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HEPATOPROTECTIVE POTENTIAL OF *SAPINDUS MUKOROSSI* AGAINST CCL_4-INDUCED LIVER DAMAGE THROUGH REGULATION OF VOLTAGE DEPENDENT ANION CHANNEL EXPRESSION

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ABSTRACT

In liver diseases.(Voltage Dependent Anion Channel) VDAC had played an important role because it triggers the opening of the mitochondrial porin ion channel that leads to mitochondrial damage and induce apoptotic or necrotic hepatic cell death. In the present study, the relationship between expression of mitochondrial VDAC may underlie the protective effect of *Sapindus mukorossi* against carbon tetrachloride (CCl_4) induced liver damage in Wister rats. The protective potential of total saponin fraction of *Sapindus mukorossi* was determined by evaluating Aminotransferase activity, mitochondrial membrane potential, calcium-induced liver MPT (Mitochondrial permeability transition) and VDAC expression. Saponin administration shows significant dose dependent restoration of serum enzymes levels. The membrane potential of mitochondria dropped from -199.1 ±4.4 mv to-157. 6±4.8mv after the rats has been treated with CCL_4. Pretreatment with (*S.mukorossi* sapogenin fraction)SMSF showed significant preservation of mitochondrial membrane potential as compared to CCl_4 control demonstrated the mitochondrial protection. It exerted a dose-dependent effect against sensitivity to mitochondrial swelling induced by calcium. SMSF (150 mg/kg) significantly increased the transcription and translation of VDAC. In conclusion the study suggests that *Sapindus mukorossi* significantly prevents the damage to liver mitochondria induced by CCl_4 through regulating the expression of VDAC.

KEYWORDS: **Sapindus mukorossi, Total saponin content, mitochondria, Voltage dependent anion channels.**

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INTRODUCTION

Evidences has accepted that apoptotic cell death and necrotic cell death is involved in liver disease and liver injury.[1,2] Mitochondria play a key role in controlling cell death not only by providing chemical energy by oxidative phosphorylation in addition mitochondria involved in cell signaling, cellular differentiation and also act as a center of apoptotic regulation.[3,4] Dysfunction of mitochondria is the commitment step in liver cell death and hepatic cell death is dependent on Mitochondria .[5] Mitochondrial permeability transition (MPT) is defined as an increase in permeability of the inner mitochondrial membrane to allowing the diffusion of molecules of less than 1500 Kilo Dolton molecular weight.[6] Under certain pathological conditions opening of the mitochondrial permeability transition pore (PTP) is critical to the release of both pro and anti-apoptotic factors which result in the attenuation of mitochondrial membrane potential, swelling of mitochondria and cell death through apoptosis or necrosis. The mitochondrial PTP composed of a voltage-dependent anion channel (VDAC) has been recognized as a major complex in MPT.[7]VDAC is a key protein located in the outer mitochondrial membrane. That regulates mitochondrial function such as transduction of cellular energy, intracellular ca^{+2} homeostasis and substance metabolism as well as triggers the apoptosis by release of intermembrane space proteins.[8] The genus *Sapindus* belongs to the family Sapindaceae, which has about 2000 species. Most of the species of the *Sapindus* genus are in use for the treatment of several diseases and other commercial purposes. *Sapindus mukorossi*, commonly known as a Reetha, is a deciduous tree. The tree is indigenous to northern and central India and is widely distributed in the Himalayan region, Haryana, Uttar Pradesh, and Chhattisgarh.[9]Traditionally; it is used in the treatment of asthma, snakebite, tooth disorders, piles, dermatological disorders, and hepatic disorders. It is a rich source of potential biological activities.[10-13] A survey of the chemical literature reveals that a great deal of Phytochemical work on different parts fruit, pericarp, seeds, leaves, ripe fruit, roots, and stems of *S. mukorossi, S. saponaira, S. trifoliatus*, etc, has been carried out.[14,15]Chemically, the fruit of *S. mukorossi* is valued for the saponins. Recently, in our study, Total Saponin content of *Sapindus mukorossi* was demonstrated to possess hepatoprotective activity against ccl_4 toxicity in Wister rats.[16]However, the mechanism underlying the Hepato protective activity has not been investigated. In the present study, we evaluated Hepatoprotective potential of *S. mukorossi* against ccl4 induced Hepato toxicity, addressing the possible action of *Sapindus mukorossi* on hepatic mitochondrial and expression of VDAC to search for the possible mitochondrial mechanism underlying its Hepatoprotective activity.

MATERIALS AND METHODS

Extraction, isolation and standardization of the S. mukorossi sapogenin fraction

One kilogram (1 kg) of dried powder of fruit was extracted with cold ethanol (70%) by maceration for seven days and solvent was removed under reduced pressure. The crude ethanolic extract was resuspended in water and chloroform in HCl (50% v/v) was added to carry out acidic hydrolysis of saponin to isolate sapogenin. Chloroform phase was separated and concentrated under 40°C up to 1/3 of the original volume. Chloroform phase was exhaustively extracted three times with water-saturated n-butanol and solvent was removed under reduced pressure. Brown coloured dried powder with 2.41% of yield represents the crude sapogenin mixture and was designated as *S. mukorossi* sapogenin fraction (SMSF). It showed positive results for Salkowski and Noller's test an indicating the presence of triterpenoids in the saponins fraction.[17] The isolated saponins fraction was standardized qualitatively by TLC profile using precoated silica gel plates as stationary phase, Ethyl acetate: Methanol: Water (81:11:8) and anisaldehyde-Sulphuric acid as detecting reagent. The qualitative separation of saponins by TLC revealed the presence of 11 Compound with Rf value 0.27 confirmed the presence of terpenoid saponins. TLC profile of this investigation was similar to that reported in literature.[18] Acute oral toxicity study was carried out on *S. mukorossi* sapogenin fraction as per guidelines of the Organization for Economizing Co-operation and Development, following the up-and-down method (Guideline 425).

Effect of S. mukorossi sapogenin fraction on CCl4 Induced Hepatotoxicity in rats

The test drug was administered with normal saline (10mL/kg BW) to various treatment groups with the help of gavage. The doses for the isolated fractions of *Sapindus mukorossi* extract and Silymarin were calculated on basis of body weight for each individual animal in a group. Animals were divided into five groups with six animals in each group. Group I received single oral dose of CMC (Sodium CMC 0.3%) daily and served as control and was not treated with the toxicant. Group II received CCl_4 (1 ml/kg body weight, i.p, 30% CCl_4 suspended in olive oil1:1ratio) once in every 24 h served as CCl_4 - treated control. Acute toxicity studies were performed and the dose was fixed at low dose of 100 mg/kg body weight, high dose 150 mg/kg body weight for *S.mukorossi* sapogenin fraction and 250 mg/kg body weight for standard Silymarin, respectively. Group III received the standard Silymarin (250mg/kg b.w.) and group IV and V received a suspension of the *S. mukorossi* sapogenin fraction (100 mg/kg b.w., 150 mg/kg b.w.). The animals received these treatments by the oral route for a period of 7 days. On the seventh day except group I, all other groups received 30% CCl_4 suspended in olive oil (1 ml/kg b.w.) i.p. After 24 h of intoxication, on the 8^{th} day, blood was collected from the all groups of animals by cardiac puncture. The collected blood was

allowed to clot and centrifuged at 3000 rpm for 10 min to obtain the serum. Serum alanine aminotransferase and aspartate aminotransferase levels, markers for hepatotoxicity were determined. Meanwhile, the whole liver was excised, liver lobes intended for mRNA and protein analyses were frozen immediately and stored in liquid nitrogen before extraction.

Rat Hepatic Mitochondrial Isolation

Mitochondria were prepared from liver of Albino rats by Modified method of Apprille[14]. In brief excised rat liver was treated with ice cold buffer. Buffer contains 0.1 M Tris–MOPS of 10ml, 20 ml of 1M sucrose and1 ml of EGTA. This treatment removes the blood from the liver of albino rat. Preparation of mitochondrial isolation buffer: 225 mM D-mannitol, 10 mM Tris-HCl, 0.05 mM EDTA and 75 mM sucrose (pH: 7.4) at 4°C. Excised Liver slices were homogenized in mitochondrial isolation buffer. Centrifuged homogenate solution at, 1600 r.p.m for five minutes performed at 4^0 C. Transfer the supernatant of homogenate into polypropylene Falcon tube and centrifuged 10minute at 8800 rpm. Discard the supernatant and re suspend the pellet in isolation buffer prepare suspension. Suspension contains mitochondria's Transfer the suspension to polypropylene Falcon tube and determined protein concentration by using biuret method.[19]

Measurement of mitochondrial membrane potential

The membrane potential of mitochondria was evaluated by up taking of rhodamine dye, which accumulates into mitochondria in response to their ionic charge of the inner membrane. Isolated hepatic mitochondria were immersed in the assay buffer (1.0 mg protein/ml) containing 5mM HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonicacid), 75mMsucrose, 225mM mannitol, pH7.5. The membrane potential of mitochondria was evaluated by spectrophotometrically (Shimdzu1800, Japan) at 530 nm by addition of rhodamine. The mitochondrial membrane potential was calculated by the Nernst equation $\Delta\Psi_m = -59 \log [Rh123] / [Rh123]$. [20]

Measurement of Mitochondrial Swelling

Hepatic mitochondrial swelling was evaluated by measuring all suspensions absorbance at 540 nm. Mitochondrial suspensions were prepared in 5ml of the assay buffer (1mg protein/ml) containing10mM HEPES (N 2hydroxyethyl piperazine - *N*-2ethane sulfonic - acid), 125mM sucrose, 50mMKCl, $2mMKH_2P0_4$, 5 mM succinate. To initiate Mitochondrial swelling 50 mM Ca^{+2} was added to assay buffer at 30^0c. Various concentrations of SMSF (1, 10, 50, 80 and 100 μM) were added to mitochondrial solution 3 min before incubation with 50 μM of Ca^{+2}. After addition of 50MM of Ca^{+2} swelling of mitochondria was assayed by measuring absorbance at every 30sec for 0-10 min and the inhibitory rate of swelling of mitochondria was calculated by following equation.[21]

$$Inhibitory\ rate\ of\ mitochondrial\ sweling\ (\%) = \frac{\Delta A\ normal - \Delta A\ SMSF \times 100}{\Delta A\ normal}$$

Evaluation of VDAC mRNA Level by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) Assay

Total RNA was extracted from excised Liver by using Tripura reagent. Reverse transcription was started by incubating total RNA(2μg) at 42°C for 1hr in a 20 μl reaction mixture containing20 U RNase inhibitor, 0.5 μg Oligo(dT)15,15 U AMV reverse transcriptase and 0.25 mM each of dNTP. The reaction was bring to an end by incubation at 95°C for 5 min.PCR - Polymerase chain reaction) amplification was performed for 30 cycles, including 4 μl cDNA by adding 2.5 U Taq polymerase, 5 mM $MgCl_2$, 0.25 mM each of dNTP and β-actin and 5'-and 3'-sequence-specific oligonucleotide primers for VDAC in 1×Taq polymerase reaction buffer, respectively. Each PCR cycle was comprised of 94°C, 50 sec; 60°C, 1 min; 72°C, 1 min; and finally 72°C, 8 min. β-actin can be using as internal standard for assay of RNA. The amplified fragments were detected by agarose gel electrophoresis and visualized by using (EB) ethidium bromide staining. The oligonucleotide primers used were as follows: For VDAC, anti-sense 5'- CCC TCT TGT ACC CTG TCT TGA -3' and sense 5'- GGC TAC GGC TTT GGC TTA AT -3', yielding a deduced amplification product of 321 base pairs (bps).For β-actin, anti-sense 5'- GGA GGA GCA ATG ATC TTG A -3' and sense 5'- TGC TAT CCC TGT ACG CCT CT -3' yielding a deduced amplification product of 601 bps.

Western Blot Analysis for VDAC

Liver samples were homogenized in ice-cold lysis buffer. Homogenates were centrifuged at 12,000 g for 10 min and the supernatants were collected and the protein concentration was determined using Coomassie brilliant blue dye. The samples (40 μg/lane) were dissolved in the sample buffer and separated by 12% SDS-PAGE (sodium - dodecyl sulphate polyacrylamide gel electrophoresis) gel and electrophoretically transferred onto a PVDF (polyvinylidene-difluoride) membrane. The membrane was incubated with VDAC primary antibody (1:4,000) and β-actin antibody (1:80,000). The membrane was then exposed to ECL (enhanced chemiluminescence solution).

Statistical Analysis

The values are represented as mean ± S.E.M. Statistical analysis was carried out by one way analysis of variance (ANOVA) and comparison of mean values of different groups treated with different dose levels of *S. mukorossi* sapogenin fraction and positive control with normal was performed by Turkey's Multiple Comparison Test. With help of Graph Pad prism 5.0 software value $P < 0.05$ was considered significant.

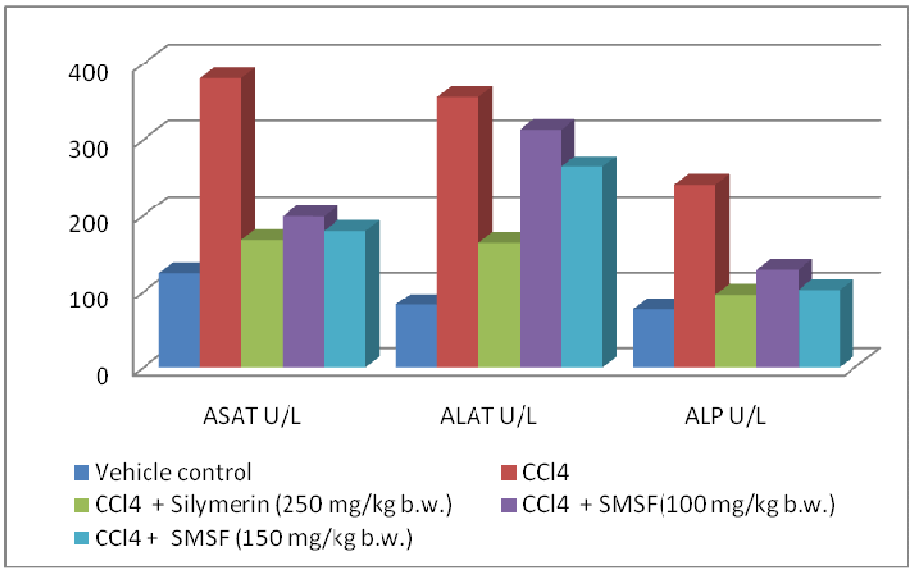
RESULT

Effect of SMSF on ALT, AST and ALP Levels

A significant increase was observed in ASAT, ALAT and ALP levels after exposed to CCl_4 when compared with normal control rats ($P < 0.001$) (Figure.1). However administration of SMSF at dose levels 100 mg/kg b.w, 150 mg/kg b.w and Standard Silymerin 250 mg/kg b.w, showed a significant restoration in the altered biochemical parameters toward the normal.

Figure 1

Effect of S. mukorossi saponin fraction on serum biochemical parameters of CCl4 treated rats. n = 6, P < 0.01, and P <0.001 when compared to negative control (CCl4 treated) group.

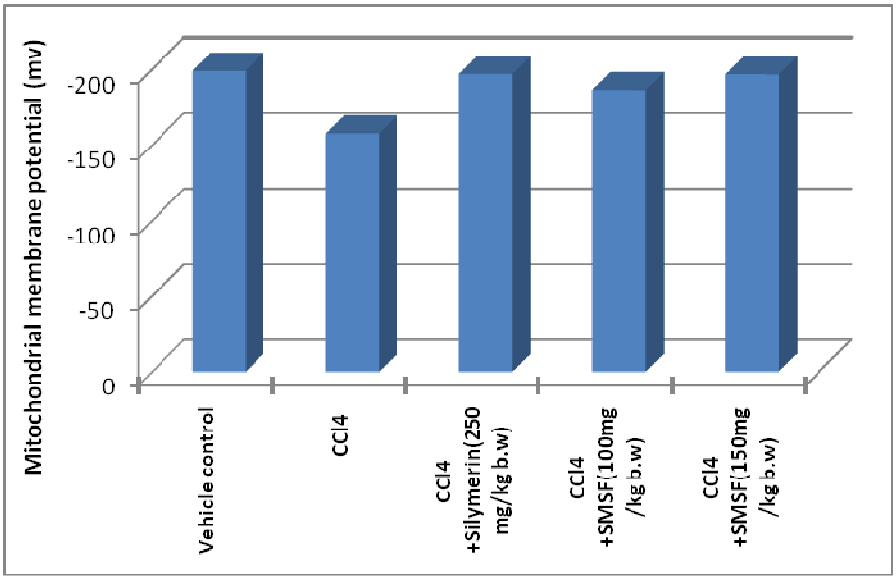


Effect of SMSF on Mitochondrial Membrane Potential Dissipation

The membrane potential of mitochondria of normal rats was − 199.1 ± 4.4 mV, which fell down to −157.6 ± 4.8 mV (16.8 %, $P < 0.01$) in the CCl_4 treated rats (Figure.2). The effect of plant extracts preserved the mitochondrial membrane potential in a dose-related manner and reverses the potential of mitochondria membrane compared to CCl_4 treated rats, at a dose of 100 mg/kg and 150 mg/kg of SMSF of *Sapindus mukorossi,* the mitochondrial membrane potential was restored to the level observed for normal rats.

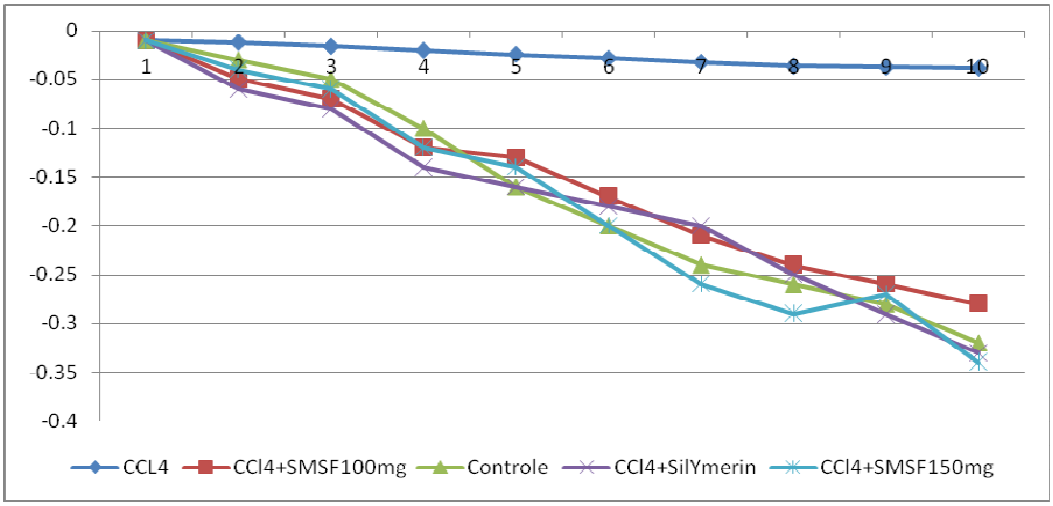
Figure 2

Inhibitory effect of SMSF on mitochondrial membrane potential dissipation induced by CCl4Inhibitory Effects of SMSF on Ca^{+2} - Induced Mitochondrial Swelling



A remarkable mitochondrial swelling induced by addition of 100 μM Ca^{+2} as shown in Fig: 3. Pretreatment with SMSF exerted a dose-dependent effect against Ca^{+2}-induced mitochondrial swelling. At 7 min, The swelling rates of SMSF52.6%, 76.1%, and94.5%, respectively, which were more sensitive than that ofCCl_4 (33.5%).

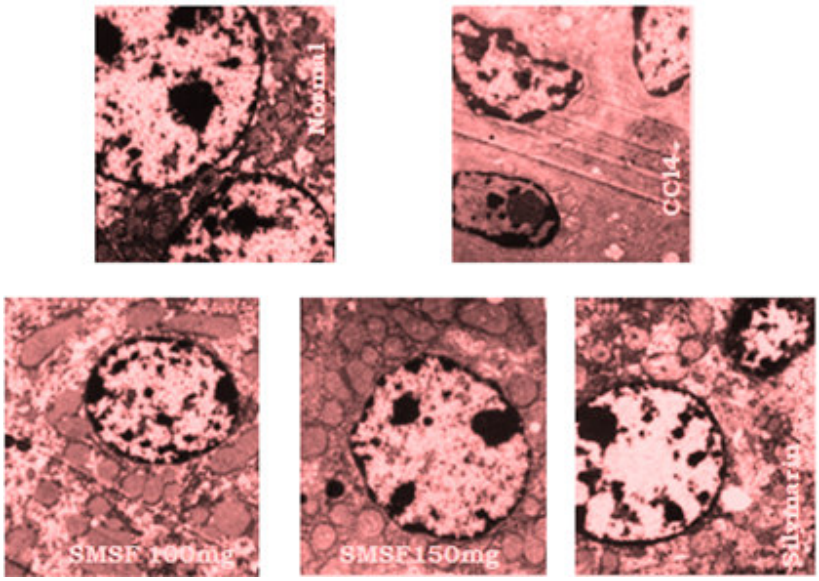
Figure 3
Effect of SMSFon Ca^{2+}-induced mitochondrial swelling.
CCl_4 can decrease the sensitivity in Ca^{2+}-induced mitochondrial swelling.



Effect of SMSF on the Ultra structure of Hepatocytes induced by CCl_4

Discernible ultra-structural changes in hepatocytes shows mitochondrial disintegration, nuclear condensation and lipid deposition as compared with CCl_4 treated rats.. However mitochondria of SMSF treated rats were morphologically different from toxicant group and exhibited a less dense, ballooned appearance compared with CCl_4 treated rats. Groups treated with Silymerin and SMSF150mg/kg b.w shows normal morphological features of mitochondria in that size and cristae structure similar to normal hepatocytes. (Figure 4).

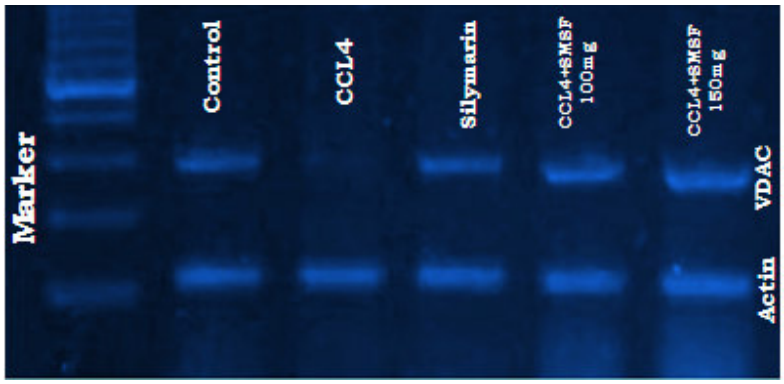
Figure 4
Effect of SMSF on the Ultra structure of Hepatocytes induced by CC



Up-regulation of liver VDAC mRNA level induced by CCl_4

The effect of SMSF on transcription of VDAC mRNA was examined by RT-PCR (reverse transcriptase). As shown in (Figure: 5A), CCl4 treated rats showing lower level of VDAC mRNA expression compared to normal control group. While treatment with 150 mg/kg. SMSF significantly blocked the CCl_4- stimulated VDAC mRNA reduction.

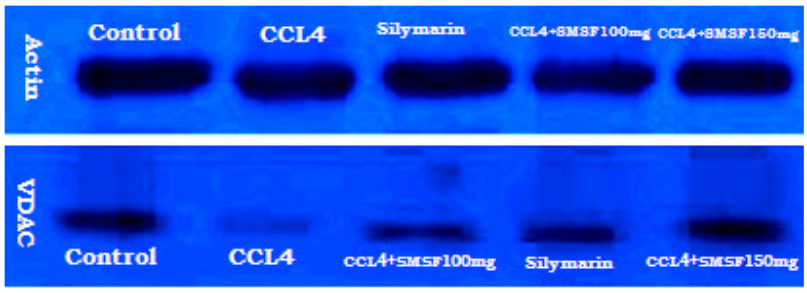
Figure: 5(A)
RTPCR analysis: Inhibitory effect of SMSF on the decrease in VDAC mRNA level induced by CCl4.



Up-regulation of liver VDAC protein level induced by CCl_4

The SMSF mediated Down-regulation of VDAC protein expression was demonstrated by Western blot analysis (Figure: 5B). Normal control animals showed a significant signal for VDAC, and rats receiving CCl_4 treated rats showing a significantly decrease signal for VDAC. In contrast, in rats pretreated with SMSF at 150 mg/kg dose, a higher level of VDAC protein signal similar to that of normal rats was evident 18 h following CCl_4 treatment compared to rats treated with CCl_4 alone.

Figure:5(B)
Western blot analysis: Inhibitory effect of SMSFon the decrease in VDAC protein level induced by CCl4.



DISCUSSION

CCl4-induced liver damage is a well-characterized perspective for acute hepatic failure and is often used to screen drugs for Hepato-protective activities.[22] CCl_4-induced acute liver injury may be involvement of covalent binding of CCl4 metabolites to cell components and per oxidative damage as the cause of injury. covalent binding of the CCl3* radical to cell components initiates per oxidation of the unsaturated fatty acids of cell membrane and lead to membrane injury and leakage of sensitive hepatic serum marker enzymes AST and ALT.[23] The result of this study revealed that a significant increase in AST and ALT levels follows exposure to CCl_4 indicating considerable hepatocellular injury which could be inhibited by the oral administration of SMSF at doses of 100 and 150 mg/kg demonstrating its hepatoprotective effect. Liver damage assessed by the levels of hepatic marker enzymes in serum released from Cytosol and especially mitochondria. ALT is one of the index for the degree of membrane damage, whereas AST is one of the index for mitochondrial damage. At the same time, the effect of SMSF on AST also suggests its possible roles on mitochondria because 80% of AST was released from mitochondria. Another sensitive marker of mitochondrial injury is the dissipation of the mitochondrial membrane potential. Also the protective effects of SMSF on liver mitochondrial membrane potential in CCl4-intoxicated rats were assessed. CCl_4 induced hepatic mitochondrial damage as characterized by the dissipation of membrane

potential of mitochondria which is conforming with previous reports of Gao *et al*., and Tang *et al*.,[24,25]Pretreatment with SMSF(100 and 150 mg/kg) could significantly preventing the collapse of the mitochondrial membrane potential confirming the protective effect of SMSF against CCl4 induced mitochondrial damage. It is very important for the cell to maintain very low levels of ca^{+2} in the Cytosol and ccl_4 can results in hepatocellular Ca^{+2} over load which can activate the mitochondrial Ca^{+2} uni-porter in the mitochondrial inner membrane and induce a mitochondrial Ca^{+2} influx. However, an excessive intra-mitochondrial Ca^{+2} can lead to the opening of mitochondrial PTP and finally damage mitochondria and it induce apoptotic or necrotic cell death. In the present study, effect of SMSF on the intra mitochondrial content in the CCl4-intoxicated rats was evaluated. Thus, Ca^{+2} - induced liver MPT has become a widely used model for evaluating the effect of drugs or other substances on mitochondria. The data revealed that SMSF could act on mitochondria PTP directly against Ca^{+2}-induced mitochondrial swelling which suggests that SMSF may protect mitochondria. Indeed, it has been believed that inhibition of mitochondrial PTP opening might constitute a relevant pharmacological approach to protect cells from death and the search for novel PTP inhibitors should be an important strategy for the treatment of liver diseases.[26,27] VDAC play a vital role in triggering the opening of the PTP. Furthermore, there was assembling evidence that changes in the expression of the mitochondrial VDAC levels. VDAC is one of the most important proteins on the outer membrane with regard to the process of apoptosis.[24,28-30] VDAC levels decreased significantly after CCl_4 administration and pretreatment of SMSF- (150 mg/kg) could inhibit the reduction of both translational and transcriptional levels of VDAC in the acute liver injury process. This suggests a protective effect of SMSF on liver mitochondria in rats that might be related to an up-regulation of the expression of mitochondrial VDAC which was up-regulated by CCl_4. In conclusion, the results of study indicated that *Sapindus mukorossi* has Hepato-protective activity and the mechanisms underlying its protective effects may be related to mitochondrial protection and especially the regulation of VDAC expression.

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