Original Article

Attenuation of Oxidative Stress and Hepatotoxicity Induced By D–Galactosamine by a Polyherbal Formulation Ambrex–*in vivo* and *in vitro* Studies

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ABSTRACT

Objective: To evaluate the hepatoprotective effect of Ambrex, a polyherbal formulation against D–galactosamine (D-GLN) induced hepatotoxicity in Swiss albino mice as well as in Chang liver cell lines. **Materials and Methods:** Ambrex was orally administered for a period of 7 days at dose levels of 250 and 500 mg/kg b.wt. D-GLN (250 mg/kg b.wt, i.p) was administered 24h prior to sacrifice the animals. The protective effect of Ambrex was evaluated by measuring plasma levels of aspartate transaminase (SGOT), alanine transaminase (SGPT), alkaline phosphatase (ALP), γ–glutamyltransferase (γGT) and total bilirubin. Its effect on antioxidants such as superoxide dismutase (SOD), catalase (CAT) and reduced glutathione (GSH) and lipid peroxide(LPO) was also determined. Histopathological evaluation of liver tissues was carried out. **Results:** Data revealed that Ambrex was able to restore the levels of antioxidants such as SOD, Catalase, and Glutathione to near normal and reduced the elevated plasma levels of SGOT, SGPT, ALP, γ – GT and total bilirubin. It also inhibited the formation of hepatic malondialdehyde induced by D - GLN. *In vitro* studies revealed that Ambrex protected D- GLN induced hepatotoxicity (30μM/ml) at dose levels of 5, 50 and 500ng/ml. Further, mRNA expression also illustrated that Ambrex inhibited the over expression of Bax, Caspase 3, TNF–α, IL–2 and CYP–450. A substantial decrease in the mRNA expression of anti-apoptotic marker Bcl2 was also observed. **Conclusion:** Results suggest that Ambrex has appreciable hepatoprotective effect which was evident from both *in vivo* and *in vitro* results.

Key words: Ambrex, D- Galactosamine, Hepatotoxicity, Chang Cells, Antioxidants.

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INTRODUCTION

D–GLN, a hepatotoxin and transcription inhibitor sensitizes hepatocytes to the cytotoxication via series of mechanisms. Diminution of uridine triphosphate (UTP) nucleotides with formation of uridine diphosphate (UDP) hexosamines and loss of intracellular calcium levels are the associated mechanisms following induction of D–GLN, ultimately leading to inhibition of RNA and DNA syntheses.[1-2] Further, inhibition of hepatocyte energy metabolism, with impairment of liver marker enzymes and alteration in the membrane phospholipid composition were also reported characteristics of D-GLN induced liver damage.[3] Javle and his workers[4] reported that D-GLN induced liver injury is associated with the development of renal failure. D–GLN also causes an elevation of TNFα with increased formation of reactive oxygen species (ROS) which might be circuitously a reason for cellular oxidative insult, thereby altering the antioxidant status.[5]

Regardless of substantial advancement in the treatment of liver disease with modern drugs, search for trail blazing drugs continues because of the side effects and limitations in the prolonged usage of existing conventional therapies.[6] Medicinal plants

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and herbal formulations derived from natural sources have antioxidant or free radical scavenging activity has turn out to be a central focus for research designed to prevent toxicity or tissue injury and may have a significant role in maintaining health.[7] Ambrex, a polyherbal formulation constitutes five medicinal plants namely *Withania somnifera* (100 mg,) *amber* (37.50 mg), *Pistacia lentiscus* (25mg), *Orchis mascula* (25mg) and *Cycas circinalis* (62.5 mg). The medicinal plants present in this formulation possess broad medicinal values individually and was mentioned under Ayurvedic text. The formulation was already reported for its potential protection against butylated hydroxyl toluene (BHT) induced toxicity,[8] gastro protective property[9] and antihyperlipidemic property.[10] The present study was designed to evaluate the hepatoprotective effect of Ambrex in *in vivo* and *in vitro* models.

MATERIALS AND METHODS

Drugs and Chemicals

All cell culture solutions and supplements were purchased from Life Technologies Inc., USA. Dulbecco's Modified Eagle Medium (DMEM) was obtained from GIBCO, USA. D–GLN was obtained from Himedia, India. LIV–52 was purchased from Himalayas, India. Biochemical kits were obtained from Merck, India. Ambrex was procured from Care and Cure Herbs Ltd., India. All other chemicals and reagents were of analytical grade obtained from Himedia, India and Sisco Research Laboratories Pvt. Ltd., India.

Standardisation of Ambrex for curcumin content

Since Ambrex is a polyherbal formulation containing five medicinal plants, we planned to standardise it using a chemical marker which possesses wider biological activity. Curcumin was known for its anti- tumor, oxidant, arthritic, amyloid, ischemic and inflammatory properties. Curcumin was found to be the ideal candidate to satisfy the above criterion. Ambrex was standardised for curcumin content by HPTLC method.[11]

In vivo Study

Animal Husbandry

Young healthy male swiss albino mice (18-22 g) were obtained from central animal facility, Sri Ramachandra University, Chennai, India. Animals were housed in colony cages (6 animals/cage) and were kept under laboratory standard conditions with 22 ± 3°C temperature, 12-h light/12-h dark cycle and 30-70% relative humidity. They were provided with rodent feed (M/s. Provimi Animal Nutrition India Pvt. Ltd, India) and purified water *ad libitum*. Animals were acclimatized at least for 7 days to the laboratory conditions prior to initiation of the experiment. Guidelines of "Guide for the Care and Use of Laboratory Animals" (Institute of Laboratory Animal Resources, National Academic Press 1996; NIH publication number #85-23, revised 1996) were strictly followed throughout the study. Institutional Animal Ethical Committee (IAEC), Sri Ramachandra University, Chennai, India approved the study (IAEC No: IAEC/XXXIII/SRU/268/2013).

Experimental Study Design

The experimental animals were divided into five groups with six animals in each. Group I served as normal control - received vehicle. Group II received single dose of D-GLN (250 mg/kg i.p.). Group III served as reference control received LIV-52 (100 mg/kg p.o.) + D-GLN (250 mg/kg i.p). Group IV & V received Ambrex at 250 and 500 mg/kg p.o. + D-GLN (250 mg/kg i.p.). Animals were pre-treated with vehicle or respective drug for a period of 7 days. D-GLN was injected on day 7, blood were collected 24 h following D-GLN injection from the over-night fasted mice through retro orbital puncture and sacrificed. Liver tissue was excised out, cleared of blood in ice cold saline and stored in -80°C.

Biochemical Assays

Plasma biochemical parameters

SGOT, SGPT, ALP, γ-GT and total bilirubin were measured using commercial diagnostic kits (Accurex, India) in semi-automatic biochemical analyser (Biosystem, India)

SOD Activity

SOD activity was performed by taking 0.05 ml of 10% tissue homogenated followed by addition of 0.3 ml of sodium pyrophosphate buffer (0.025 M, pH 8.3), 0.025 ml of phenasonium methosulphate (186 μM) and 0.075 ml of nitroblue tetrazolium (300 μM in buffer of pH 8.3) The reaction was started by addition of 0.075 ml of NADH (780 μM in buffer of pH 8.3). After incubation at 30°C for 90 seconds, the reaction was stopped by addition of 0.25 ml glacial acetic acid. Then the reaction mixture was stirred vigorously and shaken with 2.0 ml of n-Butanol. The mixture was allowed to stand for 10 minutes and centrifuged. 1.5 ml of n-butanol alone was served as blank. The colour intensity of the chromogen was read at 560 nm using Thermo Scientific multi-scan spectrophotometer, USA.[12]

LPO content

The method involved heating of 0.2 ml of 10% tissue homogenate with 0.8 ml saline, 0.5 ml of butylated hydroxyl toluene and 3.5 ml thiobarburic acid (TBA) reagent for 1½ h in a boiling water bath. After cooling, the solution was centrifuged at 3500 rpm for 10 min and the precipitate obtained was removed. The absorbance of the supernatant was determined at 532 nm using spectrophotometer (Thermo Scientific multi-skan spectrophotometer, USA) against a blank that contained all the reagents except the sample.[13]

GSH content

Glutathione content was estimated according to the method of Moren *et al*.[14] 0.25 ml of 10% homogenate was added to equal volume of ice cold 5% trichloro acetic acid. The precipitate was removed by centrifugation at 3500 rpm for 10 minutes. To 1ml of the supernatant, 0.25 ml of 0.2 M phosphate buffer, pH 8.0 and 0.5 ml of DTNB (0.6 mM in 0.2 M phosphate buffer, pH 8.0) was added and mixed well. The absorbance was read at 412 nm using Thermo Scientific multi-skan spectrophotometer, USA.

CAT activity

Catalase assay was performed as described by Sinha.[15] The reaction mixture contained H_2O_2 (2 mM), 0.2 ml of the homogenate in a final volume of 1 ml in phosphate buffer (10 mM, pH 7.4). It was incubated at 37ºC for 5 min and then Dichromate Acetic Acid reagent (5% Potassium dichromate in water, Glacial Acetic Acid mixed in 1:3 ratio) was added and absorbance was taken at 570 nm using Thermo Scientific multi-skan spectrophotometer, USA. 2 ml Dichromate Acetic acid reagent acts as blank whereas the reaction mixture without homogenate acts as control.

Histopathology

The experimental animals were sacrificed using anesthetic ether, liver was excised out and fixed in 10% neutral buffered formalin solution for 48 hours. The tissue was then trimmed, dehydrated in graded alcohol and embedded in paraffin. Paraffin sections of 3-4 micron thickness were obtained, mounted on glass slides, counter-stained with Haematoxylin and Eosin (H&E) and examined under light microscopy.

In vitro Study

Cell Proliferation Assay or MTT Assay

Cell respiration as an indicator of cell viability and proliferation was determined using a mitochondrial dependent reduction of 3-(4, 5-dimethylthiazol-2-yl)-2, 5- Diphenyl tetrazolium bromide (MTT) to formazan. Pre-confluent Chang liver cells were seeded in 96-well plates at a density of 8,000 cells/200 µl/well. Cells were treated with different concentrations of D-galactosamine hydrochloride (1, 3, 10, 30, 100, 300 and 1000 µM) after 24 h following plating and incubated at 37°C for one day. At 20 h following drug exposure, the cells were incubated at 37°C with 0.5 mg/ml MTT for 4h. At the end of the experiment, the medium was removed, and the insoluble formazan product was dissolved in DMSO (200 µl) and kept at least 15 minutes in dark. The intensity of purple blue colour developed was measured at 570 and 630 nm using Thermo scientific multi-scan spectrophotometer, USA. Percentage growth inhibitory rate of the test drug was calculated using the formula

% Growth inhibitory rate = ([Control OD – Test OD] / Control OD) * 100

D-GLN induced hepatotoxicity in Chang liver cells

Chang liver cells were seeded in 6 well plates at a density of $1x10^5$cells/well and allowed to grow for a period of 24 h. Ambrex was administered at a concentration of 5, 50 and 500 ng/ml. One hour following test drug exposure, D-GLN (30 µM/ml) was added to each well except the control and incubated for a period of 24 h. Cells were then trypsinised for measuring gene expressions of BAX, BCL-2, Caspase 3, TNF-α, IL-2 and CYP-450.

Reverse transcriptase - Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted using TRIzol Reagent (Sigma, USA).[16] After homogenizing the cells with TRIzol reagent, the tubes was incubated for 10 min and centrifuged at 1000 rpm for 5 min. 200 µl of chloroform was added to the supernatant, allowed to incubate for 5 min at room temperature and centrifuged at 12000 rcf for 20 min. Then 500 µl of isopropyl alcohol was added to the supernatant to precipitate the total RNA and centrifuged at 12000 rcf for 15 min following the incubation period of 10 min. The supernatant was decanted carefully; the pellet was washed thrice with 75% ethanol, centrifuged at 12000 rcf for 15 min. The pellet was air dried and re-suspended in 20 µl of RNase free water and stored in -80°C until use. RT-PCR was carried out using PCR master cycler gradient (Eppendorf, Germany) and semi-quantified using Bio1D software in gel documentation (Vilber Loumart, France). The primer sequence of BAX, BCL-2, CYP-450, caspase 3, IL-2 and TNF- α were provided in the Table 1.

Statistical Analysis

All the grouped data were significantly evaluated with Graph Pad version 5. Mean difference between the

groups were analysed by one-way analysis of variance (ANOVA) followed Tukey's multiple comparison test as posthoc. $P \leq 0.05$ is considered to indicate statistical significance. $^{\#,\#\#}$ denotes $P < 0.05$ and 0.01, respectively vs normal control; *, ** denotes $P < 0.05$ and 0.01, respectively vs D-GLN.

RESULTS

Curcumin quantification using HPTLC

HPTLC chromatogram of curcumin and ambrex were quantified (Figure 1). Curcumin content of ambrex was found to be 0.55%w/w.

In vivo Study

Biochemical Observations

A significant elevation ($p < 0.01$) of ALT, AST, ALP, γ – GT and total bilirubin levels were observed in D-GLN induced mice when compared to normal mice. Pre-treatment with Ambrex at two dose levels of 250 mg/kg b.wt and 500 mg/kg b.wt have significantly ($p < 0.01$) decreased the levels of the above indices. Results also clearly revealed significant decrease in the levels of CAT ($p < 0.01$), SOD ($p < 0.05$), GSH ($p < 0.01$) and increase in the levels of TBARS ($p < 0.01$) in liver of D-GLN mice in comparison to normal mice. Ambrex treatment at high dose significantly increased these alterations in comparison to D-GLN induced mice. The values were found to be comparable with that of standard drug, LIV-52 (100 mg/kg b.wt p.o.). (Figure 2-10)

Histopathology

Histological profile of normal control group revealed normal histology of liver with central vein, hepatocytes and portal triads. D-GLN induced group revealed marked degree of centrilobular necrosis along with polymorphonuclear cells infiltration, severe vacuolations in the hepatocytes and sinusoidal congestion. Pre-treatment with LIV-52 reduced galactosamine induced microscopic changes and showed marked beneficial effects in the liver architecture. Ambrex treatment at low dose showed moderate degree of necrosis, mild degree of vacuolations in the hepatocytes with minimal sinusoidal congestion. Ambrex administration at high dose revealed regeneration of hepatocytes to normal architecture, absence of sinusoidal congestion and vacuolations in the hepatocytes. On the basis of above observation, it is concluded that pre-treatment with Ambrex at high dose (500 mg/kg b.wt. p.o) have

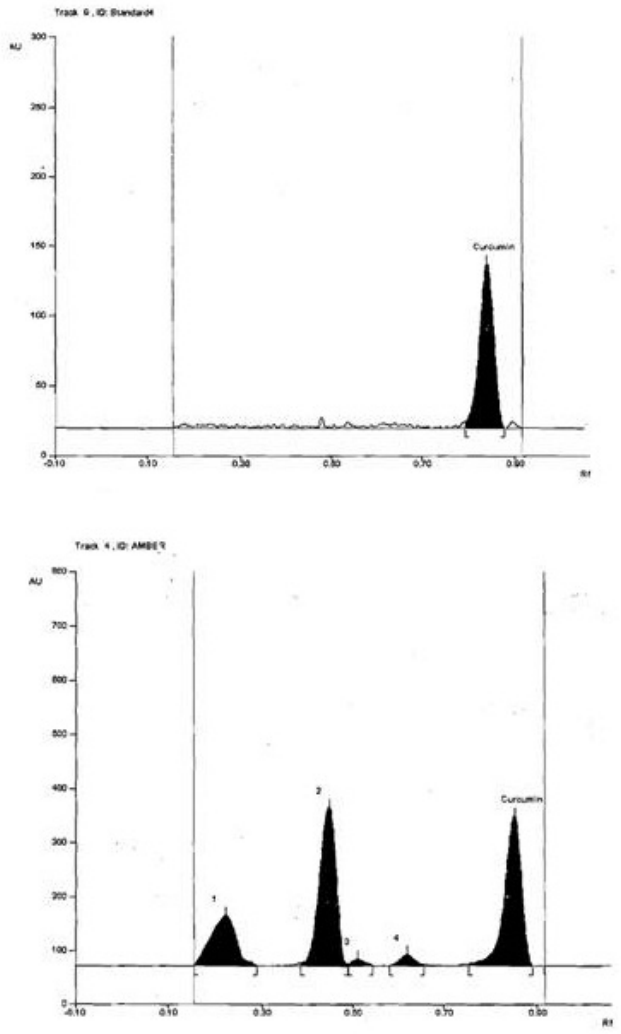

Figure 1: HPTLC chromatogram of curcumin and ambrex.

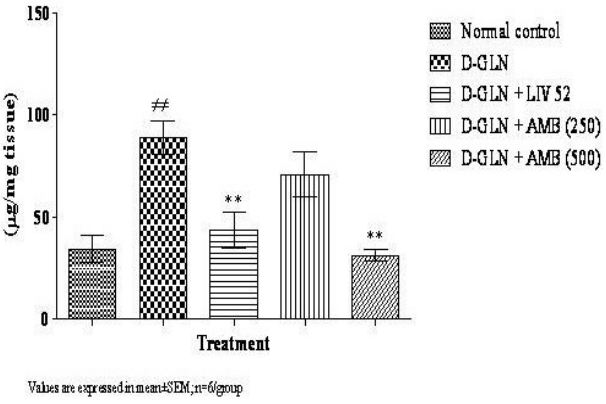

Figure 2: Effect on Ambrex on hepatic lipid peroxidation content in D-galactosamine intoxicated mice. Values are expressed in mean± SEM; n=6/group ; Mean difference between the groups were analysed by one way ANOVA followed by Tukey's multiple comparsion test as posthoc; ## denotes P<0.01 vs normal control; **denotes P<0.05 vs D-galactosamine group.

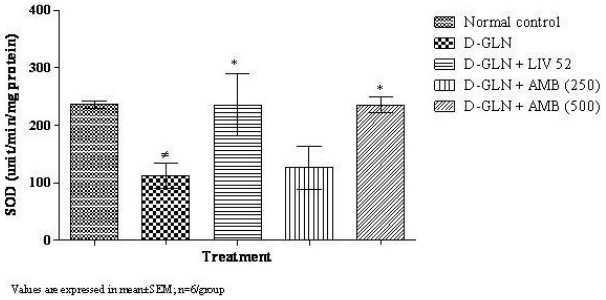

Figure 3: Effect on Ambrex on hepatic superoxide dismutase activity in D-galactosamine intoxicated mice. Values are expressed in mean±SEM; n=6/group; mean difference between the groups were analysed by one way ANOVA followed by Tukey's multiple comparsion test as posthoc; ## denotes P<0.01 vs normal control; **denotes P<0.05 vs D-galactosamine group.

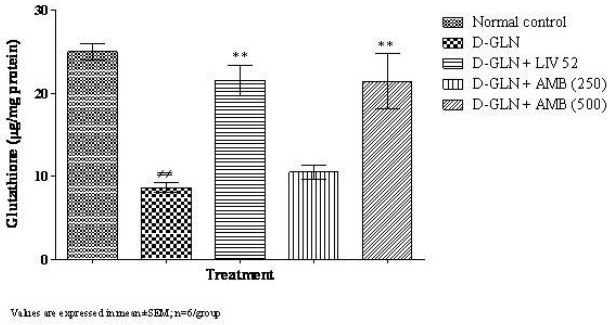

Figure 4: Effect on Ambrex on hepatic glutathione content in D-galactosamine intoxicated mice. Values are expressed in mean±SEM; n=6/group; mean difference between the groups were analysed by one way ANOVA followed by Tukey's multiple comparsion test as posthoc; ## denotes P<0.01 vs normal control; **denotes P<0.05 vs D-galactosamine group.

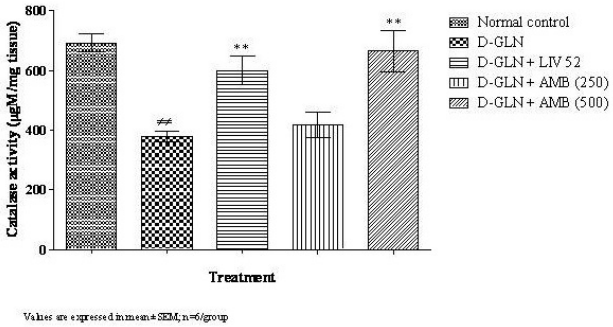

Figure 5: Effect on Ambrex on hepatic catalase activity in D-galactosamine intoxicated mice: Values are expressed in mean±SEM; n=6/group; mean difference between the groups were analysed by one way ANOVA followed by Tukey's multiple comparsion test as posthoc; ## denotes P<0.01 vs normal control; **denotes P<0.05 vs D-galactosamine group.

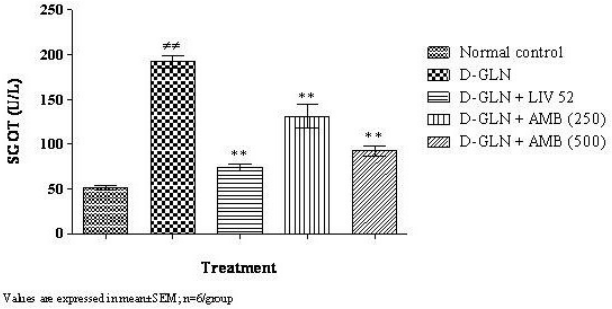

Figure 6: Effect on Ambrex on SGOT level in D-galactosamine intoxicated mice: Values are expressed in mean±SEM; n=6/group; mean difference between the groups were analysed by one way ANOVA followed by Turkey'smultiple comparsion test as posthoc; ## denotes P<0.01 vs normal control; **denotes P<0.05 vs D-galactosamine group.

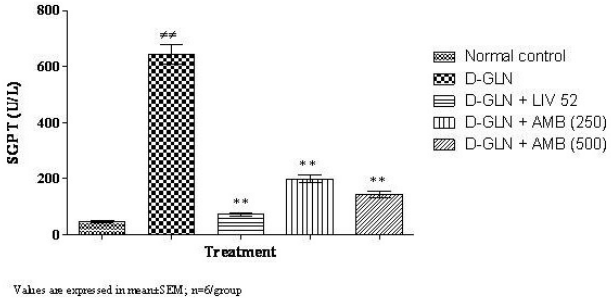

Figure 7: Effect on Ambrex on SGPT level in D-galactosamine intoxicated miceValues are expressed in mean±SEM; n=6/group; mean difference between the groups were analysed by one way ANOVAfollowed by Tukey's multiple comparsion test as posthoc; ## denotes P<0.01 vs normal control; **denotes P<0.05 vs D-galactosamine group.

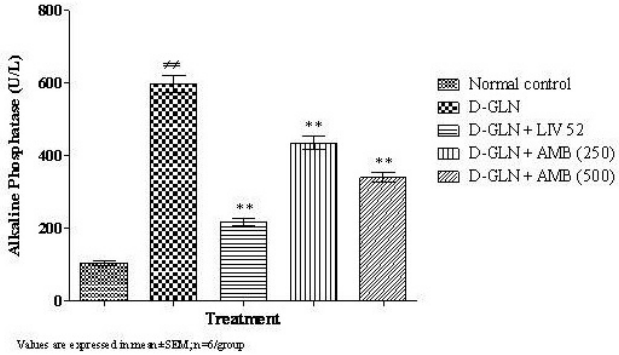

Figure 8: Effect on Ambrex on ALP level in D-galactosamine intoxicated mice: Values are expressed in mean±SEM; n=6/group; mean difference between the groups were analysed by one way ANOVA followed by Tukey's multiple comparsion test as posthoc; ## denotes P<0.01 vs normal control; **denotes P<0.05 vs D-galactosamine group.

hepatoprotective activity against D-GLN induced hepatotoxicity. (Figure 11)

In vitro Study

Cytotoxicity assay

Cytotoxicity was assessed for D-GLN and Ambrex at concentration range of 1 – 1000 µM/ml and $1X10^{-3}$-$1X10^{6}$ ng/ml, respectively following 24 h incubation. It was observed from the results that D–GLN exhibited cytotoxicity in a concentration dependent manner and its IC_{50} was found to be 30.83 µM. Ambrex exhibited an IC_{50} value of 44.73 ng when exposed in chang liver cells for a period of 24 h. Hence, hepatoprotective effect of ambrex against D-GLN induced toxicity was carried out at three different concentrations of 5, 50 and 500 ng/ml. (Figure 12, 13)

RT – PCR analysis

D-GLN induced Chang liver cells showed significant ($p < 0.01$) increase in CYP-450, BAX, Caspase 3, TNF-α and IL-2 gene expressions and decrease in BCL-2 expression when compared to normal cells. Ambrex exposure at 5, 50 and 500 ng/ml significantly and dose dependently down-regulated CYP-450 ($p < 0.01$), BAX ($p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively), Caspase 3 ($p < 0.05$, 0.01 and 0.01, respectively), TNF-α ($p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively) and IL - 2 ($p < 0.01$) gene expressions and up-regulated BCl2 ($p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively) expression in comparison to positive control cells. (Figure 14)

DISCUSSION

World-wide population from developing countries are being contingent on traditional system of Medicine. Well documented ancient literatures on traditional medicine system publicized the use of plants for various ailments and that many drugs on current use have been derived either directly or indirectly from plant sources.[17] Use of herbal formulations has gained much importance than single herb due to its better and extended therapeutic potential as well as their easy availability. Perception on polyherbalism has been dated back to 1300 AD in an Ayurvedic literature named *Sharangdhar Samhita*.[18] Herbal formulations have been claimed to be more potent as they display synergistic, potentiative, and agonistic/antagonistic actions and the mixture of species in them shows better therapeutic effect than either species on its own.[19] Hence, the present study investigated the effect of Ambrex, a polyherbal formulation on D–GLN induced hepatic toxicity.

D-GLN induced hepatotoxicity is a widely accepted experimental model as it mimics the morphological and functional alterations caused due to viral hepatitis.[20] Also, the toxin is more specific to liver because hepatocytes have high levels of galactokinase and galactose-1-uridyltransferase. Reports also showed that galactosamine does not affect other organs.[21-22] It has been reported that the mechanisms of liver damage induced by D–GLN is due to the instability of cellular membranes as a result of lipid peroxidation.[23] The present study results showed increased MDA levels in D–GLN intoxicated mice, a characteristic parameter of lipid peroxidation[24]

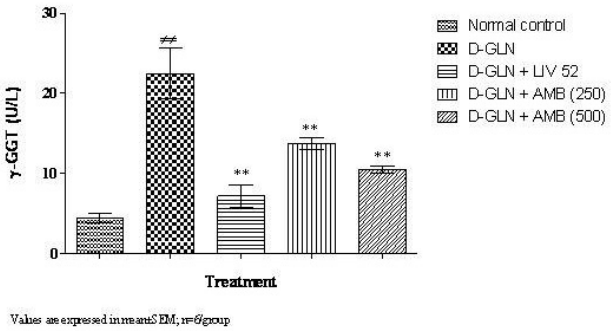

Figure 9: Effect on Ambrex on γ-GGT level in D-galactosamine intoxicated mice: Values are expressed in mean±SEM; n=6/group; mean difference between the groups were analysed by one way ANOVA followed by Tukey's multiple comparsion test as posthoc; ## denotes P<0.01 vs normal control; **denotes P<0.05 vs D-galactosamine group.

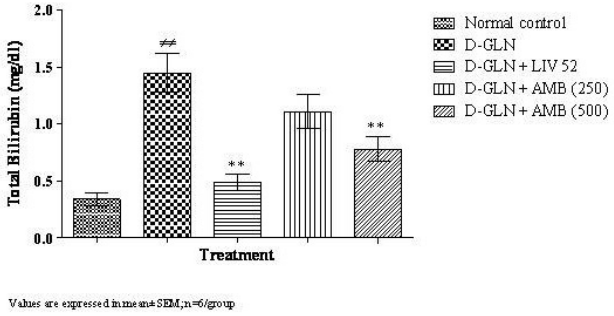

Figure 10: Effect on Ambrex on total bilirubin content in D-galactosamine intoxicated mice: Values are expressed in mean±SEM; n=6/group; mean difference between the groups were analysed by one way ANOVA followed by Tukey's multiple comparsion test as posthoc; ## denotes P<0.01 vs normal control; **denotes P<0.05 vs D-galactosamine group.

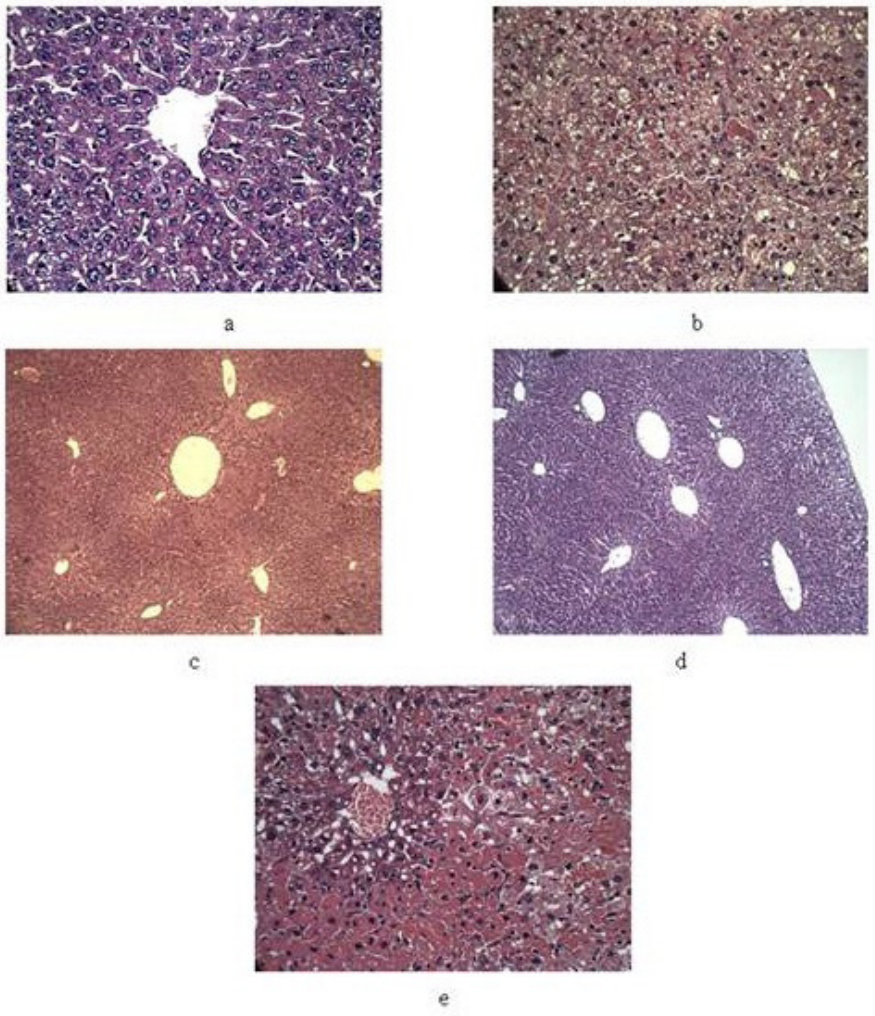

Figure 11. Representative photograph showing the effect of Ambrex on D-galactosamine induced mice liver - H & E stain. a. Normal control; b. D-Galactosamine (250 mg/kg b.wt. *i.p.*)+ Vehicle + 0.5% CMC (10 ml/kg b.wt.,*p.o*) treated group; c. D-Galactosamine (250 mg/kg b.wt. *i.p.*) + Liv 52 (100 mg/kg b.wt.,*p.o*) treated group; d. D-Galactosamine (250 mg/kg b.wt. *i.p.*) + Ambrex(250mg/kg b.wt.,*p.o.*) treated group; e. D-Galactosamine (250 mg/kg b.wt.*i.p.*) + Ambrex(500 mg/kg b.wt., *p.o.*) treated group.

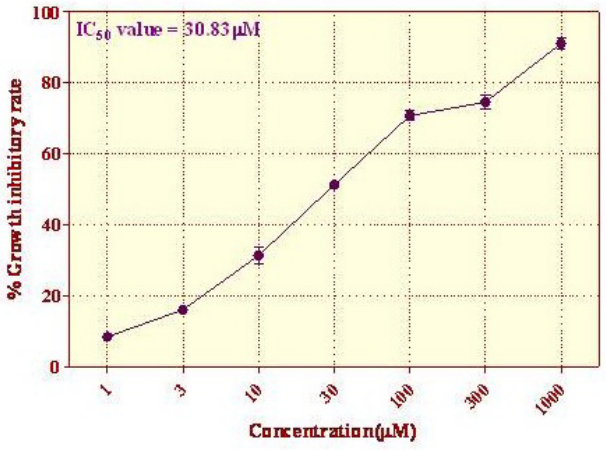

Figure 12: Cytotoxicity of D-galactosamine on chang liver cell line by MTT assay.

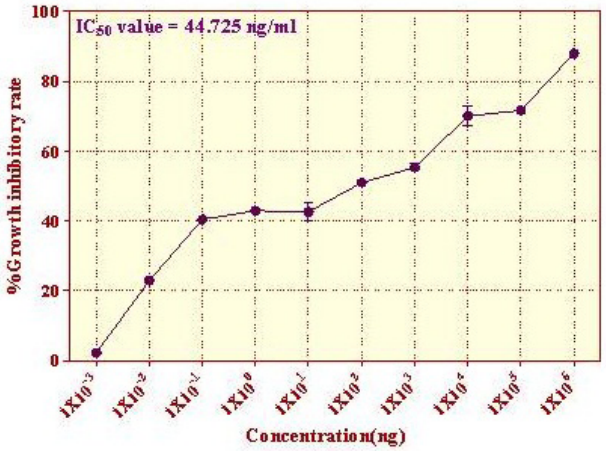

Figure 13: Cytotoxicity of Ambrex on chang liver cell line by MTT assay.

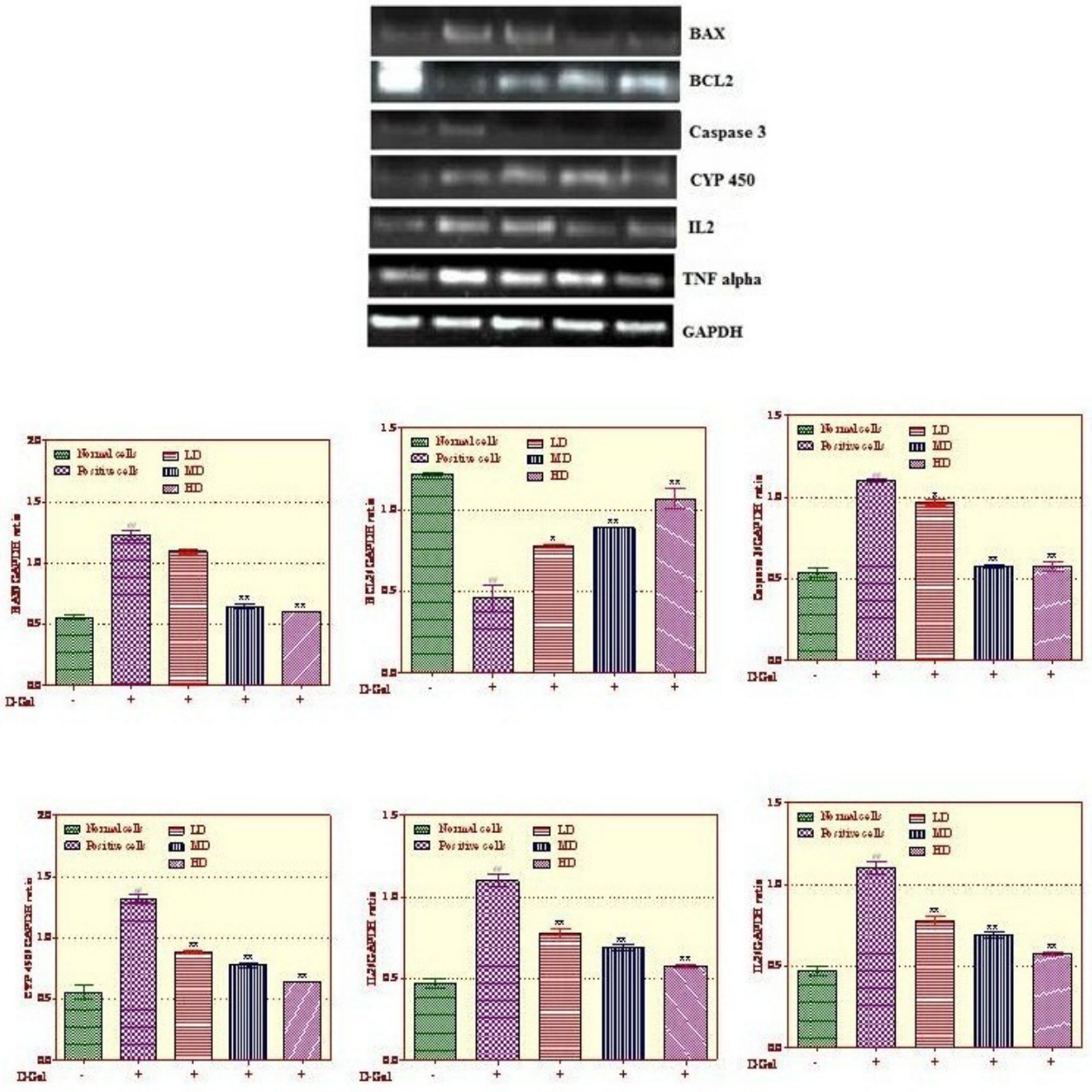

Figure 14: Effect of ambrex on D-galactosamine induced hepatotoxicity in chang liver cell line. (a). Representative bar graphs of mRNA expression.Lane I: Normal control; Lane II: Positive control; Lane III: Low dose; Lane IV: Mid dose; Lane V: High dose. Values were expressed in mean ± SEM; Statistical analysis was performed using one way anova followed by tukey's multiple comparison tests in Graph pad prism 5.0. ## represents p< 0.01 vs Normal control; *, ** represents p < 0.05 and 0.01, respectively vs positive control.

Table 1. Primer sequence for RT-PCR analysis

Primer	Forward Primer	Reverse Primer
GAPDH	5'-CGACAGTCAGCCGCATCTT-3'	5'-CCAATACGACCAAATCCGTTG-3'
BAX	5'-TTTTGCTTCAGGGTTTCATC-3'	5'-GACACTCGCTCAGCTTCTTG-3'
BCL2	5'-ATGTGTGTGGAGAGCGTCAACC-3'	5'-TGAGCAGAGTCTTCAGAGACAGCC-3'
Caspase 3	5'-TTTGTTTGTGTGCTTCTGAGCC-3'	5'-ATTCTGTTGCCACCTTTCGG-3'
TNFα	5'-ATTCTGTTGCCACCTTTCGG-3'	5'-GAAGGCCTAAGGTCCACTTGTGT-3'
IL2	5'-GTCACAAACAGTGCACCT AC-3'	5'-GAAAGTGAATTCTGGGTCCC-3'
CYP	5'-GTGATGCCCTGGCTGCAG-3'	5'-AATCGAGCTGGATCAAAGTTC-3'

whereas mice administered with Ambrex at two doses of 250 mg/kg b.wt and 500 mg/kg b.wt significantly attenuated the elevation which was comparable to standard LIV 52 treated group ($p < 0.01$).

D-GLN causes damage to the functional integrity of the liver cellular membrane leading to leakage of enzymes which ultimately elevates the liver enzyme levels.[25] Hence substantial increase in the levels of liver enzymes such as AST, ALT, ALP and γ – GT are indices of liver damage. It was found from our results that pre – treatment with Ambrex was able to protect the damage induced by D–GLN which significantly restored the levels of these enzymes to near normalization. The serum bilirubin level is an index for hepatic function and any deformity found in the levels of the same is been liable to abnormal hepatocellular function.[26] Elevated levels of total bilirubin in D–GLN intoxicated mice was in agreement with previous reports[26-27] and Ambrex mediated suppression of the increased bilirubin levels in the treatment groups suggest that the herbal formulation was able to alleviate the hepatobiliary dysfunction.

D-GLN induced hepatotoxicity enhances the production of ROS which is an indirect mechanism of toxicity induction, overwhelming the antioxidant status of the liver and ultimately proceeds to oxidative stress causing liver destruction.[28] SOD is the first line of defence in the antioxidant system against the oxidative damage mediated by superoxide radicals, catalysing the dismutation of superoxide radical to hydrogen peroxide and oxygen.[29] It has been reported that Catalase, GSH and GPx also constitute the mutually supportive defense against reactive oxygen species.[30-31] D–GLN induction has shown to decrease these anti-oxidant levels whereas pre - treatment with Ambrex has significantly increased the anti-oxidant levels compared to that of standard group. Ultra-structural studies also revealed the hepatoprotective efficacy of Ambrex.

The hepatoprotective effect of Ambrex was also evaluated in Chang liver cells. This human liver cell line is considered an appropriate model to study *in vitro* toxicity in the liver since it retains many of the specialized functions which are characteristics of normal human hepatocytes.[32] Apoptosis is a crucial pathologic alteration in liver disease, including viral hepatitis,[33] liver ischemia,[34] chemical[35] and drug-induced[36] liver injury as well as fatty liver disease.[37] The results of our present study showed that D–GLN induction has led to elevated levels of BAX and Caspase 3 as well as decreased levels of BCl-2. Also increased production of ROS sensitize the hepatocytes to tumour necrosis factor (TNF–α). Increased levels of CYP-450 and IL–2 were also indicated in our study following exposure of cells to D–GLN. The present study demonstrated that Ambrex at 5, 50 and 500 ng/ml down-regulated the pro – apoptotic markers BAX, Caspase 3 and up-regulated the anti – apoptotic marker BCl-2, including CYP-450 and IL–2. Ambrex pre–treatment reversed the changes in the levels of mRNA assessed indicates its anti–apoptotic and hepatoprotective action.

CONCLUSION

In conclusion, the present study demonstrated that Ambrex has preventive potential as a hepatoprotective agent which was evident from *in vivo* and *in vitro* studies. This preventive action of Ambrex might be attributed to the synergistic potential of the herbs present in the formulation.

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None

CONFLICT OF INTEREST

Authors declare no conflict of interest.

ABBREVIATION USED

TNF–α: Tumour necrosis factor α; **IL–2:** Interleukin 2; **CYP–450:** Cytochrome P 450; **DNA:** Deoxyribonucleic acid; **RNA:** Ribonucleic acid; **RNase:** Ribonuclease; **PCR:** Polymerase Chain reaction; **MDA:** Malondialdehyde; **GPx:** Glutathione peroxidase.

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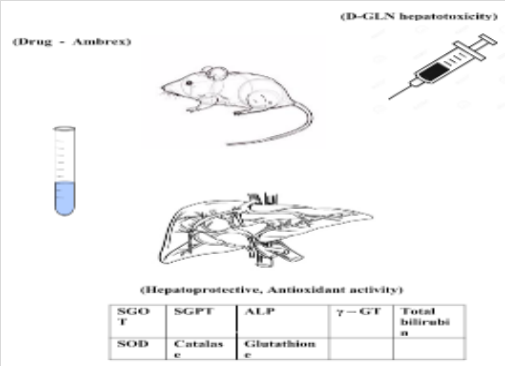
PICTORIAL ABSTRACT



SUMMARY

- The in-vitro study revealed that ambrex was found to be non-toxic at various concentrations tested and possess potent hepatoprotective activity.
- Ambrex at higher concentrations restores hepatic damage in D-galactosamine induction in Swiss Albino mice as evidenced by biochemistry and histology evaluation.
- Ambrex showed potent hepatoprotective effect against toxin induced liver damage may be through anti-oxidant capacity. This may be considered for future molecular studies and clinical trial.

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