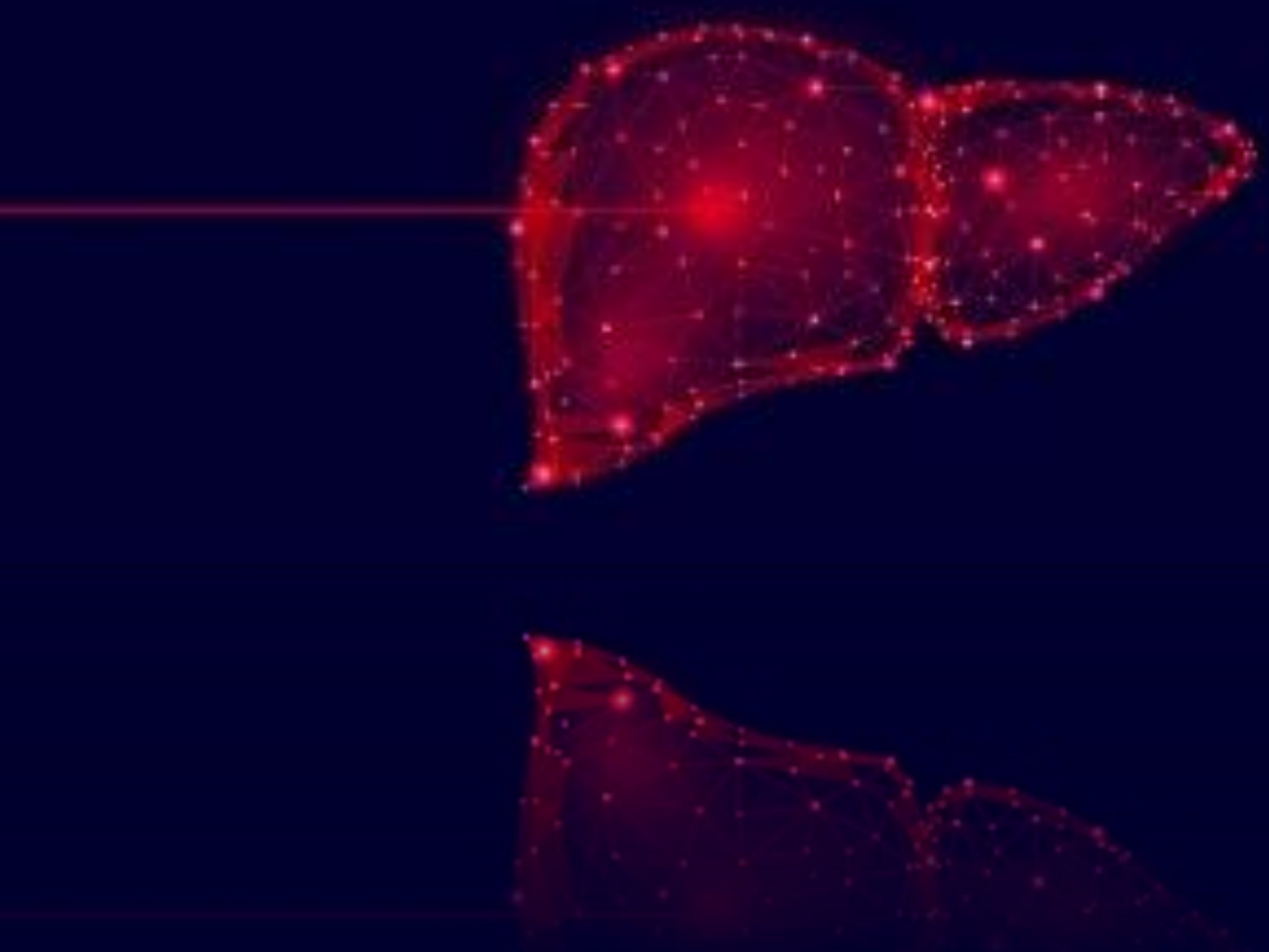
Chapter-2

CORM-A1 improves acetaminophen (APAP) induced hepatotoxicity in swiss albino mice via Nrf2-ARE pathway.



Introduction

Acute liver injury (ALI) can be caused due to alcohol, hepatitis virus infections, autoimmune responses, sepsis and drug/chemicals (Bernal *et al.*, 2010). World is experiencing a constant rise in consumption of therapeutic drugs recommended and prescribed by several pharma companies and clinicians. Drugs administered or consumed alleviates the medical condition on one hand but on the other hand generates a toxic load on liver. Liver damage caused by over dose of such toxins is called drug induced liver injury (DILI) (Zhu *et al.*, 2012). Acetaminophen (N-acetyl-p-aminophenol, APAP), an anti-inflammatory drug is extensively consumed across the world and its overdose is one of the commonly reported cause of hepatotoxicity (Lee, 2012, Morsy *et al.*, 2010, Nourjah *et al.*, 2006, Urrunaga *et al.*, 2015). N-acetyl cysteine (NAC), a precursor for cellular glutathione (GSH) biosynthesis, is the only FDA-approved pharmacologic therapy against APAP overdose (Heard *et al.*, 2012). However, NAC has also been reported for its side-effects like vomiting, nausea and shock (Network, 2010). Also, lack of hepatoprotection reported in some NAC treated cases is because of a limited rescue window that is between APAP ingestion and initiation of therapy (Kerr *et al.*, 2005, Whyte *et al.*, 2007). In APAP overdose, the sulfation and glucuronidation pathways are saturated leading to hepatic CYP-450 activation that metabolizes APAP to N-acetyl-p-benzoquinone imine (NAPQI). Under conditions of depleted GSH, NAPQI binds to cellular proteins and causes activation of JNK pathway and phosphorylation of transcription factors (p53, NF-κB) that accelerates inflammation (Jaeschke *et al.*, 2012, Knight *et al.*, 2002, Saito *et al.*, 2010). These

sequences of events culminate in hepatic necrosis. APAP mediated cellular redox imbalance has been accredited to depletion of cellular GSH. Hepatoprotective agents that protect cellular antioxidant defense system, scavenge free radicals and reduce the risk of ROS induced damage are the key in management of various types of ALI.

Nuclear erythroid 2-related factor 2 (Nrf2) is transcriptional factor and key regulator of oxidative stress in numerous cell types including hepatocytes. Nrf2 protects against APAP induced hepatotoxicity by increasing the expression of both drug metabolizing enzymes and antioxidants. Nrf2 activation promotes transcription of its target genes, viz. NQO1, GCLC, GCLM and HO-1 (Knight *et al.*, 2002, Mani *et al.*, 2013). Therefore, a variety of Nrf2 activators such as phytochemicals, drugs or gases have been reported till date with an aim to develop their potential into marketable therapeutic options against a variety of ailments including liver diseases (Jadeja *et al.*, 2016). Nitric oxide (NO) and hydrogen sulphide (H_2S) have been reported to be hepatoprotective via elevating intracellular GSH levels in APAP treated experimental models thus providing evidences on the use of gasotransmitters as a novel therapeutic approach (Liu *et al.*, 2003, Morsy *et al.*, 2010).

Unlike other gasotransmitters such as NO and H_2S, CO is more stable, and its action is specific to transitional metals. Carbon monoxide releasing molecules (CORMs) with a core containing of third transitional metal such as manganese or ruthenium were found to be fast releasers of CO. Whereas, CORM-A1 is readily soluble in water and consists of boron core which makes it a slow releaser of CO ($t_{1/2}$= 21

min; at physiological pH and temperature). Boron core make CORM-A1 less toxic in biological systems and therefore has evoked scientific interest in investigating its pharmacological role against variety of disease model such as diabetes (Nikolic *et al.*, 2015), myocardial infarction (Varadi *et al.*, 2007), posterior uveitis (Fagone *et al.*, 2015) and neurogenesis (Almeida *et al.*, 2016). Apart from preclinical studies, 5 clinical trials have been initiated to examine safety and efficacy of CO in humans against idiopathic pulmonary fibrosis, pulmonary arterial hypertension, lung inflammation and acute respiratory distress syndrome (ClinicalTrials.gov., Record as of May 25, 2015a, ClinicalTrials.gov., Record as of May 25, 2015b, ClinicalTrials.gov., Record as of May 25, 2015c, ClinicalTrials.gov., Record as of May 25, 2015d). Also, safety and tolerability study of inhaled CO in kidney transplant patients has been reported thus emphasizing the importance of CO in regulating cellular functions. In the light of available background information, present study investigates the mechanism of hepatoprotective potential of CORM-A1 against experimentally induced (by APAP) hepatotoxicity whereas; the Nrf2-Keap1 pathway forms the epicenter of our investigation.

Experimental Design

Experimental Animals

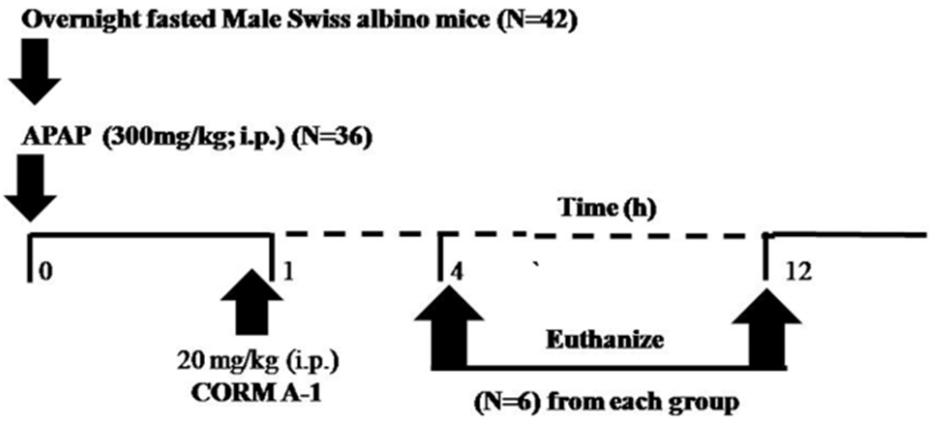
Swiss albino male mice (n=42, 6-8 weeks old, 25-30 gm body weight) were obtained from Flair lab, Surat, Gujarat, India and maintained as per standard guidelines (23±2˚C, LD 12:12, laboratory chow and water *ad libitum*). Experiment was started after 1week of acclimatization. Protocol was approved by Institutional Animal Ethical Committee (IAEC) (Approval no. MSU-Z/IAEC/04-2017) and experiments were conducted in CPCSEA approved *(827/GO/Re/S/04/CPCSEA)* animal house facility of Department of Zoology, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India.

Acetaminophen (APAP) induced liver injury and CORM-A1 treatment

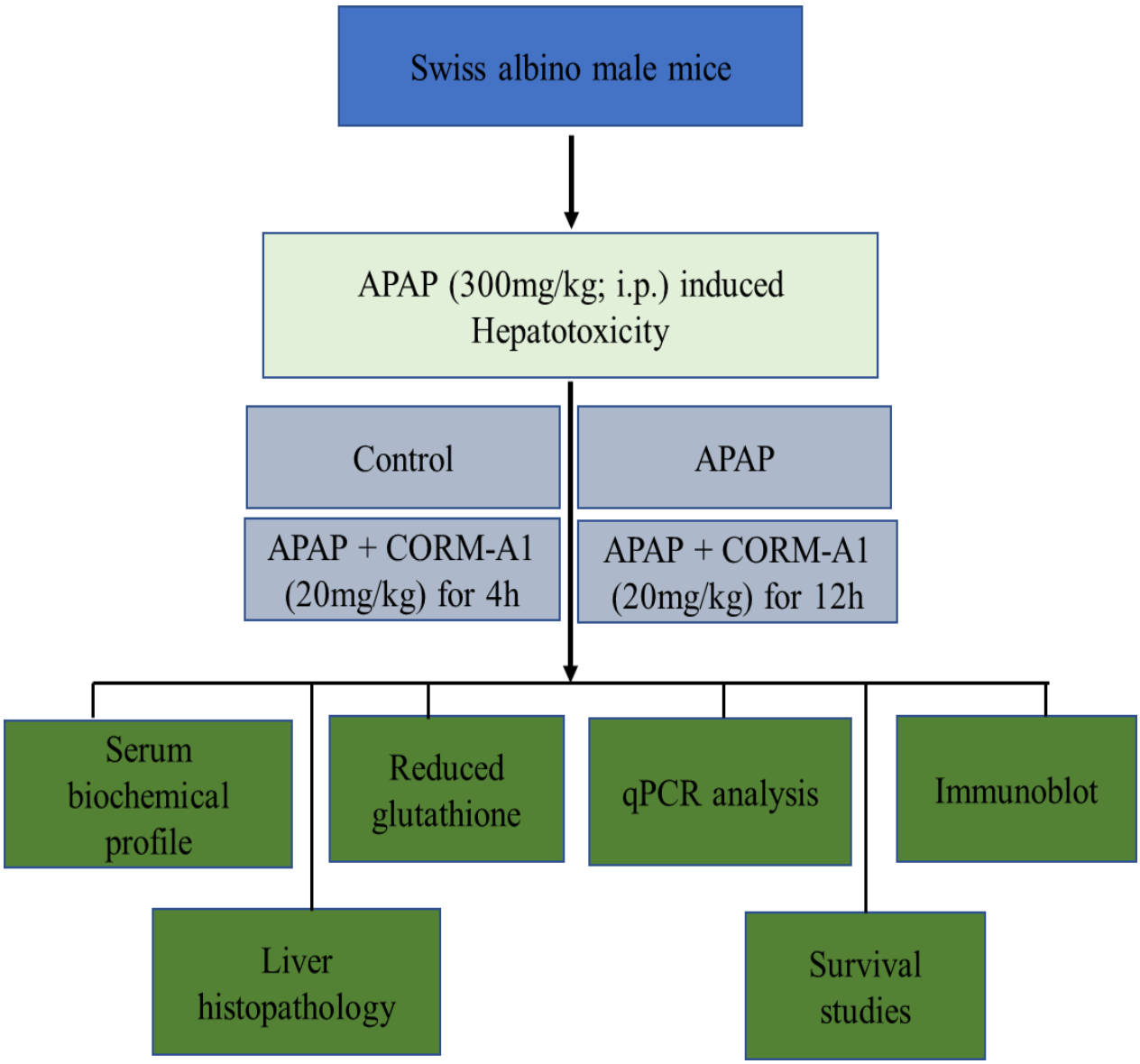
APAP dose (300 mg/kg bodyweight; i.p.) and time point selection was as per (James *et al.,* 2003). Overnight fasted Mice were divided into seven groups (n=6).

- Group 1: Untreated
- Group 2: APAP (4h)
- Group 3: APAP + CORM-A1 (4 h)
- Group 4: APAP (12h)
- Group 5: APAP + CORM-A1 (12 h)
- Group 6: APAP + iCORM-A1 (4h).
- Group 7: APAP + iCORM-A1 (12h).

Intraperitoneal APAP (300 mg/kg) was injected in the said groups and CORM-A1 (20 mg/kg) was injected after 1 h of APAP overdose (Fig. 2.1). Literature suggests different doses of CORM-A1 (2 mg/kg every day/twice a day or a single dose of 60 mg/kg) in mice depending on the experimentally induced disease scenario (Hosick *et al.*, 2014, Mangano *et al.*, 2018, Nikolic *et al.*, 2015). In a pilot experiment, we used single dose of 2 mg/kg of CORM-A1 that had minimal effect on APAP induced liver injury in mice (data not shown here). Since CORM-A1 has a very short half-life (21 min), to achieve antioxidant effect, following administration of APAP one log greater dose of 20 mg/kg was employed to achieve a robust antioxidant effect. Mice were euthanized at 4 h or 12 h wherein livers were dissected, stored in 10% formalin, RNAlater and at −80 °C for histology, RNA isolation and protein respectively.



Fig, 2.1. Study design depicting treatment schedule of swiss albino male mice injected intraperitoneally with APAP (300 mg/kg). CORM-A1 (20 mg/kg, i.p.) was injected after 1 h. Experimental groups include control, APAP treated or APAP+CORM-A1 treated mice (n=6/group). Mice were euthanized at 4 And 12 h followed by liver autopsy.


Swiss albino male mice
APAP (300mg/kg; i.p.) induced Hepatotoxicity
Control
APAP
APAP + CORM-A1 (20mg/kg) for 4h
APAP + CORM-A1 (20mg/kg) for 12h
Serum biochemical profile
Reduced glutathione
qPCR analysis
Immunoblot
Liver histopathology
Survival studies

Results

CORM-A1 reduces APAP-induced elevation in circulating markers of liver injury

Swiss albino mice injected with APAP (300mg/Kg B.W.) showed significant increment in AST and ALT activity levels at 4 and 12 h. Similar increment was also recorded in activity levels of serum ALP. However, CORM-A1 injection (after 1 h of APAP administration; 20 mg/kg bodyweight) accounted for significant decrement in serum titers of AST, ALT and ALP. But the levels of said parameters were significantly higher than the control at the end of 12 h of treatment (Fig.2.2). However, no significant changes were observed with iCORM-A1 group. Hence, the same was not studied for further investigation (Fig.2.3).

CORM-A1 reduces APAP-induced hepatocyte necrosis

Microscopic observations of H&E stained sections of liver of control and treated mice were observed, scored and photographed (2.5X and 40X, Leica DMRB 2000). Observations revealed focal necrosis in the portal area, hepatocytes ballooning and nuclear condensation sinusoidal enlargement, disruption of endothelial integrity and focalized fatty infiltration (Fig.2.4). Control and treated slides were scored for necrosis and vacuolation manually by investigator blinded to the study. Results revealed that APAP treatment accounted for increased indices of necrosis (~1.5 fold) and vacuolation (~4 fold) of hepatocytes whereas, APAP+CORM-A1 treated group recorded lesser (~0.3 or ~1 increment respectively) prominent changes

(Fig.2.5). These patterns of changes were consistent at both 4 and 12 h stages of treatment.

CORM-A1 facilitates Nrf2 translocation and improves antioxidant status of liver in APAP treated mice.

Tissue level oxidative stress is reduced either directly by scavenging ROS or by upregulating antioxidant defenses. Hence, protective effect of CORM-A1 against liver injury with changes in Nrf2 and related enzymes was investigated. mRNA levels of Nrf2 were significantly decreased at 4 and 12 h following APAP administration. However, treatment with CORM-A1 orchestrated hepatoprotective effect by upregulating Nrf2 expression. Expression of Nrf2 protein in nuclear fraction confirms translocation at 4 and 12 h following CORM-A1 treatment (Fig.2.7-A). Keap1 and CYP2E1 mRNA levels was also reduced with CORM-A1 treatment. Whereas, HO-1, a downstream regulator of Nrf2 gene recorded an increase in mRNA and protein levels in APAP and APAP+CORM-A1 treated groups with increment being more pronounced in the latter group (Fig.2.6-B&Fig.2.7-B). NQO1, a key antioxidant enzyme recorded significant increment at 12 h in APAP+CORM-A1 treated group. Activity levels of GCLC and GCLM revealed moderate to significant decrement at 4 and 12 h following APAP treatment. CORM-A1 administration to APAP treated mice recorded significant improvement in GCLC and GCLM mRNA expression. Hepatic GSH showed significant decrement at both 4 and 12 h stages following APAP treatment, but a reciprocal increment was recorded in CORM-A1 treated group (Fig.2.6). Taken

together these results suggested that CORM-A1 protect liver against APAP induced injury through upregulating ARE genes.

CORM-A1 alleviates APAP-induced upregulation of inflammatory cytokines

APAP-mediated sterile inflammation is initiated and perpetuated by damage-associated molecular patterns (DAMPs). RNA levels of NF-κB and downstream markers of inflammation were investigated in control and experimental groups. mRNA levels of NF-κB and IL-1β were significantly increased following APAP treatment at both 4 and 12 h. The same were significantly decreased in CORM-A1 treated groups. TNF-α level markedly increase in liver of APAP treated mice; in mice treated with CORM-A1 recorded a significant decrement. mRNA levels of IL-6 were unchanged in both APAP and APAP + CORM-A1 treated groups at 4 and 12 h (Fig.2.8). Overall, the results indicate that treatment with CORM-A1 reduces hepatic cytokine production, possibly via downregulation of NF-κB, thus reducing the sterile inflammation.

CORM-A1 improved percentage survival of APAP treated mice

For survival studies mice were divided into 3 groups (n=10); control, APAP dose and APAP + CORM-A1 treated group. Acute dose of APAP (600 mg/kg bodyweight) was administered and mice were monitored for 24 h and results were represented in Kaplan-Meier survival curves (Fig.2.9). APAP treatment accounted for pronounced mortality of 50% (n=5) at 4 h followed by 100% mortality by 10 h (additional 5) as compared to the control. APAP+CORM-A1 treated group had only

20% mortality (n=2) at 4 h followed 50% (Additional 3) by the end of 6 h. Later, there was no mortality recorded in this group till the end of study. Briefly, mice treated with APAP + CORM-A1 showed an improved survival percentage as compared to APAP treated group. Cage-side observations revealed that general activity levels of mice in both the experimental groups were greatly reduced but no diarrhea or convulsions were observed.

Table-2.1. Effect of CORM-A1 on serum AST, ALT and ALP against APAP induced hepatotoxicity.

	Control	**APAP(4h)**	**APAP+CORM-A1(4h)**	**APAP (12h)**	**APAP+CORM-A1(12h)**
AST	159.66±23.38	603.75±30.037	498.50±29.50	524.00±32.659	349.00±50.204
ALT	36.50±2.601	416.66±38.940	69.50±45.030	384.50±71.500	184.00±54.249
ALP	140.00±24.378	244.00±19.056	174.00±56.00	411.50±13.472	227.25±38.206

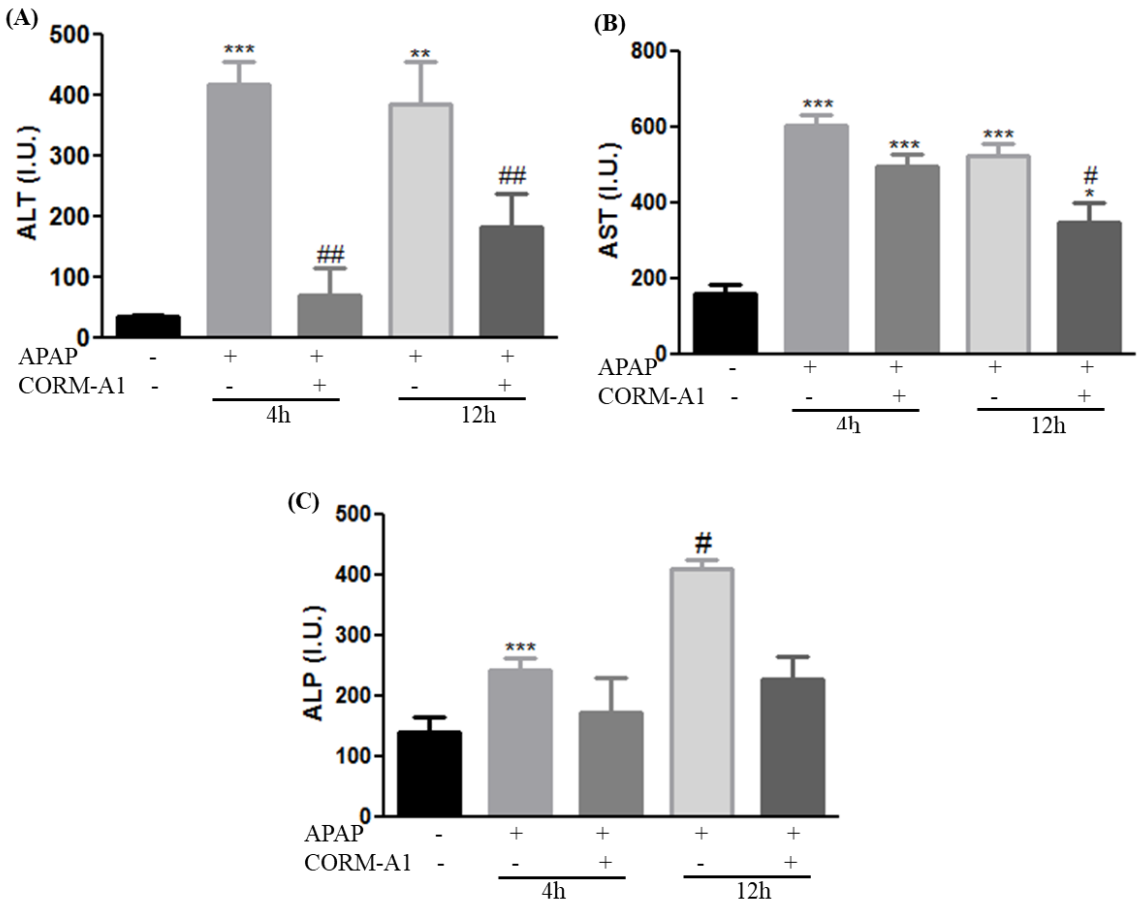


Fig.2.2. CORM-A1 reduces (A) ALT (B) AST and (C) ALP in APAP induced hepatotoxicity. Results expressed as Mean ± SEM. *P<0.05, **P<0.01 and *** P<0.001 as compared to control group whereas; #P<0.05, ##P<0.01, and ###P<0.001 is when compared with respective APAP treated group.

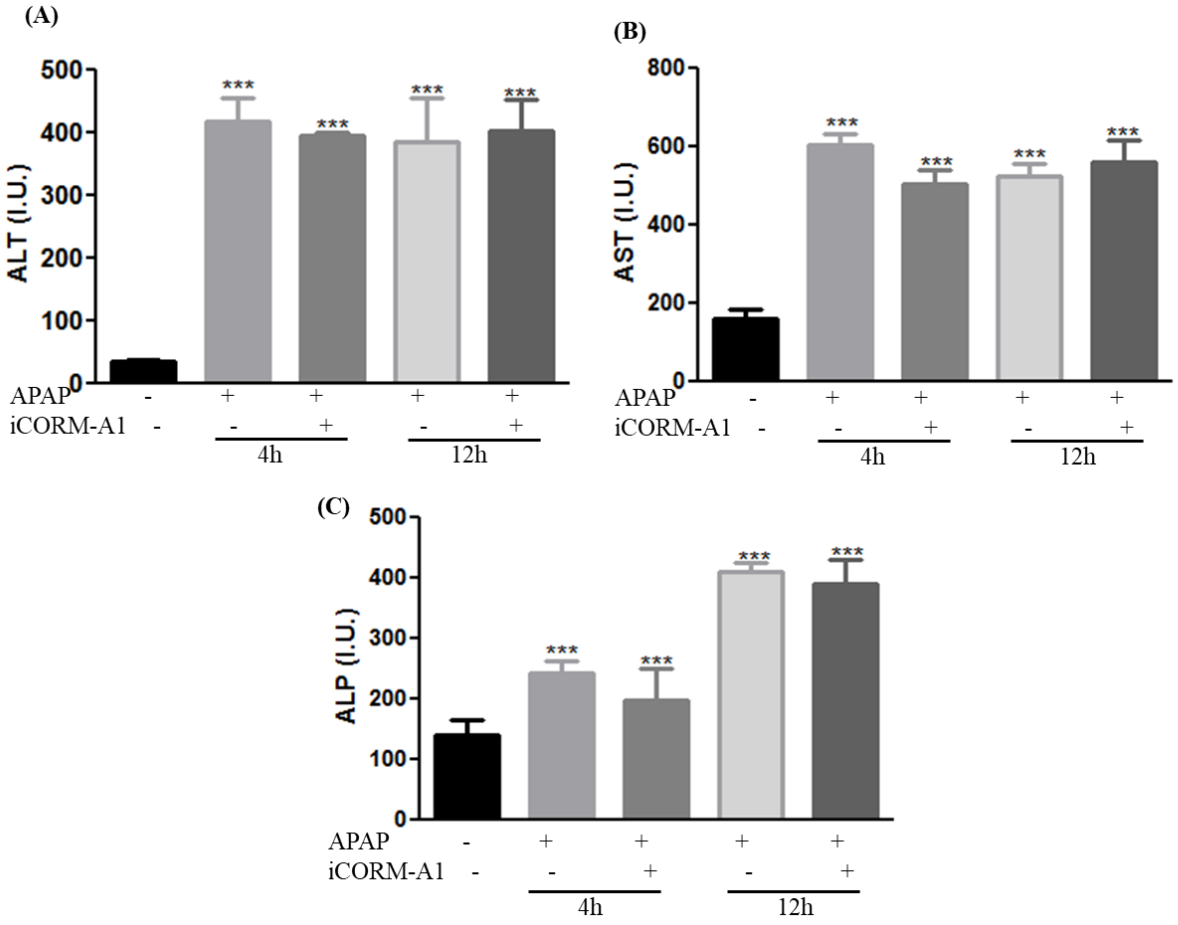


Fig.2.3. Effect of iCORM-A1 on liver function enzymes (A) ALT (B) AST and (C) ALP in APAP induced hepatotoxicity. Results expressed as Mean ± SEM. *P<0.05, **P<0.01 and *** P<0.001 as compared to control group whereas; #P<0.05, ##P<0.01, and ###P<0.001 is when compared with respective APAP treated group.

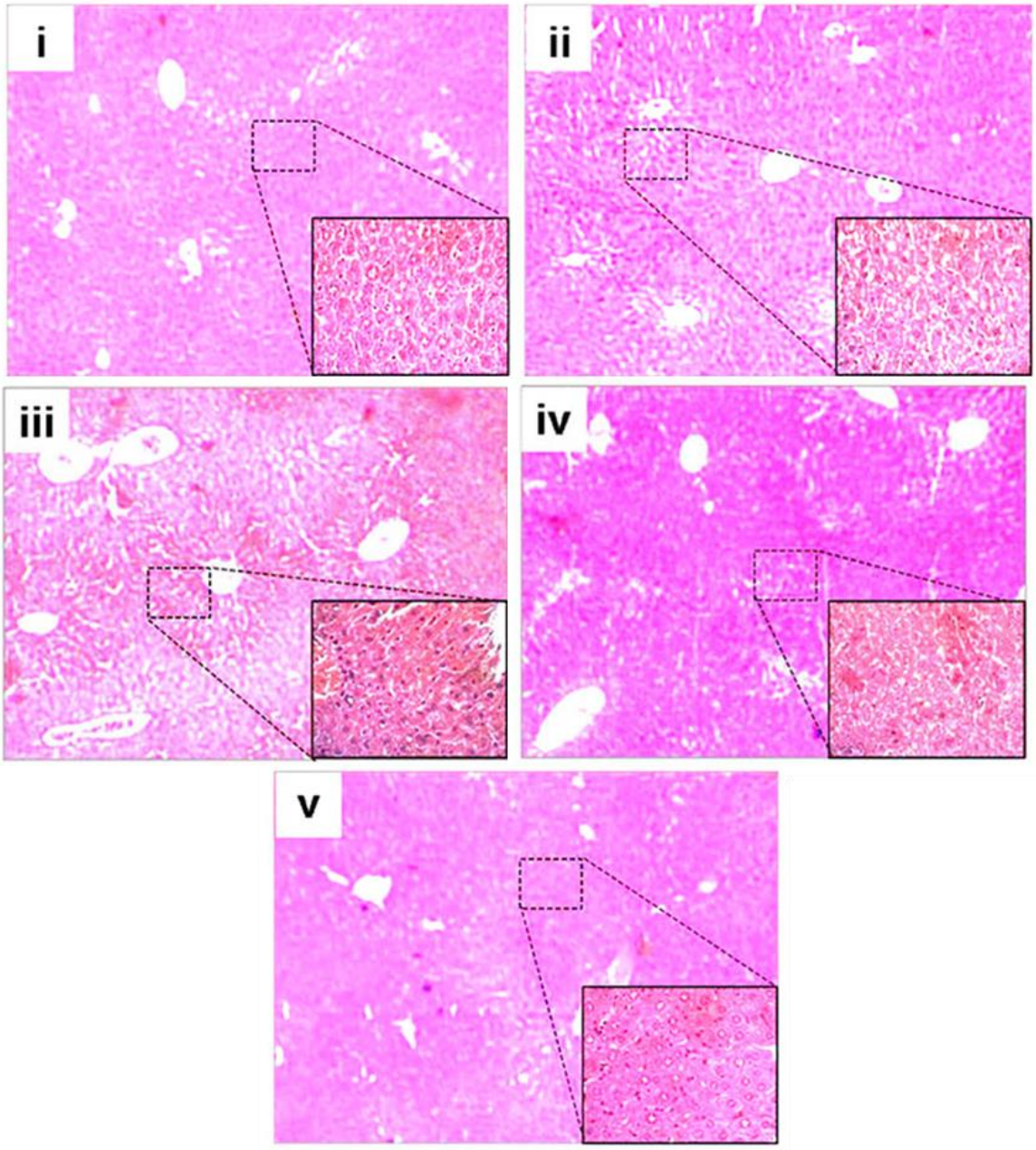


Fig.2.4. Microscopic evaluations of H&E-stained liver sections (2.5X and insert 40X). (i) Control, (ii) APAP (4h), (iii) APAP + CORM-A1 (4h), (iv) APAP (12h) and (v) APAP + CORM-A1 (12h).

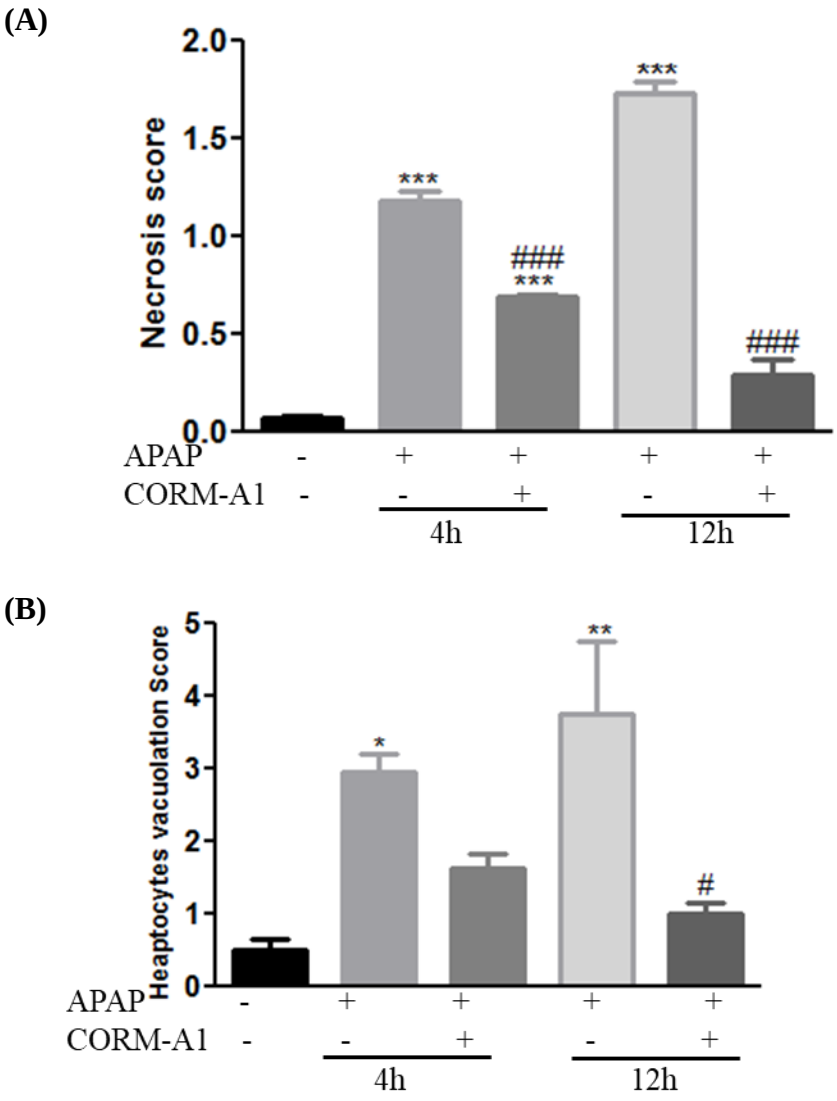
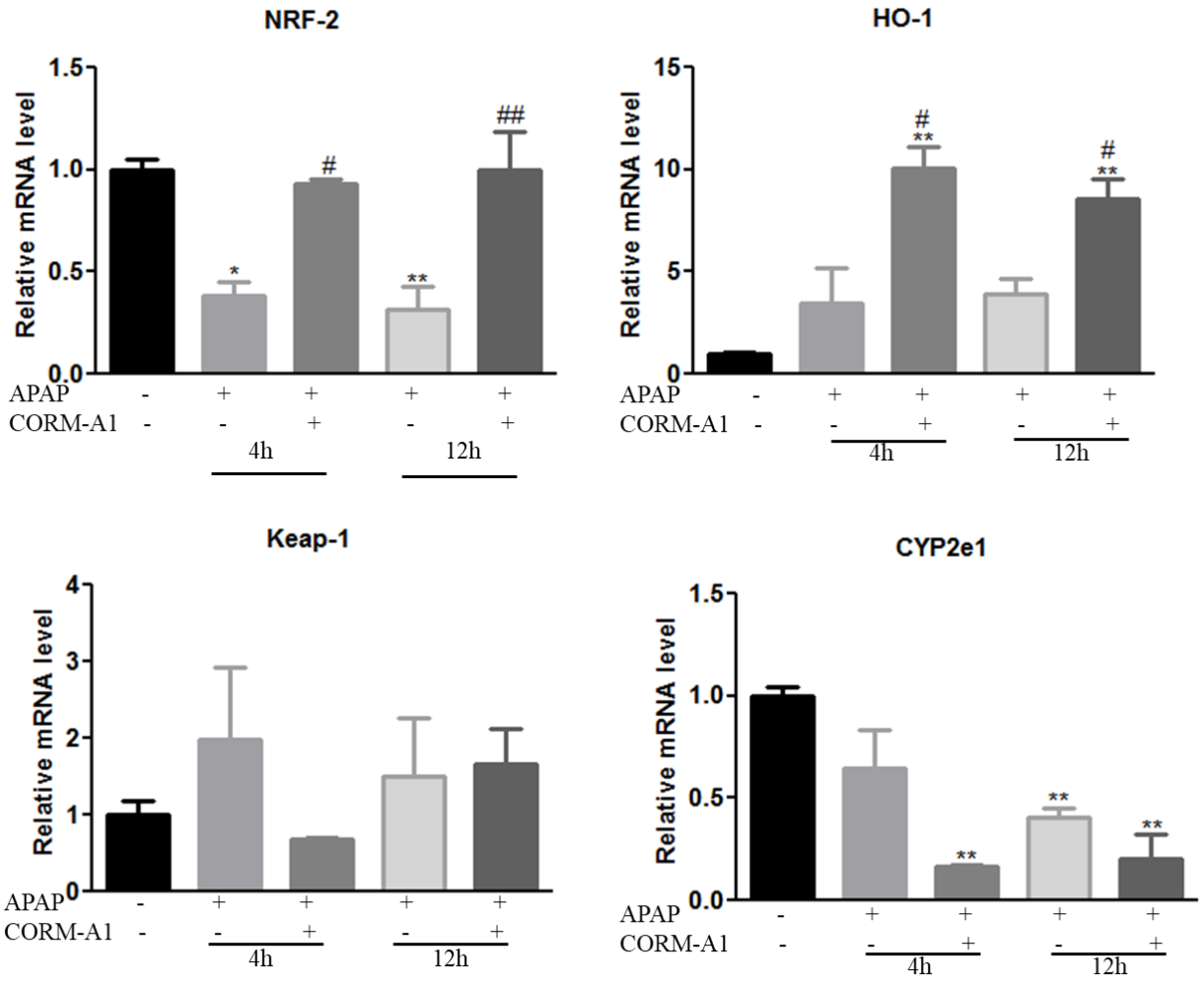


Fig.2.5. CORM-A1 improved hepatocyte necrosis and vacuolations. 3 slides per liver scored for toxicity viz. (A) hepatocyte necrosis (B) hepatocyte vacuolation. Results expressed as Mean ± SEM. *P<0.05, **P<0.01 and *** P<0.001 as compared to control group whereas; #P<0.05, ##P<0.01, and ###P<0.001 is when compared with respective APAP treated group.


NRF-2
Relative mRNA level
1.5
1.0
0.5
0.0
*
#
**
##
APAP - + + + +
CORM-A1 - - + - +
4h
12h
HO-1
Relative mRNA level
15
10
5
0
#
**
#
**
APAP - + + + +
CORM-A1 - - + - +
4h
12h
Keap-1
Relative mRNA level
4
3
2
1
0
APAP - + + + +
CORM-A1 - - + - +
4h
12h
CYP2e1
Relative mRNA level
1.5
1.0
0.5
0.0
**
**
**
APAP - + + + +
CORM-A1 - - + - +
4h
12h

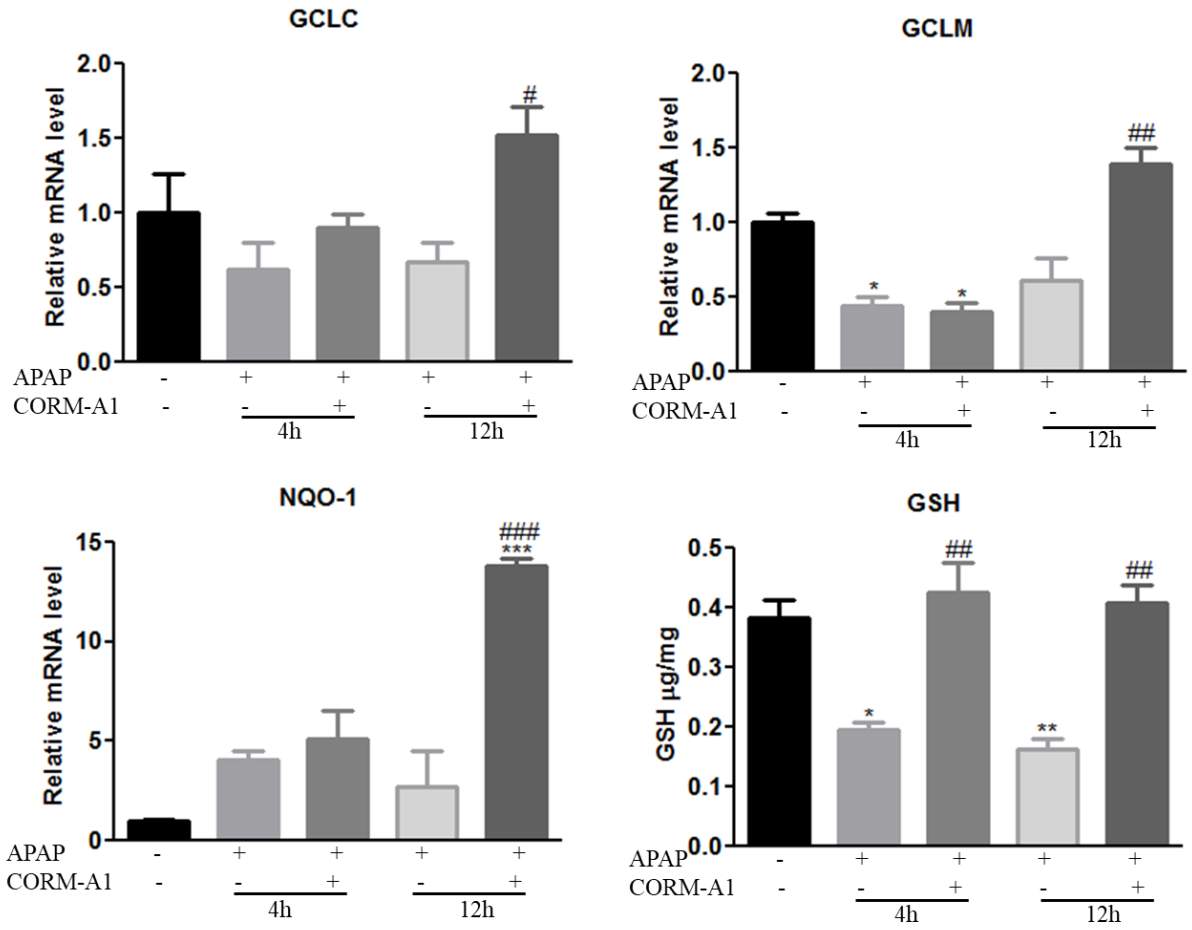


Fig.2.6. mRNA levels of Nrf2-ARE genes viz. (A) Nrf2, (B) HO-1 (C) Keap1, (D) CYP2E1, (E) GCLC (F) GCLM (G) NQO-1 and (H) hepatic GSH content of control and treated mice. Results expressed as Mean ± SEM. *P<0.05, **P<0.01 and *** P<0.001 as compared to control group whereas; #P<0.05, ##P<0.01, and ###P<0.001 is when compared with respective APAP treated group.

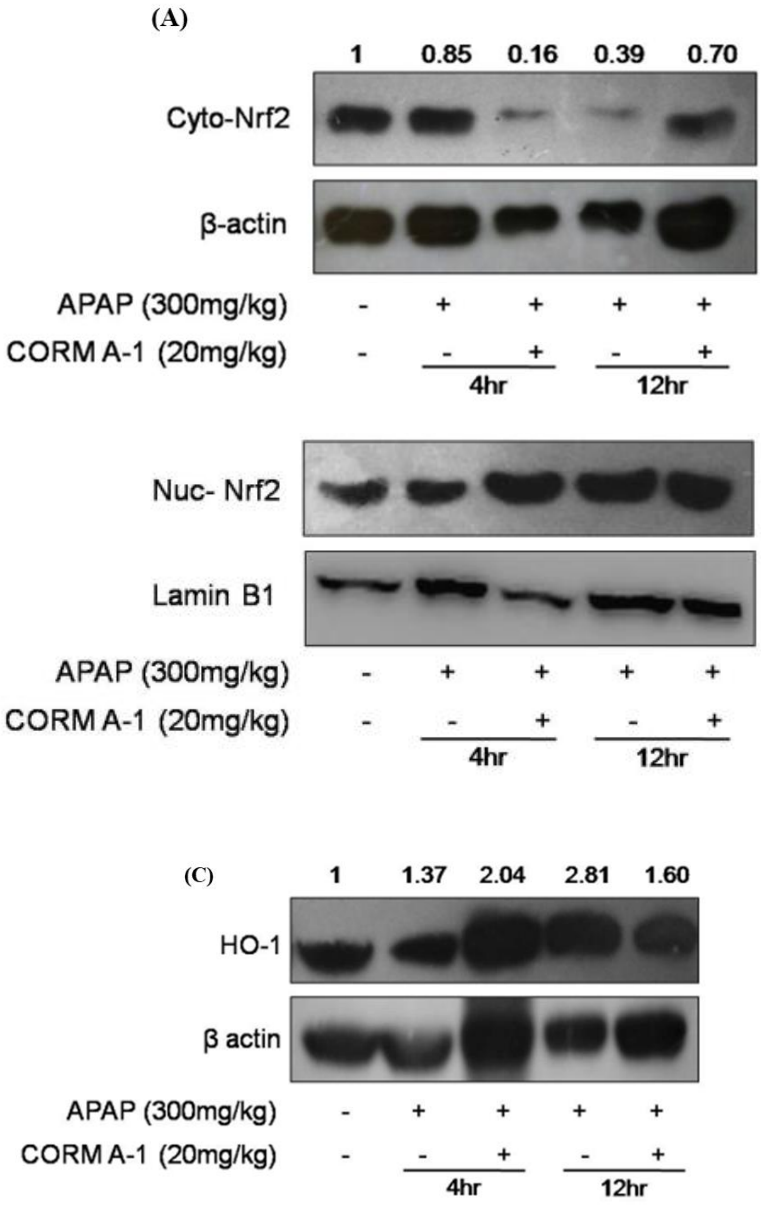


Fig.2.7. Immunoblots analysis of (A) Nrf2 in cytosolic fraction and in nuclear fraction and (B) HO-1 in cellular fraction of control and treated mice.

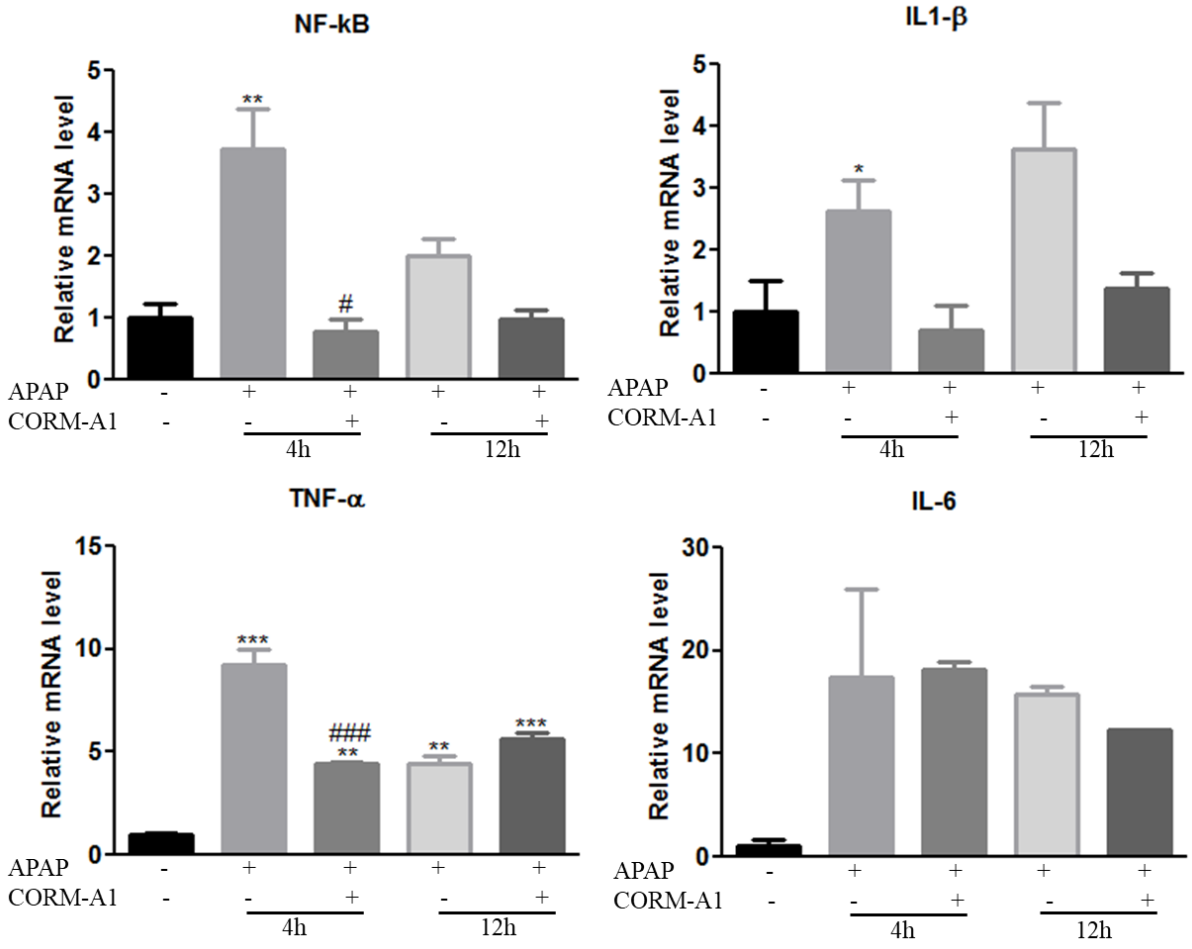


Fig.2.8. mRNA levels of inflammatory marker genes: Nf-κB, IL-1β, TNF-α, IL-6 in liver of control and treated mice. Results are expressed as Mean ± SEM. *P<0.05, **P<0.01 and *** P<0.001 as compared to control group whereas; #P<0.05, ##P<0.01, and ###P<0.001 is when compared with respective APAP treated group.

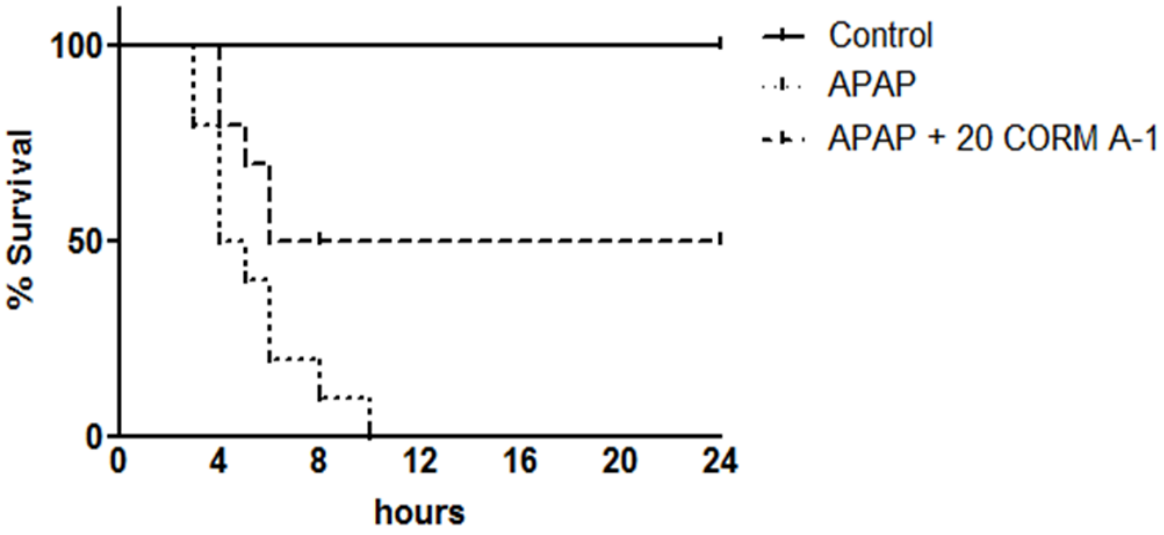


Fig.2.9. Survival Study following lethal dose of APAP (600 mg/kg) and effect of CORM-A1 (20 mg/kg after 1 h). Kaplan–Meier survival curve shows improved survival in CORM-A1 treated mice.

Discussion

Hepatotoxicity associated to DILI is related with oxidative stress mediated hepatocyte death. In this study, we investigated the therapeutic potential of CO releasing CORM-A1 in alleviating oxidative stress-induced hepatotoxicity. In this regard, APAP induced acute liver injury model of Swiss albino mice was employed. A recent study had shown that Concanavalin A-induced hepatitis in mice is reduced with CORM-A1 pretreatment (Mangano *et al.*, 2018). Hence, an important aspect of our experimental strategy was administration of CORM-A1 an hour after APAP overdose. This approach was employed for various reasons. (i) It mimicked the clinical scenarios wherein, patients are treated after the APAP overdose. (ii) This strategy reduced the possibility of interference with CYP-450 activity (Du *et al.*, 2015), since APAP is metabolized by CYP-450 into NAPQI, a critical step before the necrotic signaling is initiated. (iii) NAPQI-mediated protein adduct formation is a key step in initiating hepatocyte necrosis. APAP protein adduct formation peaks in about 0.5–1 h and then declines there after along with GSH recovery in mice (McGill *et al.*, 2013). Administration of CORM-A1 after 1 h of APAP overdose, prevented interference with above mentioned crucial signaling events.

The circulating titers of serum transaminases AST, ALT and ALP are indicators of hepatic function. In this study, the same were found to be significantly altered following APAP treatment suggesting development of hepatotoxicity. Microscopic evaluation revealed progressive necrosis, cellular vacuolation, hepatocyte ballooning, distorted hepatic ports, intrahepatic hemorrhage and infiltration of inflammatory cells in liver of APAP treated mice. These observations were in

agreement with the reported effect of APAP (Jadeja *et al.*, 2015). Single dose of CORM-A1 (20 mg/kg) injected following 1 h after APAP injection restored circulating titres of AST, ALT and ALP closer to control group. Also, histoarchitecture of APAP+CORM-A1 treated group appeared to be comparable to that of control with a less number of cells showing the said toxic manifestation (>0.5 fold).

The GSH antioxidant system plays a key role in elimination of toxic substances and failure of the same results in hepatotoxicity (Chan *et al.*, 1992). Pioneering studies have opined that hepatic necrosis begins after a marked depletion in GSH stores (Yuan *et al.*, 2009). Our results suggest that CORM-A1 modulate later events such as GSH recovery and activate ARE genes leading to improvement in antioxidant milieu in liver. Activation of Nrf2 contributes in stimulating cellular defense mechanism by upregulation of genes in ARE promotor region (viz. HO-1, GCLC, GCLM and NQO-1) that reduces oxidative stress and maintain the ratio of oxidant: antioxidant in a cell. APAP induced downregulation of Nrf2 and related genes resulting in GSH depletion is well documented in murine model of hepatotoxicity (Reisman *et al.*, 2009) and the same was observed in our study. Significantly high mRNA levels of Nrf2, HO-1, GCLC, GCLM and NQO-1 in APAP + CORM-A1 treated group corroborates with the increment in GSH levels. Our data regarding CORM-A1 induced upregulation of Nrf2 is similar to an effect seen in a mouse model of autoimmune hepatitis (Mangano *et al.*, 2018). Nrf2 $^{-/-}$ mice have been reported to be more susceptible to a variety of experimentally induced liver injuries including APAP toxicity due to lowered expression of drug metabolizing enzymes

and antioxidants that underlines the importance of Nrf2-ARE pathway in hepatoprotection (Enomoto *et al.,* 2001). CORM-A1 induced upregulation of Nrf2 and related genes observed herein, supports hepatocellular antioxidant machinery to counteract the action of APAP.

Previous studies had suggested that APAP-mediated liver injury is followed by sterile inflammation (Woolbright *et al.,* 2017). In liver injury, the role of NFκB in modulating tissue level inflammatory response is via release of cytokines (IL-1α, IL-1β, IL-6, TNFα or Fasl), recruitment of inflammatory cells and activation of neutrophils (Dambach *et al.*, 2006). Other studies had indicated that IL-1β, TNF-α, and IL-6 are involved in liver regeneration. Also, role of inflammatory and immune cells in modulating APAP-mediated liver injury is under active investigation wherein, NFκB upregulation and histoarchitectural changes are well-established (Ju, 2012). Lowered hepatic inflammation (reduced expression of NFκB, TNF-α and IL-1β) along with an improved functional status of liver observed in our study is comparable with a similar study conducted by other group wherein hepatoprotection is observed in APAP treated mice administered with saponin rich extract of Rosa laevigata michx fruit or Dioscin respectively (Dong *et al.*, 2014, Zhao *et al.*, 2012). APAP-dosed mice treated with CORM-A1 had less hepatic injury as compared to those that did not receive CORM-A1.

Till date, NAC is the only FDA-approved antidote for APAP-induced hepatotoxicity. In humans, higher effect of NAC was observed when administrated in early hours of APAP overdose (i.e. 8-10 h) (Lee *et al.*, 2009). However, according to AASLD guidelines irrespective of the time of APAP ingestion NAC

can be administered in all acute liver failure patients (Ryter *et al.*, 2006). Epidemiological studies indicate that most patients with APAP-induced liver injury either spontaneously recover or die if liver is not transplanted within 4 days (Reddy *et al.*, 2016). Liver transplantation is not feasible in many patients due to shortage of organs and psychosocial reasons. Hence newer therapies are needed in patients who present 10 h after APAP overdose. Also, in case of a toxic insult, extending the therapeutic window is the key for an effective treatment (Carvalho *et al.*, 2013). Therefore, we had included, survival study following an acute lethal dose of APAP (600 mg/kg). The said dose of APAP accounted for 50% mortality within first 4 h followed by 100% mortality by 12 h. However, CORM-A1 treatment to APAP induced hepatotoxic mice delayed the onset of toxic phase by improving (30%) survival at 4 h stage that was followed by an improved survival (50%) at the end of 12 h. Carvalho *et al*., 2013 had reported 100% mortality in swiss albino mice at ~8 h following APAP injection whereas, APAP+NAC treated mice had recorded 45-50% survival at the end of 12 h. These results are comparable to the ones obtained in our study (APAP+CORM-A1 treated group) that recorded ~50% survival at the end of 12 h. It is interesting to note that no further mortality was recorded in APAP+CORM-A1 treated group in our study. But, APAP+NAC accounted for further mortality with an overall survival of ~25% at the end of 24 h. These results underline the effectiveness of NAC in negotiating toxicity in the primordial phase only. whereas, CORM-A1 was found to be effective even in the later stages as it had accounted for an overall higher percentage survival. This is an important observation and first report on hepatoprotective potential of CORM-A1.

Overall, it can be concluded that CORM-A1 can modulate experimentally induced hepatotoxicity by upregulation of Nrf2 and related genes, reducing inflammation and preventing GSH depletion resulting in improved survival rate. These results also underline the importance of slow releasing molecule in CORM-A1 in intracellular signaling and negotiating hepatotoxicity