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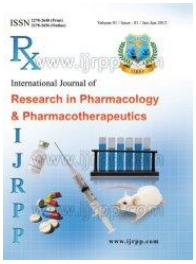

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Research article ***Open Access***

Hepatoprotective potential of *Rheum emodi* against ccl_4-induced liver damage through regulation of voltage dependent anion channel expression

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ABSTRACT

Aim

In liver diseases VDAC has play as important role because it triggering the opening of the mitochondrial porin ion channel that leads to mitochondrial damage and induce apoptic or necrotic hepatic cell death. The present study, the relationship between expression of mitochondrial VDAC may underlie the protective effect of *Rheum emodi* against carbon tetrachloride (CCl_4) induced liver damage in Wister rats.

Methods

The protective potential of the total Anthraquinone glycoside fraction of *Rheum emodi* (TAGF *Rheum emodi*) was determined by evaluating Aminotransferase activity, mitochondrial membrane potential, calcium-induced liver MPT (Mitochondrial permeability transition) and VDAC expression.

Results

Pretreatment with a total Anthraquinone glycoside fraction of *Rheum emodi* showed significant preservation of mitochondrial membrane potential as compared to CCl_4 control demonstrating the mitochondrial protection. In addition, pretreatment with TAGF *Rheum emodi* at various concentrations exerted a dose-dependent effect against sensitivity to mitochondrial swelling induced by calcium. In addition, TAGF (400 mg/kg) significantly increased the transcription and translation of VDAC.

Conclusion

The result obtained from the study suggests that TAGF of Rheum emodi significantly prevents the damage to liver mitochondria induced by CCl_4through regulating the expression of VDAC.

Keywords: *Rheum emodi*, Total Anthraquinone glycoside content, mitochondria, Voltage dependent anion channels.

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 committed 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 weights (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). Voltage dependent anion channel (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 inter membrane space proteins (8).

Rheum emodi is an important medicinal plant which is extensively used in the Ayurveda and Unani systems of medicines. *Rheum emodi* is commonly known as Indian rhubarb/ Revanchini belongs to the family Polygonaceae (9). It has the property of purgative, hemostatic, antipyretic, anthelmintic, laxative, atonic indigestion, constipation, diarrhea, dysentery, antibacterial, antitumor, antifungal, diuretic, hemostatic, cholagogue, antihypertensive, lowers serum cholesterol, anti-inflammatory and antioxidant activity (10-12). Recently, in our study, Total anthraquinone glycoside fraction of *Rheum emodi* was demonstrated to possess hepato protective activity against ccl4 toxicity in Wister rats (13). However, the mechanism underlying the Hepato protective activity has not been investigated.

In the present study, we have evaluated the hepatoprotective potential of the Total anthraquinone glycoside fraction of *Rheum emodi* against ccl_4 induced hepatic toxicity, addressing the possible action of *Rheum emodi* on hepatic mitochondrial and expression of VDAC to search for the possible mitochondrial mechanism underlying its hepatoprotective activity.

MATERIAL AND METHODS

Extraction, isolation and standardization of the Total Anthraquinone glycosides

Five hundred grams (500gm) of the powdered rhizome of *Rheum emodi* were extracted with 3x 3 L of 70% ethanol using a soxhlet apparatus. The extract was evaporated in a rotary evaporator till completely evaporation and dried ethanol extract was weighed. A portion of the crude ethanol extract (75 g) was further extracted by adding 170 ml of water and 30 ml of 70% ethanol. After 10.5% w/v ferric chloride hexahydrate solution (50 ml) was added, the mixture was refluxed for 30 min before adding 20 ml concentrated hydrochloric acid (HCL) and refluxed for another 30 min and allow the mixture to cool. When the mixture was cooled, it was filtered and the filtrate was extracted with 5 x 200 ml of chloroform (CHCL3). The collected extracts from chloroform layer were evaporated after aqueous layer was added to 0.1 g of sodium bicarbonate ($NaHCO_3$) to adjust to neutral pH. The whole solution was centrifuged at 4000 RPM for 20 min. The supernatant which contained Anthraquinone glycoside was evaporated finally brown dried power with 2.25% of yield represented a total Anthraquinone glycoside mixture and it was designated as total Anthraquinone glycoside fraction of *Rheum emodi* (TAGF of *Rheum*

emodi). It showed positive results for Borntragers test, indicating the presence of Anthraquinone glycosides.

TAGF of *Rheum emodi* was standardized by TLC profile using precoated silica gel plates (60 F254) the stationary phase, Petroleum ether: Ethyl acetate: Formic acid (75:25: 1), and 10% methnolic KOH as the spray reagent. TLC revealed the presence of four spots on the plate with R_f values of 0.52, 0.38, 0.27 and 0.84 confirmed the presence of Anthraquinone glycosides The TLC profile of this investigation was similar to that reported in the literature. An acute oral toxicity study was carried out on the total Anthraquinone glycoside fraction of *Rheum emodi* as per guidelines of the Organization for Economizing Co- operation and Development, following the up-and-down method (Guideline 425).

Effect of the total Anthraquinone glycoside fraction of Rheum emodi 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 *Rheum emodi* extract and silymarin were calculated on the 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 oil1:1 v/v mixture of CCl4and olive oil) once in every 24 h served as CCl_4 -treated control. Acute toxicity studies were performed and the dose was fixed at lower dose of 200 mg/kg body weight, high dose 400mg/kg body and 250 mg/kg body weight for anthraquinone glycoside fraction and standard Silymarin, respectively. Group III received the standard Silymarin (250mg/kg b.w.) and group IV and V received a suspension of the total anthraquinone glycoside fraction of *Rheum emodi* (200 mg/kg b.w., 400 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. The 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 the 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 homogeneous solution at, 1600 r.p.m for five minutes performed at 4^0 C. Transfer the supernatant of homogenate into the polypropylene Falcon tube and centrifuged 10minute at 8800 RPM. Discard the supernatant and resuspend the pellet in the isolation buffer prepares suspension. Suspension contains mitochondria's Transfer the suspension to polypropylene Falcon tube and determined protein concentration by using biuret method (15).

Determination 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 the addition of rhodamine. The mitochondrial membrane potential was calculated by the Nernst equation (16) $\Delta\Psi_m = -59 \log [Rh123] / [Rh123]$.

Determination 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 sulfonicacid), 125mM sucrose, 50mMKCl, 2mMKH_2P0_4, 5 mM succinate. To initiate Mitochondrial swelling 50 mm Ca^{+2} was added to assay buffer at 300c. Various concentrations of TAGF of *Rheum emodi* (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 (17).

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

Assessment 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 brought 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 used 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 was 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 (sodiumdodecyl 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 total Anthraquinone glycoside fraction and positive control with normal was performed by Turkey's Multiple Comparison Test. With the help of Graph Pad prism 5.0 software value $P < 0.05$ was considered significant.

RESULTS

Effect of TAGF of Rheum emodi on ALT, AST and ALP Levels

A significant increase was observed in ASAT, ALAT and ALP levels after exposing to CCl_4 when compared with normal control rats ($P < 0.001$) (Table:1). However the administration of TAGF of *Rheum emodi* at dose levels 200 mg/kg b.w, 400 mg/kg b.w and Standard Silymerin 250 mg/kg b.w, showed a significant restoration in the altered biochemical parameters toward the normal.

Table 1: Hepatoprotective effect of Total anthraquinone glycoside fraction of *Rheum emodi* on CCl4 intoxicated rats

Treatment /Concentration	**ASAT U/L**	**ALAT U/L**	**ALP U/L**
Vehicle control	89.48 ± 0.537	60.65 ± 13.54	326.42 ± 2.106
CCl_4	170.04 ± 2.04[a]	116.28 ± 12.29[a]	596.14 ± 3.604[a]
CCl_4 + Silymerin (250 mg/kg b.w.)	80.87 ± 1.47[b]	62.15 ± 12.16[b]	358.06± 2.43[b]
CCl_4 + *R.emodi* TAGF(200 mg/kg b.w.)	96.64 ± 2.36[b]	79.46 ± 10.89[b]	398.12 ± 2.65[b]
CCl_4 + *R.emodi* TAGF (400 mg/kg b.w.)	86.92± 1.29[b]	64.52 ± 12.08[b]	370.09 ± 2.38[b]

Note

Values expressed as mean ± SEM (n=6); a= P < 0.001, when compared to the normal group, b=P < 0.001, c=P < 0.001 when compared to the CCl 4 - treated group

Effect of *TAGF of Rheum emodi* on Mitochondrial Membrane Potential Dissipation

The membrane potential of mitochondria of normal rats was − 199.1 ± 8.2 mV, which fell down to −157.62 ± 6.2 mV (19.2 %, $P < 0.01$) in the CCl_4 treated rats (Figure.1). 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 200 mg/kg and400 mg/kg of TAGF of *Rheum emodi*, the mitochondrial membrane potential was restored to the level observed for normal rats.

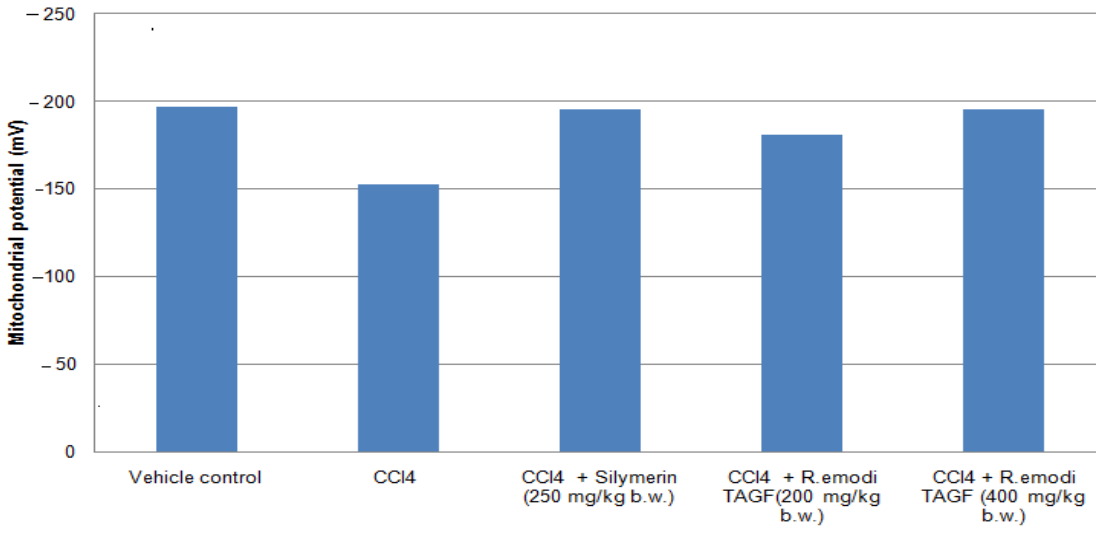

Figure 1: Inhibitory effect of TAGF of Rheum emodi on mitochondrial membrane potential dissipation induced by CCl4

Inhibitory Effects of TAGF of Rheum emodi on Ca^{+2} - Induced Mitochondrial Swelling

A remarkable mitochondrial swelling induced by the addition of 100 μM Ca^{+2} as shown in Fig: 2. Pretreatment with TAGF of *Rheum emodi* exerted a dose-dependent effect against Ca^{+2}-induced mitochondrial swelling. At 7 min, The swelling rates of TAGF of *Rheum emodi* 58.6%, 79.1%, and 92.5%, respectively, which were more sensitive than that ofCCl_4 (34.5%).

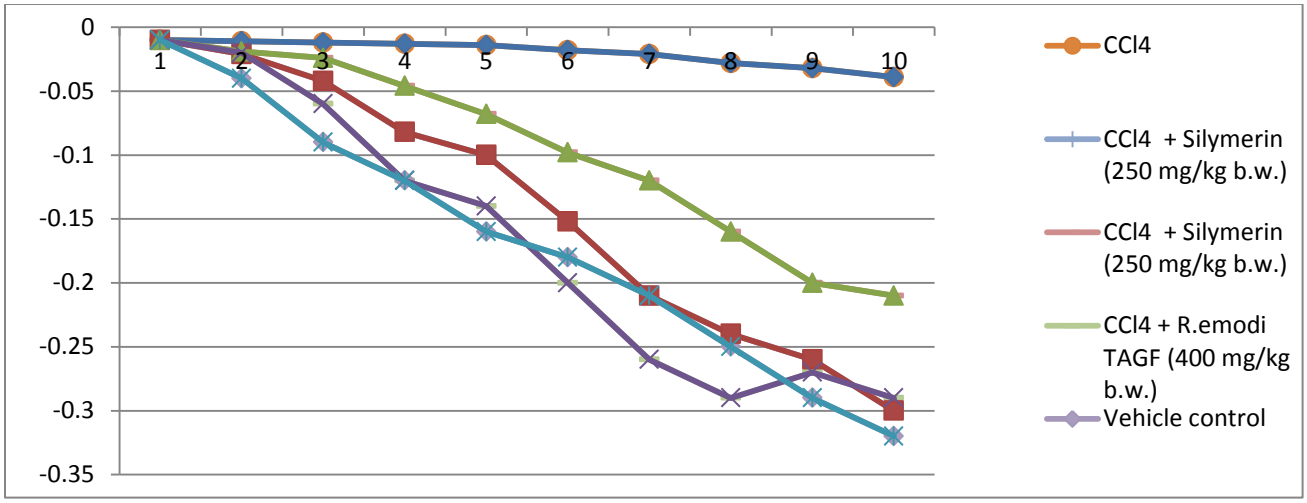

Figure 2: Effect of TAGF of Rheum emodi on Ca2+-induced mitochondrial swelling. CCl4 can decrease the sensitivity in Ca2+-induced mitochondrial swelling.

Regulation of liver VDAC mRNA level induced by CCl_4

The effect of TAGF of *Rheum emodi* on transcription of VDAC mRNA was examined by RT-PCR (reverse transcriptase). As shown in (Figure: 3A), CCl_4 treated rats showing lower levels of VDAC mRNA expression compared to a normal control group. While treatment with 400 mg/kg TAGF of *Rheum emodi* significantly blocked the CCl_4-stimulated VDAC mRNA reduction.

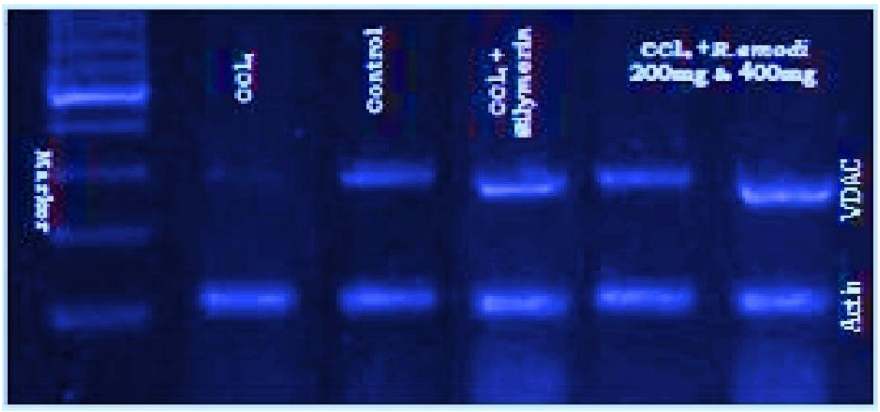

Figure: 3 (A) RTPCR analysis: Inhibitory effect of TAGF of Rheum emodi on the decrease in the VDAC mRNA level induced by CCl4.

Regulation of liver VDAC protein level induced by CCl_4

The TAGF of *Rheum emodi* mediated up-regulation of VDAC protein expression was demonstrated by Western blot analysis (Figure: 4B). Normal control animals showed a significant signal for VDAC, and rats receiving CCl_4 treated rats showing a significant decrease signal for VDAC. In contrast, in rats pretreated with TAGF of *Rheum emodi* at 400 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.

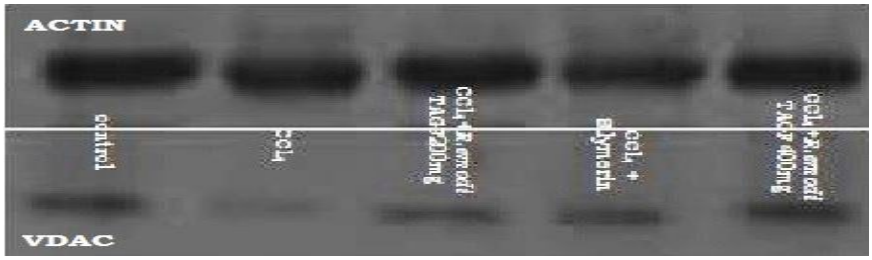

Figure: 3 (B) Western blot analysis: Inhibitory effect of TAGF of Rheum emodi on the decrease in the VDAC protein level induced by CCl_4.

DISCUSSION

CCl4-induced liver damage is a well-characterized perspective for acute hepatic failure and is often used to screen drugs for hepatic protective activities. (18) CCl_4-induced acute liver injury may be involved of covalent binding of CCl4 metabolites to cellular components and per oxidative damage as the cause of injury. Covalent binding of the CCl3* radical to cell components initiates peroxidation of the unsaturated fatty acids of cell membrane and lead to membrane injury and leakage of sensitive hepatic serum marker enzymes AST and ALT. (19)

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 TAGF of *Rheum emodi* at doses of 200 and 400 mg/kg demonstrating its hepato protective 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 TAGF of *Rheum emodi* on AST also suggests its possible roles on mitochondria because 80% of AST were released from mitochondria.

Another sensitive marker of mitochondrial injury is the dissipation of the mitochondrial membrane potential. In the present study, the protective effects of TAGF of *Rheum emodi* 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*., (20,21) Pretreatment with TAGF of *Rheum emodi* (200 and 400 mg/kg) could significantly prevent the collapse of the mitochondrial membrane potential confirming the protective effect of TAGF of *Rheum emodi* against CCl4 induced mitochondrial damage. It is very important for the cell to maintain very low levels of ca^{+2} in the Cytosol and ccl4 can results in hepatocellular Ca^{+2} overload 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 induces apoptotic or necrotic cell death.

In our present study, we evaluated the effects of TAGF of *Rheum emodi* on the intra mitochondrial content in the CCl4-intoxicated rats. Thus, Ca^{+2} -induced liver MPT has become a widely used model for evaluating the effect of drugs or other substances on mitochondria. Our data reveal that TAGF of *Rheum emodi* could act on mitochondria PTP directly against Ca^{+2}-induced mitochondrial swelling which suggests that TAGF of *Rheum emodi* 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. (22, 23)

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 in the outer membrane with regard to the process of apoptosis. (20,24-26) VDAC levels decreased significantly after CCl_4 administration and pretreatment of TAGF of *Rheum emodi* (400 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 TAGF of *Rheum emodi* on liver mitochondria in rats might be related to a up-regulation of the expression of mitochondrial VDAC which was up-regulated by CCl_4.

In conclusion, the results of study indicate that *Rheum emodi* 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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