with less side-effect [29]. Eastern countries have been using herbal drugs to treat liver diseases since ancient times [30].

As per the WHO report, around three-quarters of the world's population uses herbs and other traditional medicines to cure various diseases, including liver disorders [31, 32]. Medicinal plants such as Guduchi [33, 34], *Elephanto pusscaber* [35], *Picro rhizakurroa* [36], *Silybum marianum* [37], *Andrographis paniculate* [38], *Azadira chtaindica* [39] and *Glycyrrhiza glabra* [40] have proven hepatoprotective properties and are used to treat liver disorders. Guduchi (*Tinospora* sp.) is one of the most versatile rejuvenating shrubs, also known as 'Giloya' in Indian vernacular, and is reported to have many therapeutic applications [40], and has been described as "one which protects the body" [4].

Tinospora belongs to family Menispermaceae. *Tinospora* is a climbing or twining shrub [41] and is found mostly in tropical and subtropical areas of India with different names [42]. More than 32 species of Guduchi are found all over the world [43]. Four different forms of *Tinospora* occur in India viz. *Tinospora cordifolia* (Wild.) Miers ex Hook. F. & Thoms, *Tinospora sinensis* (Lour.) Merr., *Tinospora crispa* (L.) Miers ex Hook. f. & Thoms and *Tinospora glabra* (Burm f.) Merrill [44, 45]. In this study, we selected three different forms of Guduchi: *Tinospora cordifolia* (Willd.) Miers ex

Hook. F. & Thoms., *Tinospora sinensis* (Lour.) Merrill., and *Neem-giloe* (*T. cordifolia* plant growing on *Azadirachta indica* (Neem tree).

Our earlier reports showed hepatoprotective activity of satwa of three different *Tinospora* forms against acetaminophen and alcohol-induced hepato-toxicity through normalization of biochemical parameters and hepatic architecture [46, 47].

In the present study, we have analyzed modulation in the hepatic expression levels of genes from animals treated with satwa of three different *Tinospora* forms against acetaminophen and alcohol-induced hepatotoxicity. To the best of authors' knowledge, this is the first report analyzing the changes in gene expression in animals treated with the intervention of Guduchi satwa.

MATERIALS AND METHODS:

Procurement and Authentication of Plant Materials: The mature stems of *Tinospora cordifolia, Tinospora sinensis*, and *Neem-giloe* (Guduchi plant growing on *Azadirachta indica (Neem*)) were collected during February-April 2012 from Pune and Dapoli, Maharashtra, India [46, 47]. The plant material was identified, and a voucher specimen was deposited at the herbarium of the Medicinal Plants Conservation Centre (MPCC), Pune [46, 47], (*Tinospora cordifolia* (Willd.) Miers ex Hook. F. & Thoms (MPCC 3483), *Tinospora sinensis* (Lour.) Merr. (MPCC 3529) and *Neem-giloe* (*T. cordifolia* (Willd.) Miers ex Hook. F. & Thoms) (MPCC 3526) [46, 47].

Preparation of Satwa Three Tinospora Species: [46, 47] Fresh stems of three Tinospora species were used for the preparation of Guduchi Satwa (Residual marc of aqueous extract). The preparation, as defined in Ayurveda literature, is a sediment extract predominantly starchy in nature. The preparation of satwa was done as per the procedure described by Khandal (1992) [46, 47, 48]. Five kilograms of freshly collected stem pieces were washed thoroughly with water. The stem peel was removed, and the stem was cut into pieces of 1.5-2 inches, having 1.6-2.0 cm diameter **Fig. 1**. The stem pieces thus obtained were pounded slightly **Fig. 1A, 1B,** and **1C**. The crushed stem pieces of three species were separately suspended in a quantity of water 4 times of their weight **Fig. 1D**. This mixture was kept undisturbed for 24 h. The next day, Guduchi was hand-rubbed till it was slimy with the appearance of foam on water **Fig. 1E**. This homogenized mixture was then filtered through several layers of sterile muslin cloth, and the filtrate was left undisturbed for 24 h.

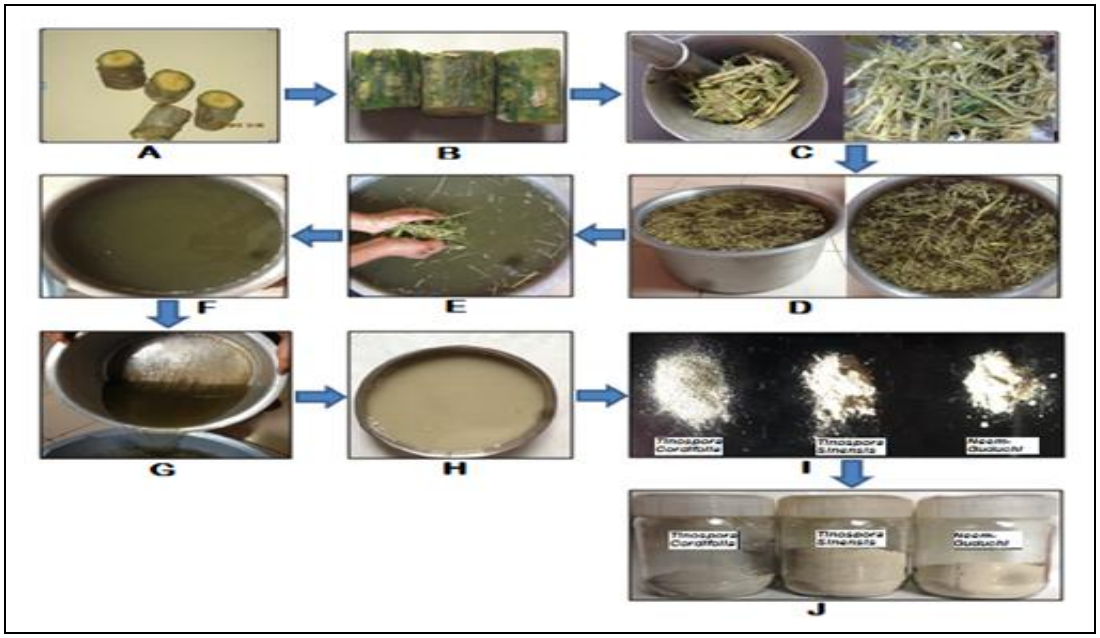

FIG. 1: PREPARATION OF GUDUCHI SATWA. A-GUDUCHI STEM, B-GUDUCHI STEM WITH OUTER BROWNISH WHITE COLOURED PEEL REMOVED, CPOUNDING, D-OVERNIGHT SOAKING, E-RUBBING OF SLIMY, CRUSHED STEM PIECES, F- SEDIMENTATION, G-REMOVAL OF SUPERNATANT, H-COLLECTION AND DRYING OF WHITE SEDIMENT, I-COMPLETELY DRIED GUDUCHI SATWA OF THREE GUDUCHI FORMS AND J-GUDUCHI SATWA STORED IN AIR TIGHT CONTAINERS

On the next day, the water was decanted carefully without disturbing the sediment **Fig. 1F**. The sediment was again suspended in half liter water and kept undisturbed for two hours. The water was then carefully decanted **Fig. 1H**, and the sediment was collected and sun-dried for 48 h. The sun-dried residue thus obtained, is termed as 'satwa'. Satwa was stored in airtight containers until further use of **Fig. 1I** and **1J**.

Drugs/Chemicals: Acetaminophen tablets (1000 mg/kg b.w./day, p.o.) (Paramol; Ranbaxy Laboratories Ltd.) were purchased from the local pharmacy and dissolved in sterile water to make the stock solution convenient [46] and 30% alcohol (Ethanol) (1ml/100g b.w./day, p.o.) [46] was obtained from Changshu Yangyuan Chemical; China was used as hepatotoxicant for animal administration as inducing agents for hepatotoxicity. Silymarin tablets (100mg/kg b.w./day, p.o.) (Silybon-140; Micro Labs) were purchased from a local pharmacy and dissolved in sterile water to make the stock solution convenient for animal administration as standard [46, 47].

Experimental Animals: The studies were carried out as per the CPCSEA guidelines and after approval of the Institutional Animal Ethical Committee (Ref. No. BVDUMC/443/2012-2013). Three months old male albino Wistar rats weighing between 150-200 gm were procured for the study from institutional animal house. The animals were acclimatized for seven days and were maintained under standard husbandry conditions (Temperature 25 ± 2 °C, 12-h light: 12-h dark cycle) throughout the experimentation. The animals were fed with standard pellet diet (Nutrivet life science, Pune, M.S., India), and water was supplied ad-libitum.

Selection and Preparation of Dose Satwa of Three *Tinospora* Species: The dose of satwa was finalized based on previous studies carried out in the Laboratory [46, 47]. The quantity of satwa for administration to each animal was calculated based on the weight of the animal.

The required quantity of satwa was weighed and suspended in water for administration to animals. The satwa from three forms of *Tinospora* species (200mg/kg b.w./day, p.o.) was administered to rats to study their hepatoprotective activity.

Experimental Design: The animals were divided into six groups by random assignment of six animals per group. The variation in the average weight of the animals in and between the groups was less than 20%. The treatment protocol to assess the hepatoprotective potential of satwa of three different species of *Tinospora* (*T. cordifolia*, *T. sinensis*, and *Neem-giloe*) against acetaminophen and 30% alcohol-induced liver injury is outlined below:

Hepatoprotective Activity of Satwa against Acetaminophen Induced Hepatotoxicity: [46]

Group I: Healthy Control (n=6); received feed and water normally for 15 days.

Group II: Negative Control (n=6); rats were administered acetaminophen (1000mg/kg b.w./day, p.o.), daily for 15 days.

Group III: Positive Control (n=6); the rats in this group were treated daily with acetaminophen (1000mg/kg b.w./day, p.o.), 30 min after administration of silymarin (100mg/kg b.w./day, p.o.), for 15 days.

Group IV: Treatment group 1 (n=6); the rats in this group were treated daily with acetaminophen (1000mg/kg b.w./day, p.o.), 30 min after administration of *Tinospora cordifolia* satwa (200mg/kg b.w./day, p.o.), for 15 days.

Group V: Treatment group 2 (n=6); the rats in this group were treated daily with acetaminophen (1000mg/kg b.w./day, p.o.), 30 min after administration of *Tinospora sinensis* satwa (200mg/kg b.w./day, p.o.), for 15 days.

Group VI: Treatment group 3 (n=6); the rats in this group were treated daily with acetaminophen (1000mg/kg b.w./day, p.o.), 30 minutes after administration of *Neem-giloe* satwa (200mg/kg b.w./day, p.o.), for 15 days.

Hepatoprotective Activity of Satwa against Alcohol-Induced Hepatotoxicity: [45]

Group I: Healthy Control (n=6); received feed and water normally for 15 days.

Group II: Negative Control (n=6); administrated 30% alcohol (1ml/100g b.w./day, p.o.), for 15 days.

Group III: Positive Control (n=6); the rats in this group were treated daily with 30% alcohol

(1ml/100g b.w./day, p.o.), 30 min after administration of silymarin (100mg/kg b.w./day, p.o.), for 15 days.

Group IV: Treatment group 1 (n=6); the rats in this group were treated daily with 30% alcohol (1ml/100g b.w./day, p.o.), 30 min after administration of *Tinospora cordifolia* satwa (200mg/kg b.w./day, p.o.), for 15 days.

Group V: Treatment group 2 (n=6); the rats in this group were treated daily with 30% alcohol (1ml/100g b.w./day, p.o.), 30 min after administration of *Tinospora sinensis* satwa (200mg/kg b.w./day, p.o.), for 15 days.

Group VI: Treatment group 3 (n=6); the rats in this group were treated daily with 30% alcohol (1ml/100g b.w./day, p.o.), 30 min after administration of *Neem-giloe* satwa (200mg/kg b.w./day, p.o.), for 15 days.

During the period of the experiment, animals were observed daily for any signs of infection and/or discomfort. After completion of the experiment (15 days), all animals were fasted overnight and were humanely sacrificed. The liver was excised from the dissected animals immediately, washed with saline, and snap-frozen in liquid nitrogen. Frozen tissues were stored at -80 °C till further use for gene expression analysis.

RNA Extraction: RNA extraction was performed by the TRIzol method (Sigma-Aldrich, USA) [49]. Each frozen liver sample (~100mg of tissue) was crushed in liquid nitrogen with mortar and pestle and made into a fine powder. The powdered tissue was added in 1mL of TRIzol reagent before thawing and vortexed vigorously for 15 sec.

Chloroform (200µl) was added in these tubes, and the contents were gently mixed by inverting the tubes. The tubes were incubated for 2 to 3 min at room temperature. The mixture was centrifuged at 12000 rpm for 15 min at 4 °C. The aqueous phase was transferred carefully to a new tube without disturbing the interphase. Chilled isopropyl alcohol was added to the aqueous phase in a new tube and incubated overnight at -20 °C. The next day, the mixture was kept for 10 min at room temperature. The mixture was centrifuged at 12000 rpm for 15 min at 4 °C.

The pellet was washed with freshly prepared 75% chilled ethanol (500µl) and centrifuged at 7500 rpm for 5 min at 4 °C. The supernatant was discarded, and the pellet was suspended in 30µl diethyl-pyrocarbonate-treated water (DEPC) water. RNA samples (2µl) were loaded on 0.8% agarose gel. Quantification of RNA was performed with a UV spectrophotometer (NanoDrop; Eppendorf). The isolated RNA with a 260nm/280nm ratio between 1.5 to 2.0 is a dimensionless parameter for RNA purity.

cDNA Synthesis: Total RNA was reverse transcribed using the Super Script First-Strand cDNA synthesis kit (Invitrogen, USA) according to the manufacturer's instructions. First-strand synthesis of complementary DNA (cDNA) was done by reverse transcription. Briefly, 4µg RNA was mixed with 3µl of random hexamer (50ng/µl) and 1µl of dNTP (10mM) in a total volume of 12µl. The mixture was incubated at 65 °C for 5 min. After the incubation, the reaction was cooled rapidly on ice for 1 min, followed by addition of 4µl 5x first strand buffer (Promega, USA), 2µl 0.1M DTT (Invitrogen, USA) and 1µl RNaseOUT™ Recombinant ribonuclease inhibitor (40 units/µl, Invitrogen, USA). The tubes were incubated at 37°C for 2 min followed by the addition of 0.5µl M-MLV RT (200 units, Promega, USA). The contents of the reaction were mixed gently by pipetting up and down. Reverse transcription included the following three phases: The reaction was incubated at 25 °C for 10 min for RT enzyme activation followed by 50 min at 37 °C for reverse transcription, and the reaction was inactivated by heating at 70 °C for 15 min. The synthesized cDNA was stored at -80 °C.

Semi-Quantitative Polymerase Chain Reaction: The cDNA was diluted with 1:40 Tris buffer (T10E1 buffer) (10 mM, Tris (pH 8.0), 1mM EDTA (pH 8.0) and used for semi-quantitative polymerase chain reaction (SQ-PCR). For amplification in a 25µl reaction consisting of 2.5µl 10X PCR buffer (Sigma- Aldrich, USA), 2µl 2.5mM dNTPs (GeNei, India), 0.3µl *Taq* DNA polymerase (5U/µl, Sigma-Aldrich, USA), 0.5µl each of forward and reverse KiCqStart® primers (10pM/µl stock) (Sigma-Aldrich, USA). The temperature profile for semi-quantitative PCR was as below: Initial denaturation at 94 °C for 10 min,

followed by 25 cycles, each comprising 1-min denaturation at 94 °C, 30-sec annealing temperature at 60 °C and 1-min extension at 72 °C with final extension at 72 °C for 5 min followed by incubation at 4 °C. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used as a control (endogenous or housekeeping gene) for normalization. Expression analysis of fatty acid-binding protein 1 (FABP1), peroxisome proliferator-activated receptor-gamma (PPARγ), sterol regulatory element-binding protein 1 (SREBP1), nuclear factor-kappa β (NF-κβ) and tumor necrosis factor-alpha (TNF-α) was done from all the samples. Sigma KiCqStart® primers were used to study the modulation of gene expression. The primer sequences are listed in **Table 1**. The amplified 25µl PCR products were resolved by electrophoresis on 1.5% agarose gel (Low EEO Genei). The image was captured under a UV-transilluminator (Image Lab^{TM} software 4.1, Bio-Rad Laboratories, Inc). Gene expression levels were normalized to those of GAPDH. The bands were quantified or compared by densitometry using 'Image J' analysis software V 1.41o (National Institute of Health, Washington).

TABLE 1: LIST OF PRIMERS USED FOR THE STUDY

Gene	Primer sequence	Amplified fragment	Annealing temperature
GAPDH	F 5'-AGTTCAACGGCACAGTCAAG-3' R 5'-TACTCAGCACCAGCATCACC-3'	136	60ºC
FABP1	F 5'-TGGAGGGTGACAATAAAATG-3' R 5'-TCATGGTATTGGTGATTGTG-3'	86	60ºC
PPARγ	F 5'-AAGACAACAGACAAATCACC-3' R 5'-CAGGGATATTTTTGGCATACTC-3'	195	60ºC
SREBP1	F 5'- AAACCTGAAGTGGTAGAAAC-3' R 5'-TTATCCTCAAAGGCTGGG-3'	142	60ºC
NF-κβ	F 5'- AAAAACGAGCCTAGAGATTG-3' R 5'-ACATCCTCTTCCTTGTCTTC-3'	157	60ºC
TNFα	F 5'- CTCACACTCAGATCATCTTC-3' R 5'-GAGAACCTGGGAGTAGATAAG-3'	194	60ºC

Statistical Analysis: The data were presented as Mean ± Standard Error (SE). The Dunnett Multiple Comparison Test and One-Way Analysis of Variance (ANOVA) were done to estimate the statistical significance between groups. Graphs were plotted using GraphPad Prism (Trial Version 5.0, GraphPad Software, San Diego, CA, USA) was used for statistical analysis.

RESULTS: In the present study, the comparative hepatoprotective potential of *T. cordifolia, T. sinensis* and *Neem-giloe* satwa was evaluated by modulation in the expression levels of the genes regulating the lipid metabolism and inflammation. Satwa of *T. cordifolia, T. sinensis* and *Neem-giloe* had a differential effect on expressions of these genes in rats treated with acetaminophen and 30% alcohol.

Effect of Satwa of *T. cordifolia, T. sinensis* and *Neem-giloe* on Gene Expression in Acetaminophen Induced Hepatotoxicity:

Genes Involved in Lipid Metabolism: Fig. 1 depicts the modulation of expression levels of the genes from liver tissues of animals treated with the satwa of three different *Tinospora* forms. Expression levels of FABP1 **Fig. 2A** and PPARγ **Fig. 2B** was found to be decreased in acetaminophen-induced hepatotoxicity as compared with healthy control. Treatment with the satwa of *Neem-giloe* significantly improved (P≤0.05) the expression of FABP1. The increase in the expression level of PPARγ observed in satwa treated groups was statistically not significant. The expression of SREBP1 was up-regulated in acetaminophen treated rats while it was significantly down-regulated (P≤0.01) in groups treated with *T. cordifolia, T. sinensis* and *Neem-giloe* **Fig. 2C**.

Genes Involved in Inflammation: NF-κβ and TNF-α were up-regulated in acetaminophen treated rats as compared to the healthy control group. NF-κβ was significantly down-regulated (P≤0.01 or P≤0.001) in groups treated with *T. cordifolia,T. sinensis* and *Neem-giloe* **Fig. 2D**. Interestingly, NF-κβ was significantly down-regulated (P≤0.001) in groups treated with *Neem-giloe* **Fig. 2D**. Expression of TNF-α, though down-regulated in treatment groups, was not found to be statistically significant than that of negative control **Fig. 2E**.

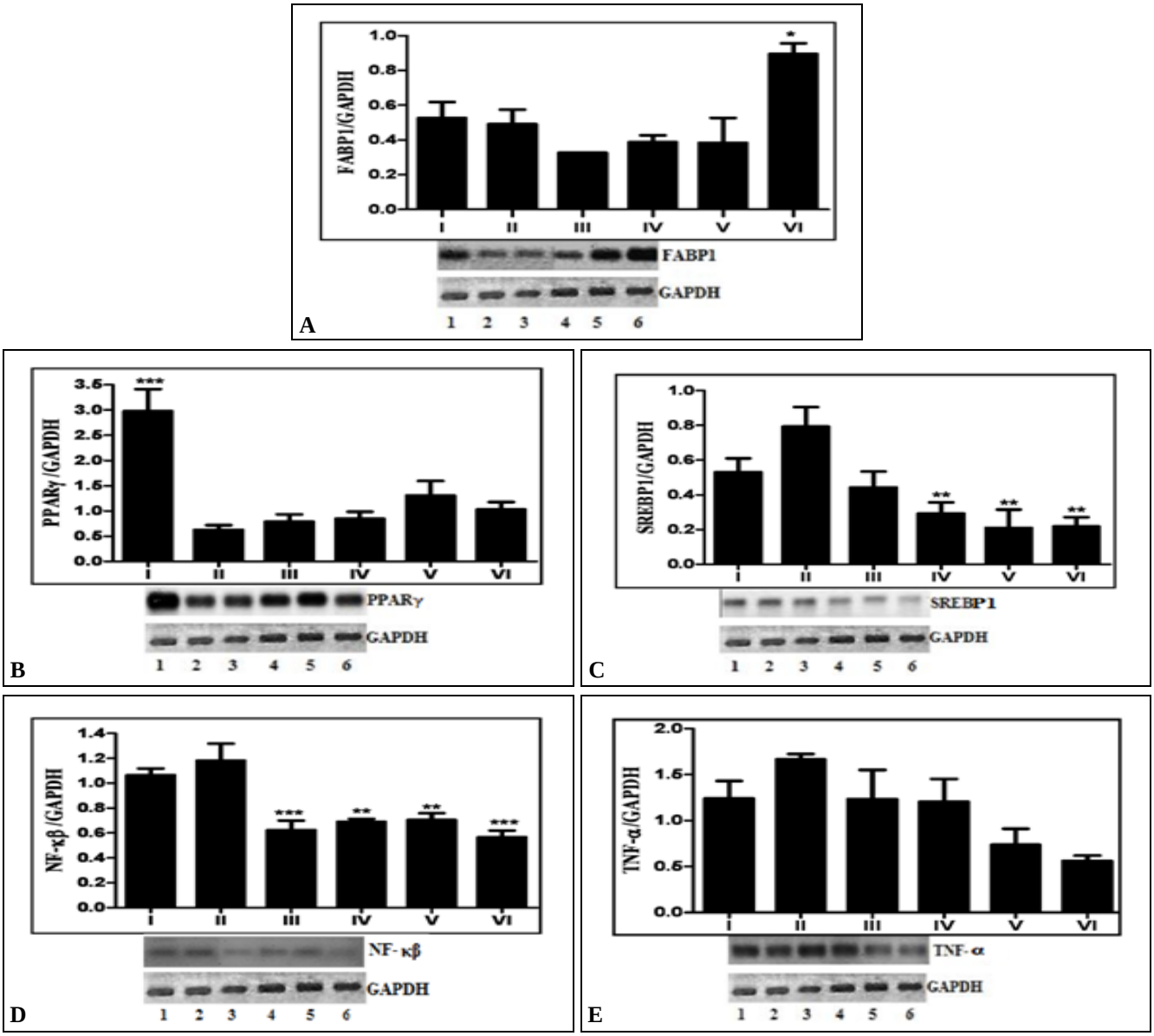

FIG. 2: EFFECT OF SATWA OF *T. CORDIFOLIA, T. SINENSIS,* AND *NEEM-GILOE* ON GENE EXPRESSION IN ACETAMINOPHEN INDUCED HEPATOTOXICITY. DENSITOMETRIC ANALYSIS OF EXPRESSION WAS DONE BY USING GAPDH AND GENE SPECIFIC EXPRESSION DATA. *P≤0.05; **P≤0.01; ***P≤0.001 cOMPARED WITH NEGATIVE CONTROL RATS. LANES: HEALTHY CONTROL (GROUP I) -1; NEGATIVE CONTROL (GROUP II) -2; POSITIVE CONTROL (GROUP III) -3; *T. CORDIFOLIA* SATWA TREATED RATS (GROUP IV) -4; *T. SINENSIS* SATWA TREATED RATS (GROUP V) -5; NEEM-GILOE SATWA TREATED RATS (Group VI) -6. GAPDH (INTERNAL STANDARD). A: FABP1, B: PPARγ, C: NF-κβ, D: SREBP1, E: TNF-α.

Effect of Satwa of *T. cordifolia, T. sinensis* and *Neem-giloe* on Gene Expression in Alcohol Induced Hepatotoxicity:

Genes Involved in Lipid Metabolism: The expression levels of FABP1 **Fig. 3A** were down-regulated while, PPARγ **Fig. 3B** and SREBP1 **Fig. 3C** were increased in alcohol-treated rats as compared to the healthy control group. FABP1 expression was significantly up-regulated (P≤0.001) by treatment with *T. sinensis* and *Neem-giloe* **Fig. 3A**. Treatment with *Neem-giloe* lead to significant down-regulation, but treatment with *T. sinensis* showed up-regulation of PPARγ as compared to the negative control group **Fig. 3B**. Treatment with *Neem-giloe* leads to significant down-regulation (P≤0.05) of SREBP1 than negative control group **Fig. 3C**.

Genes Involved in Inflammation: NF-κβ **Fig. 3D** and TNF-α, **Fig. 3E** was increased in alcohol-treated rats as compared to the healthy control group. Expression of NF-κβ was not altered in the positive control group while all treatment groups exhibited significantly reduced (P≤0.01 or

P≤0.001) expression of NF-κβ. The expression of TNF-α was reduced in the positive control group (P≤0.01). In contrast, the three treatment groups showed a significant decrease (P≤0.001) in the expression levels of TNF-α as compared to the negative control group **Fig. 3D**.

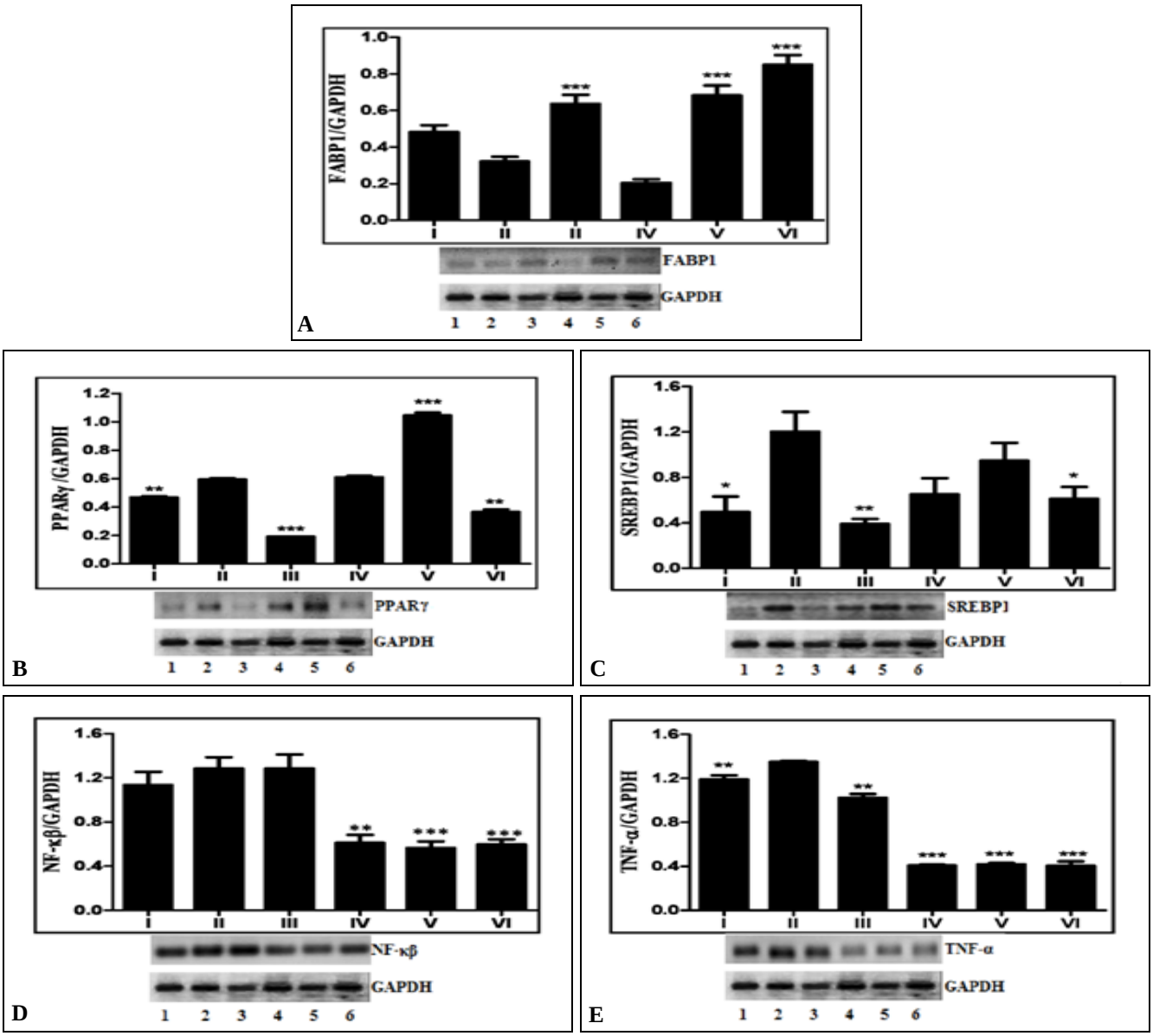

FIG. 3: EFFECT OF SATWA OF *T. CORDIFOLIA, T. SINENSIS,* AND NEEM-GILOE ON GENE EXPRESSION IN ALCOHOL INDUCED HEPATOTOXICITY. DENSITOMETRIC ANALYSIS OF EXPRESSION WAS DONE BY USING GAPDH AND GENE SPECIFIC EXPRESSION DATA. *P≤0.05; **P≤0.01; ***P≤0.001 COMPARED WITH NEGATIVE CONTROL RATS; LANES: HEALTH CONTROL (GROUP I) -1; NEGATIVE CONTROL (GROUP II) -2; POSITIVE CONTROL (GROUP III) -3; T. CORDIFOLIA SATWA TREATED RATS (GROUP IV) - 4; T. SINENSIS SATWA TREATED RATS (GROUP V) -5; NEEM-GILOE SATWA TREATED RATS (GROUP VI) -6. GAPDH (INTERNAL STANDARD). A: FABP1, B: PPARγ, C: NF-Kβ, D: SREBP1, E: TNF-α.

DISCUSSION: Understanding the exact mechanism of xenobiotic hepatotoxicity is one of the major challenges for hepatologists. Recent advances in the studies of toxicogenomic have been useful in elucidating several different pathways of hepatotoxicity [49]. Further research is needed to confirm these results to gain a mechanistic understanding of toxic changes that occur in the liver. PPARγ and SREBP1 are transcription factors and regulators of lipid homeostasis in hepatocytes and a target for fatty acids and hypolipidemic drugs [28]. The proteins encoded by different PPAR genes (PPARα, PPARδ, and PPARγ) have the ability to induce hepatic peroxisome proliferation in response to xenobiotic stimuli [50] and PPARγ is believed to play a central role in regulation of carbohydrate and lipid metabolism, fatty acid metabolism and the PPARs are also assumed to possess anti-

inflammatory activity [51]. Dysregulation of PPAR isoforms contributes to the development of a wide range of liver diseases [52]. The majority of studies deal with PPARγ in diabetic and obese animals [53, 54, 55,] but the mechanistic relationship of increase of PPARγ expression in hepatotoxicity remains unclear to date. Pioglitazone, a PPARγ agonist, inhibits CCl_4 (Carbon tetrachloride) induced hepatic fibrosis through inhibition of inflammation and hepatic stellate cell proliferation, indicating the protective role of PPARγ in hepatotoxicity [55]. In chronic liver injury induced by CCl_4, PPARγ expression was downregulated in hepatocytes, while increased levels of these transcription factors were found in Kupffer cells associated with inverse correlation to levels of activated NF-κβ [56]. Treatment of albino rats with 8β-Glycyrrhetinic acid has been shown to exert hepatoprotective effects against cyclophosphamide-induced hepato-toxicity through the up-regulation of PPARγ [57]. Several animal experiments have shown the effect of ethanol through the regulation of hepatic expression of PPARγ and PPARγ agonists are known to prevent alcohol-induced liver injury [58, 59, 60]. Downregulation of PPARγ mRNA expression has been reported in isoniazid induced hepatotoxicity [58].

In the present study, the expression of PPARγ was reduced in acetaminophen and alcohol-treated rats as compared to healthy animals. The mechanism of action of hepatoprotection by several secondary metabolites from plants is observed through a reduction in oxidative stress due to the activation of PPARγ [61]. Alcohol intoxicated mice supplemented with *Aloe vera* polysaccharides exhibited a remarkable increase in mRNA levels of PPARα, which otherwise is down-regulated after alcohol treatment leading to liver damage [13]. Treatment of albino rats with 8β-Glycyrrhetinic acid has been shown to exert hepatoprotective effects against cyclophosphamide-induced hepatotoxicity through the up-regulation of PPARγ [57].

Though, statistically insignificant, the present study showed marginal improvement in PPARγ expression in the livers of acetaminophen intoxicated rats treated with *Neem-giloe* and *T. sinensis* satwa while PPARγ expression was significantly higher in *T. sinensis* treated group in alcohol-intoxicated rats.

SREBP1 specifically activates several key genes involved in lipogenesis [62] like fatty acid synthase (FAS), and Acetyl-CoA carboxylase alpha (ACACA) [63]. SREBP1 gene expression was observed to be downregulated in animals treated with a single high dose of acetaminophen, carbon tetrachloride, tetracycline amiodarone [64]. A thorough literature search indicated that the effects of repeated acetaminophen dosing on SREBP1 expression are not yet available. In the present study, SREBP1 expression was reported to be higher in the animals, which were repeatedly treated with a high dose of acetaminophen for 15 days as compared to healthy control. Scanty references are available on the effect of herbal interventions on SREBP1 expression in animal models for hepatotoxicity. Acute ethanol (A single oral dose of 0.5 or 5g/kg of body weight) affects the expression levels of SREBP1 and many other SREBP1 target genes, thereby increasing fatty acid synthesis in male ICR mice [26]. Cui *et al*., (2014) showed that alcohol consumption decreases AMPK-α2 expression and elevates SREBP1c levels in mice[13].

The present study also reports a higher expression of SREBP1 in alcohol-treated rats as compared with the healthy control group. The studies on traditional Chinese medicines like *Schisandra chinensis* [65,] and *Gentiana manshurica* [66] have demonstrated the prevention of alcohol-induced liver damage through decreased expression of SREBP1 regulated fatty acid synthesis.

In the present study, expression of SREBP-1 was significantly decreased in animals treated with the satwa of *T. cordifolia, T. sinensis,* and *Neem-giloe* as compared to acetaminophen treated rats and also reports higher expression of SREBP-1 in alcohol-treated rats with a significant reduction in its expression after treatment with *Neem-giloe*satwa.

A study carried out in this lab on alcohol-induced liver damage in rats has shown normalization of serum lipid profile by treatment with *Neem-giloe* satwa while *T. sinensis* satwa normalized the hepatic lipid profile as well as liver function tests. Intervention of *T. sinensis* satwa also showed significant improvements in antioxidant status of the alcohol-treated animals [46].

The previous report showed that the treatment of rats with *Neem-giloe* (200 mg/kg) decreased levels of SGOT, bilirubin, and *T. sinensis* showed a specific effect on improvements in serum SGPT and ALP. *T. cordifolia* satwa exhibited improvements in the serum levels of total cholesterol, HDL, and LDL, *T. sinensis* satwa showed improvement in VLDL and triglyceride levels while *Neem-giloe* satwa showed significant improvements in total protein and lipid profile (HDL, LDL, VLDL, Triglyceride) in liver tissues [47].

FABPs comprise a superfamily of lipid-binding proteins that are involved in the fatty acid uptake, intracellular transport, and in regulating lipid metabolism, cellular signaling pathways, and other lipid ligands [67]. FABP is highly expressed in adipocytes, liver, muscle, heart, brain, and macrophages, and the expression and activation of FABP1 have been reported to contribute to the pathogenesis of obesity, metabolic syndrome, and associated inflammation [68]. There was a dose-dependent increase in oxidative stress induced by acetaminophen with significantly low FABP1 expression [69]. FABP1 also plays an early protective role in acetaminophen-induced mitochondrial impairment through scavenging free radicals within the mitochondria itself as well as in the cytosol [69.] FABP1 has been reported to possess strong antioxidant properties [69]. FABP1 prevents free fatty acid-induced lipotoxicity and is known to be down-regulated in the pathogenesis of the non-alcoholic fatty liver disease (NAFLD) in animal models as well as in NAFLD patients [70]. Administration of Radix platycodi (RP), the roots of *Platycodon grandiflorum* (Traditional Oriental Medicine) significantly prevented alcohol-induced elevation of serum and liver lipids by normalizing the FABP expression in alcohol-treated rats [71]. Protein, as well as mRNA expression of L-FABP, showed significant decrease following ethanol consumption in mice [26]. In accordance with this role of FABP, the present study observed decreased expression levels of FABP1 in acetaminophen and alcohol-treated animals while treatment with *Neem-giloe* satwa increased the expression in these animals. The animals treated with *T. sinensis* satwa also exhibited a significant increase in the FABP1 expression in alcohol-treated animals.

NF-κβ (Nuclear factor-κβ) is one of the most important transcription factors, and it is activated by inflammatory cytokines like TNF-α (Tumor necrosis factor-alpha) [72]. The NF-κβ pathway is complex and is activated by phosphorylation, ubiquitination, and proteolysis of the inhibitory protein IκB (I kappa B), which nominally binds NF-κβ in the cytosol in the inactive form [73].

Song *et al*., (2014) recently reported significantly up-regulated expression of TNF-α and NF-κβ in acetaminophen-induced hepatotoxicity in mice [74]. mRNA and protein expressions of TNF-α and NF-κβ were significantly upregulated in D-galactosamine -induced hepatotoxicity [75].

Tu *et al*., (2012) observed a significant increase in TNF-α in carbon tetrachloride intoxicated rats [76]. The serum levels of pro-inflammatory cytokines, such as TNF-α and NF-κβ, were significantly elevated in isoniazid induced hepatotoxicity in albino rats [58]. NF-κβ is also thought to play a major role in liver regeneration [77]. Acute ethanol administration causes prominent hepatic microvesicular steatosis with mild necrosis and increased levels of SGPT and TNF-α in mice [78].

In the present study, NF-κβ and TNF-α expressions were higher in acetaminophen and alcohol-treated animals as compared to healthy animals. The diabetic rats treated for 24 weeks with *T. cordifolia* extract (250 mg/kg) exhibited a significantly reduced number of inflammatory markers such as TNF-α and IL-1β [79, 80]. The NF-κβ and TNF-α inhibitory activity are attributed to a variety of chemical constituents such as alkaloids, diterpenoid lactones, steroids, glycosides, aliphatic compounds, and polysaccharides from different parts of *T. cordifolia* [80]. Improvement in the expression of NF-κβ and TNF-α has also been reported in isoniazid or cyclophosphamide or alcohol-intoxicated rats when treated with different secondary metabolites of medicinal plants [81, 28.]

Even treatment with polysaccharides from *Aloe vera* is reported to decrease the expression of TNF-α in alcohol-intoxicated mice [13]. Silymarin, a standard drug used in the present study, has been reported to suppress NF-κβ gene expression in the hepatoma cell line HEPG2 [82].

Apart from the intervention groups in the present study, NF-κβ gene expression was also found to be significantly decreased in the rats treated with silymarin (positive control).

In the present study, NF-κβ gene expression was found to be significantly decreased in satwa of *T. cordifolia*, *T. sinensis* and *Neem-giloe* while there was statistically insignificant decrease in TNF-α gene expression in *Neem-giloe* satwa treated rats as compared with acetaminophen treated group. In alcohol-intoxicated animals, expression of NF-κβ and TNF-α was increased while it was significantly decreased in the animals treated with satwa of *T. cordifolia*, *T. sinensis* and *Neem-giloe*.

The probable mechanism of action of these satwa in acetaminophen and alcohol-intoxicated rats is shown in **Fig. 4** and **5**.

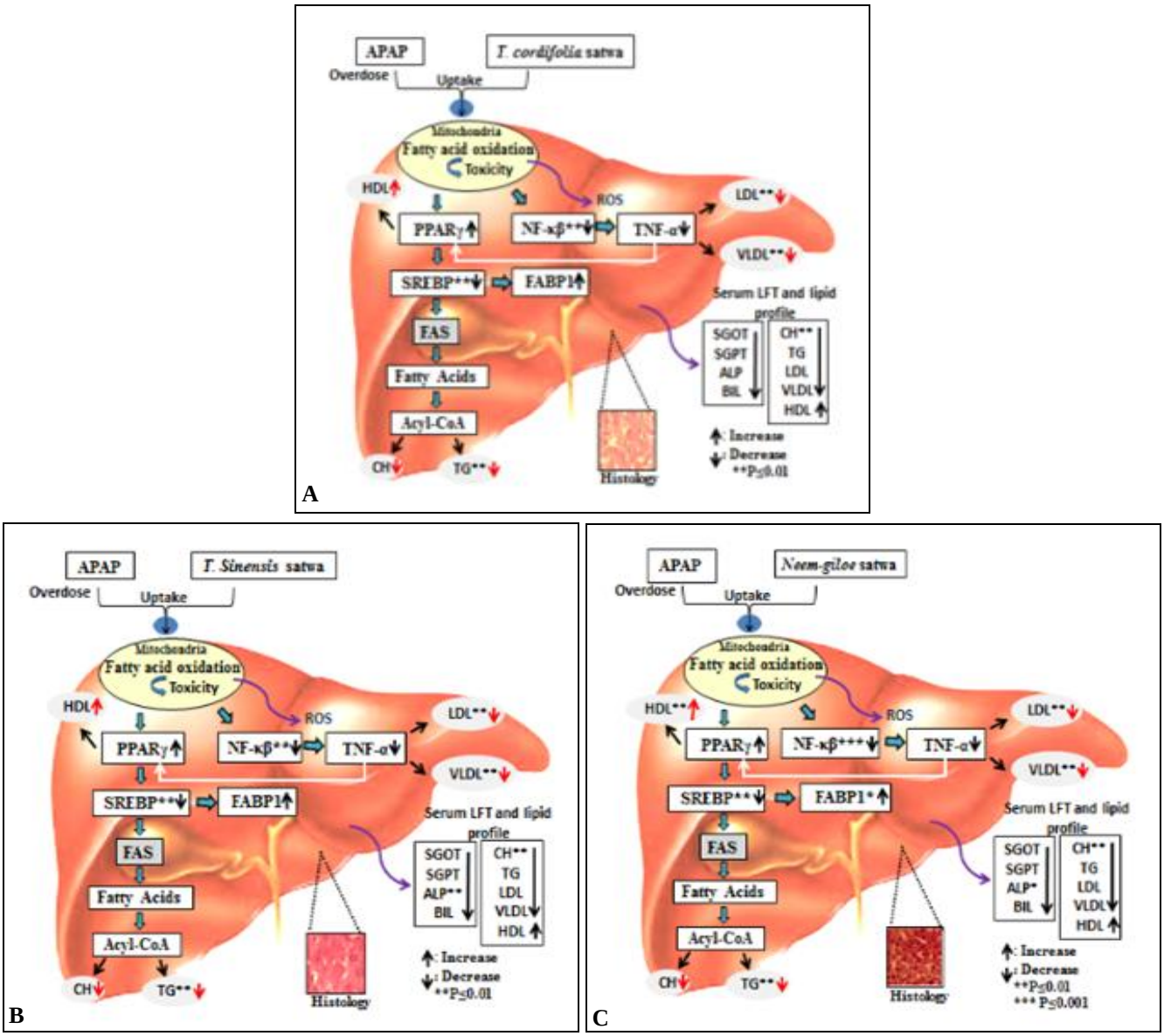

FIG. 4: A MODEL FOR PROBABLE MOLECULAR MECHANISM OF ACTION OF SATWA FROM THREE DIFFERENT FORMS OF TINOSPORA AGAINST ACETAMINOPHEN INDUCED HEPATOTOXICITY. A: EFFECT OF *T. CORDIFOLIA*, B: EFFECT OF *T. SINENSIS*, C: EFFECT OF NEEM-GILOE. APAP: ACETAMINOPHEN; PPARΓ: PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR γ; SREBP: STEROL REGULATORY ELEMENT BINDING PROTEIN; NF-Kβ: NUCLEAR FACTOR KAPPA β; ACYL-COA: ACETYL COENZYME A; FAS: FATTY ACID SYNTHASE; TNF-α: TUMOUR NECROSIS FACTOR A; SGOT: SERUM GLUTAMIC OXALOACETIC TRANSAMINASE; SGPT: SERUM GLUTAMIC PYRUVIC TRANSAMINASE; ALP: ALKALINE PHOSPHATASE; BIL: TOTAL BILIRUBIN; HDL: HIGH-DENSITY LIPOPROTEIN; LDL: LOW-DENSITY LIPOPROTEIN; VLDL: VERY LOW-DENSITY LIPOPROTEIN; TG: TRIGLYCERIDES: CH: TOTAL CHOLESTEROL

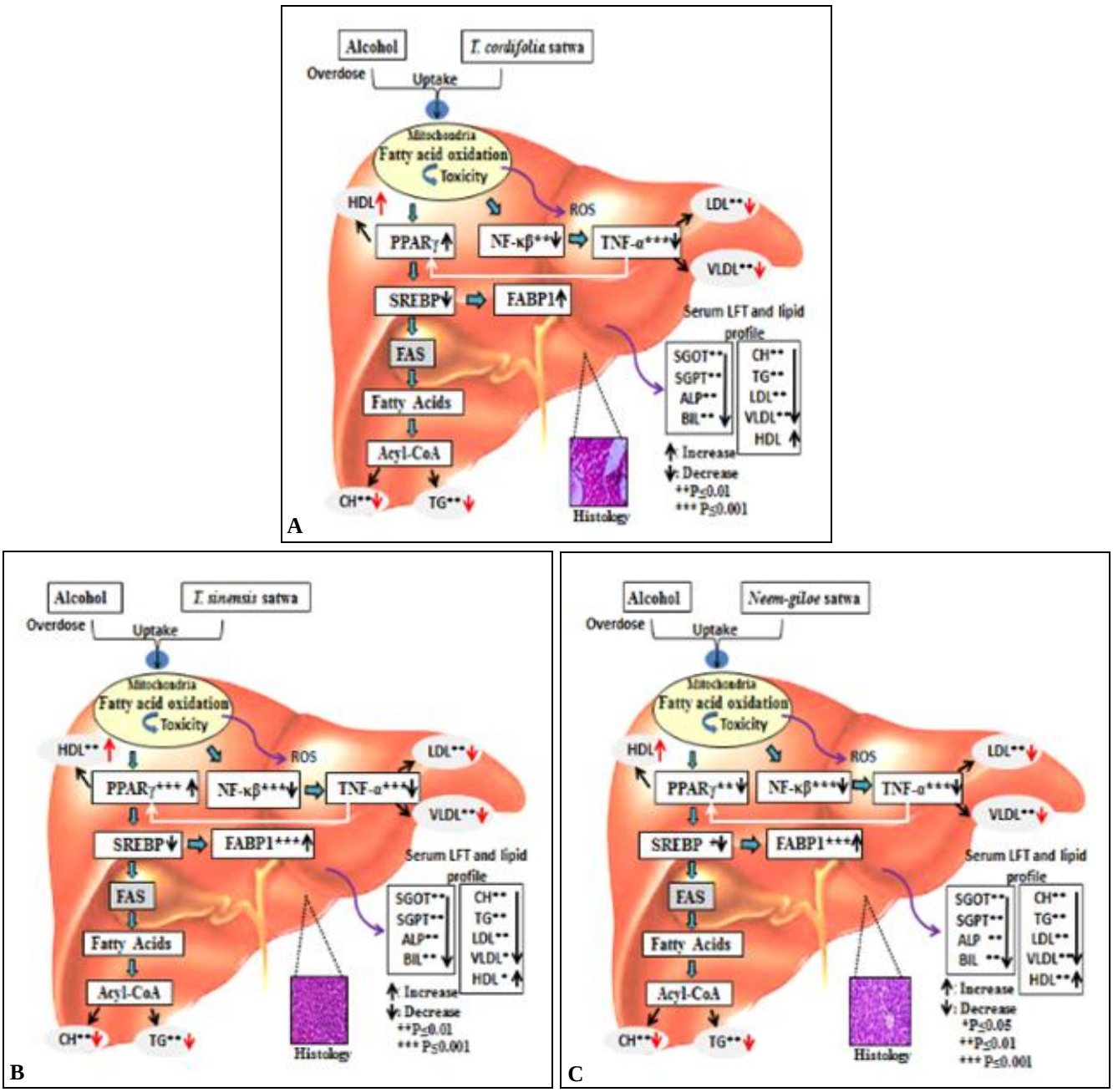

FIG. 5: A MODEL FOR PROBABLE MOLECULAR MECHANISM OF ACTION OF SATWA FROM THREE DIFFERENT FORMS OF TINOSPORA AGAINST ALCOHOL INDUCED HEPATOTOXICITY. A: EFFECT OF *T. CORDIFOLIA*, B: EFFECT OF *T. SINENSIS*, C: EFFECT OF NEEM-GILOE. PPARΓ: PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR Γ; SREBP: STEROL REGULATORY ELEMENT BINDING PROTEIN; NF-Kβ: NUCLEAR FACTOR KAPPA β; ACYL-COA: ACETYL COENZYME A; FAS: FATTY ACID SYNTHASE; TNF-α: TUMOUR NECROSIS FACTOR A; SGOT: SERUM GLUTAMIC OXALOACETIC TRANSAMINASE; SGPT: SERUM GLUTAMIC PYRUVIC TRANSAMINASE; ALP: ALKALINE PHOSPHATASE; BIL: TOTAL BILIRUBIN; HDL: HIGH-DENSITY LIPOPROTEIN; LDL: LOW-DENSITY LIPOPROTEIN; VLDL: VERY LOW-DENSITY LIPOPROTEIN; TG: TRIGLYCERIDES: CH: TOTAL CHOLESTEROL.

CONCLUSION: In conclusion, the current study provides novel information on the protective mechanisms of satwa of three different forms of Tinospora against acetaminophen and alcohol-induced hepatotoxicity. Our findings suggest that this satwa attenuated inflammation and improved lipid metabolism in acetaminophen and alcohol-intoxicated rats. Further, the hepatoprotective effects of *Neem-giloe* and *T. sinensis* satwa can be attributed to its ability to upregulate FABP1 and PPARγ and suppression of SREBP1, NF-kβ and TNF-α. The results suggest that the satwa may be used in combination as a hepatoprotective tonic.

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