Original Article

Ameliorative Effect of Multi Herbal Formulation on Lipid Peroxidation and Redox Dysfunction in Ethanol Induced Hepatic Imbalance

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ABSTRACT

Objectives: Traditional medicine is a potent antioxidant. In the present study, we developed a multi herbal formulation and examined its anti-oxidative activities and possible protective effect of it on serum and liver lipid peroxidation and glutathione in ethanol-induced hepatic dysfunction. **Materials and Methods:** In this experimental study 40 Swiss albino mice were divided into 4 groups randomly; group I as control, group II as sham treated with multi herbal formulation (MHF) (300mg/kg orally, daily), group III as liver damage control and group IV as ethanol treated with multi herbal formulation (MHF) (300mg/kg orally, daily) after induce liver damage, respectively. Liver damage was induced in the 3rd and 4th groups by ethanol administration (50% v/v). **Results:** After sixty days, animals were anaesthetized, liver was then removed immediately and used fresh or kept frozen until analysis. Blood samples were also collected before killing of the mice to measure the lipid peroxidation and antioxidant enzymes level. Serum and Liver lipid peroxidation level (MDA level) were significantly increased in ethanol treated group compared with untreated group. However, content of superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) and glutathione peroxidase (GPx) in serum and liver tissue were significantly decrease in ethanol treated group compared with untreated group. Alteration of serum and liver lipid peroxidation and antioxidant enzymes content were significantly inhibited by the administration of developed multi herbal formulation (MHF) (300mg/kg orally, daily). **Conclusion:** In conclusion the study showed that developed multi herbal formulation (MHF) might be a potent antioxidant and exert beneficial effects on the lipid peroxidation level and maintained the antioxidant levels in ethanol-induced liver dysfunction.

Key words: Alcohol, Liver disease, Lipid Peroxidation, Mice, Herbal Formulation, Antioxidant enzymes.

INTRODUCTION

Oxidation is a chemical reaction in the body that produces free radicals. These free radicals lead to chain reactions within the body that damage other cells. Commonly the consecutive reduction of oxygen through adding electrons cause the formation of a variety of ROS, which include superoxide (O_2^-), hydroxyl radical (·OH), hydroxyl ion (OH^-) and hydrogen peroxide (H_2O_2). In general, the harmful effects of ROS comes in the form of DNA damage, lipid preoccupation (oxidation of polyunsaturated fatty acids), protein amino acid oxidation and inactivation of specific enzymes through oxidation of their cofactors.[1-3] These damages can be contributing factor to many general and specific problems and diseases such as Parkinson's, Alzheimer's, asthma, aging, cancer, Rheumatoid Arthritis, Liver disorder etc. Therefore, antioxidant parameters and oxidative stress indices are considered

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potential biomarkers and are frequently used as screening tools to assess the impacts of environmental stress. Important antioxidant enzymes are catalase (CAT), superoxide dismutase (SOD), glutathione S-transferees (GST) and glutathione peroxidase (GPx). In addition, glutathione, vitamins and carotene also help the organism to mitigate the external pollutants and help the protective enzyme system of the organism.[4-6]

Liver is a major organ attacked by ROS.[7] Parenchymal cells are primary cells subjected to oxidative stress induced injury in the liver. When the ROS is excessive, the homeostasis will be disturbed, resulting in oxidative stress, which plays a critical role in liver diseases and other chronic and degenerative disorders.[8] The oxidative stress not only triggers hepatic damage by inducing irretrievable alteration of lipids, proteins and DNA contents and more importantly, modulating pathways that control normal biological functions. Since these pathways regulate genes transcription, protein expression, cell apoptosis and hepatic stellate cell activation; oxidative stress is regarded as one of the pathological mechanisms that results in initiation and progression of various liver diseases, such as chronic viral hepatitis, alcoholic liver diseases and non-alcoholic steatohepatitis.[9-11] Moreover, systemic oxidative stress arising during liver disease can cause damage to extra-hepatic organs, such as brain impairment and kidney failure.[12]

Worldwide, alcoholic liver disease (ALD) is a major cause of illness and mortality. ALD, a common effect of prolonged and heavy alcohol intake, is one of the leading health problems after cancer and cardiovascular diseases. In the modern way of life, intake of alcoholic beverages is a common characteristic and nowadays alcoholism ranks as a major health problem.[13,14] Experimental and epidemiologic studies confirmed that the duration and the degree of alcohol consumption promote the progression and genesis of liver damage. The liver is the major site of ethanol metabolism. Liver executes several important mechanisms which play crucial roles in digestion, storage, assimilation and detoxification.[15] Various study reports illustrate that ethanol causes the accumulation of reactive oxygen species (ROS) such as hydroxyl radical, superoxide radical and hydrogen peroxide in the hepatocytes that leads to the oxidation of DNA, protein and cellular membranes, resulting in the depletion of reduced glutathione and liver damage.[16,17]

Antioxidant a molecule that inhibits or stops the oxidation of other molecules in the body. So, antioxidant protect the body from cell damage. The potent sources of natural antioxidants are medicinal herbs and spices. Phenolic components in herbs and spices have been reported to be effective as natural antioxidants.[18] Intake of alcohol is associated with increase susceptibility of membranes to peroxidation and an increased requirement of antioxidant. The flavonoids are a large group of naturally occurring compounds that are found in plants and are frequently consumed as part of the human diet. Flavonoids are receiving much attention now a day for their potential pharmacological properties.[19] The antioxidant activity of flavonoids has been demonstrated by their ability to inhibit enzymes such as lipoxygenase, cyclooxygenase, along with chelating metal ions and scavenging free radicals.[20,21]

Based on previous reports, we developed a multi herbal formulation (MHF) containing nine Indian medicinal plants out of which six were medicinal plants and three were medicinal spices. Therefore, the present study was undertaken to evaluate the oxidative stress induction and tissues injury in liver of adult mice followed exposure to ethanol at different concentrations and its deleterious recovery by the application of multi herbal formulation.

MATERIALS AND METHODS

Chemicals

Disodium hydrogen phosphate (Na_2HPO_4), sodium chloride (NaCl) and trichloroacetic acid were purchased from Sigma–Aldrich (St. Louis, MO, USA). All solvents used were of analytical grade. 2-thiobarbituric acid (TBA) was obtained from Fluka Chemie (Buchs SG, Switzerland).

Collection and Authentication of the Herbs

All raw medicinal plants were collected from registered local herbal suppliers and authenticated by pharmacognosist. They were further identified by taxonomists of Department of Botany, Uluberia College, University of Calcutta, India and kept as voucher specimen. The identification was based on Ayurvedic parameters such as Varna (color), Gandha (odor), Ruchi (taste), Akriti(shape) and Parimana (size). The plants and plant parts used in preparation of the extract are listed in Table 1.

Preparation of Extract

Plant parts were air dried after cleaning with double distilled water and kept in oven at 80°Cfor 10 min and 60°C for 30 min. Then they were ground by a blade mill to fine powder. Subsequently, the extraction of the polar fraction was performed according to the method of Taamalli *et al.* (2015) with some modifications.[22] 5 gm. of dry plant parts were dissolved using 10 ml of methanol, sonicated at room temperature for 30 min

using an ultrasonic bath, centrifuged at 3000 rpm for 15 min and finally the supernatant was removed. This procedure was repeated four times, collecting all the supernatants, which were finally evaporated in rotary evaporator under reduced pressure at 35°C. Finally, the residue was re-constituted in 3 ml of methanol, filtered using what man filter papers (GE Healthcare and Life Sciences, MA, USA) and kept at 4°C for further use.

Animals

In the present study 40 male mature Swiss albino mice (25-30 g) were obtained from the CPCSEA approved animal house of Jadavpur University, Kolkata. The animals were maintained at 12 h light/dark cycle, at constant temperature (20±2°C) and humidity (50±5%). Mice were feed standard pellet diet (Purchase from Hind liver India Limited, Mumbai) containing 19.4% protein, 5.5% fiber, 11.1% water, 54.6% carbohydrates, 6.7% essential mineral mixture and 2.6% by weight of lipids and water *ad libitum*. Mice were kept under observation for one week before the onset of the experiment for acclimatization and to exclude any pathogenic infection. All the experimental procedure were carried out according to the guidelines of CPCSEA, Govt. of India, New Delhi and approved by the Institutional Animal Ethics Committee (IAEC).

Experimental Design

The mice were divided into four groups (10 per each). The studied groups were as follows: group I as control, group II as sham treated with multi herbal formulation (MHF) (300mg/kg orally, daily), group III as liver damage control and group IV as ethanol treated with multi herbal formulation (MHF) (300mg/kg orally, daily) after induce liver damage, respectively (Table 2). Liver damage was induced in the 3rd and 4th groups by ethanol administration (50% v/v).

Blood Collection

At the end of the respective fasting period, blood was collected from each mouse by retro orbital venous puncture. 200 µL of blood sample were collected into micro-centrifuge tubes with and without EDTA (2%). Collected bloods were placed in slanting position at room temperature for 2 hrs. Then, they were centrifuged at 3500 g for 10 min. Serum was separated and used for further analyses.

Preparation of Tissue Homogenate

Prior to biochemical analysis, liver samples (100 mg/mL) was homogenized in 50 mM phosphate buffer (pH 7.0); the homogenate was then centrifuged at 10000 rpm for 15 mins[23] and the supernatant obtained was collected and used for further studies. Protein concentrations of

Table 1: Composition of ingredient(s) present in novel multi herbal formulation (MHF).

Sl. No.	Botanical Name	Common Name	Part Used	Quantity used in extract
1.	*Tinospora cordifolia*	Guduchi	Stem	20 mg
2.	*Terminalia chebula*	Haritaki	Fruit	20 mg
3.	*Azadirachta indica*	Neem	Leaves	50 mg
4.	*Andrographis paniculata*	Kalmegh	Leaves & Steam	50 mg
5.	*Aloe barbadensis miller*	Aloe vera	Leaves & Steam	50 mg
6.	*Curcuma longa*	Curcuma, Haldi	Rhizome	20 mg
7.	*Trigonella foenum-graecum*	Methi	Seed	10 mg
8.	*Piper nigrum*	Black pepper	Seed	10 mg
9.	*Elettaria cardamomum*	Cardamom	Seed	10 mg

* Amount required for preparation of 5 ml extract.

Table 2: Experimental Design.

Groups	Treatment
I	Normal control received only the normal drinking water for 60 days.
II	Sham treated control received multi herbal formulation (300mg/kg-bw/day) for 60 days.
III	Animals treated with ethanol (50% v/v, single dose/day) for 60 days.
IV	Treatment with ethanol (50% v/v) for 30 days and then pre-treated with multi herbal formulation - MHF (300 mg/kg-bw/day) for next 30 days.

liver supernatant were determined using commercially available kit (Span Diagnostics Ltd, India) following procedure prescribed by manufacturer.

Levels of Malonedialdehyde (MDA)

Lipid peroxidation was estimated in the serum samples by measuring the Malondialdehyde (MDA) formation using the thiobarbituric acid method.[24] Briefly, 100 µL of animals was mixed with 500 µL of 150 mM Tris-HCl and 1.5 mL of 0.375% TBA and vortexed for 10 sec. The reaction mixture was then incubated at 100°C for 45 min in a water bath. At the end of incubation, the samples were centrifuged at 1000×g for 10 min. The MDA content was calculated from the absorbance measurement at 532 nm and using a Shimadzu spectrophotometer (Tokyo, Japan) an absorption coefficient = $1.56 \times 105\ cm^{-1}\ M^{-1}$.

Determination of ROS activity

Amount of ROS in blood was measured using 2', 7'-dichlrofluorescein diacetate (DCF-DA) that gets converted into highly fluorescent DCF by cellular peroxides (including hydrogen peroxide). The assay was performed as described by Socci *et al*. 20 Fluorescence was determined at 488 nm excitation and 525 nm[25] emission using a fluorescence plate reader (Tecan Spectra Fluor Plus).

Determination of Glutathione (GSH) and GPx Content

Reduced glutathione (GSH) was determined from liver homogenate spectrophotometrically according to Ellman's method. GPx activity was assayed using a modified method of Lawrence and Burk.[26]

Determination of Superoxide Dismutase (SOD)

The activity of SOD was measured according to a reported method.[27] In brief, 2.8 mL reagent solution (xanthine 0.3 mM, EDTA 0.67 mM, 150 µM NBT), sodium carbonate 0.4 M and bovine albumin (30 mg/30 mL) was added to 0.1 mL sample and 50 µL xanthine oxidase (10 µL in 2 M ammonium sulphate), incubated at 25°C for 20 min and mixed with, 0.1 mL 8 M copper chloride. The color reaction was measured at 560 nm.

Determination Catalase (CAT)

Catalase activity was measured according to the method of Maehly (1955).[28] Briefly, after addition of 5µL liver homogenate to 0.995 mL 30 mM H_2O_2 solution in potassium phosphate buffer (pH 7.0), change in absorbance at 240 nm was monitored for 1 min to determine catalase activity. The enzyme activity was expressed as U/mg protein.

Statistical analysis

All quantitative data are expressed as mean ± standard deviation (SD) unless otherwise stated. One-way analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison *t* tests were executed for comparison of different parameters between the groups using a computer program GraphPad Prism (version 5.00 for Windows), Graph Pad Software, California, USA. P and amp; lt; 0.05 was considered significant.

RESULTS

Determination of serum and liver lipid peroxidation

In the present study we developed a novel multi herbal formulation (MHF) containing six Indian medicinal herbs and three medicinal spices (Figure 1) enrich with high flavonoids and polyphenols. The medicinal effect of multi herbal formulation (MHF) on serum and liver MDA level of mice was determined. The levels of MDA in serum and liver are shown in Figure 2. The level of serum MDA in the untreated liver damage mice was significantly (87%) higher ($P<0.001$) than that of control animals. The level of MDA in the serum of ethanol treated liver dysfunction mice with multi herbal formulation (MHF) was very low, similar to the level (92% recovery) found in the control animals. The treatment of hepatic damage animal with MHF could significantly (92%) inhibit the elevation of MDA in comparison with the untreated experimental animals. The level of liver MDA in the untreated hepatic damage animal was significantly (84%) higher than that of control animals. The treatment of liver damage animal with multi herbal formulation (90%) inhibit the

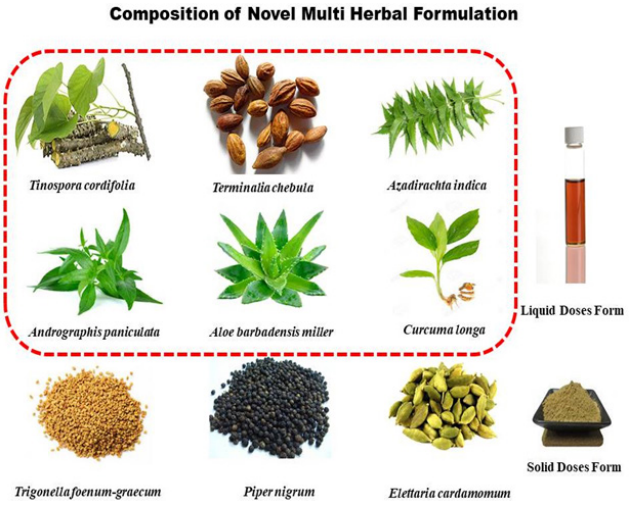

Figure 1: Ingredients present in the novel multi herbal formulation containing six medicinal herbs and three medicinal spices.

increasing of MDA (P<0.001) in comparison with the untreated hepatic damage animals. The level of MDA in the serum and liver of sham mice treated with multi herbal formulation were low, similar to the level found in the control animals.

Determination of serum and liver ROS content

Beneficial effect of multi herbal formulation (MHF) on serum and liver ROS level of mice was determined. The levels of ROS in serum and liver are shown in Figure 2. The level of serum ROS in the untreated liver damage mice was significantly (89%) lower (P<0.001) than that of control animals. The treatment of hepatic damage animal with MHF could slightly increase of ROS in comparison with the untreated experimental animals. The level of liver ROS in the untreated liver damage mice was significantly (87%) lower (P<0.001) than that of control animals. The multi herbal formulation (MHF) treated ethanol induced liver damage animals showed significantly (P<0.001) elevation (93%) in ROS level compared with the untreated control animals.

Determination of serum and liver SOD and CAT content

Beneficial effect of multi herbal formulation (MHF) on serum and liver SOD and CAT level of mice was determined. The levels of SOD and CAT in serum and liver are shown in Figure 3. The level of serum SOD and CAT in the untreated liver damage mice was significantly (86%) lower (P<0.001) than that of control animals. The treatment of hepatic damage animal with MHF could slightly increase of SOD and CAT in comparison with the untreated experimental animals. The level of liver SOD and CAT in the untreated liver damage mice was significantly (89%) lower (P<0.001) than that of control animals. The multi herbal formulation (MHF) treated ethanol induced liver damage animals showed significantly (P<0.001) elevation (96%) in SOD and CAT level compared with the untreated control animals.

Determination of serum and liver GSH and GPx content

Effect of multi herbal formulation (MHF) on serum and liver GSH and GPx level of mice was determined. The levels of GSH and GPx in serum and liver are shown in Figure 4. The level of serum GSH and GPx in the untreated liver damage mice was significantly (86%) lower (P<0.001) than that of control animals. The treatment of hepatic damage animal with MHF could slightly increase of GSH and GPx in comparison with the untreated experimental animals. The level of liver GSH and GPx in the untreated liver damage mice was significantly (89%) lower (P<0.001) than that of control animals. The multi herbal formulation (MHF) treated ethanol induced liver damage animals showed significantly (P<0.001) elevation (96%) in GSH and GPx level compared with the untreated control animals.

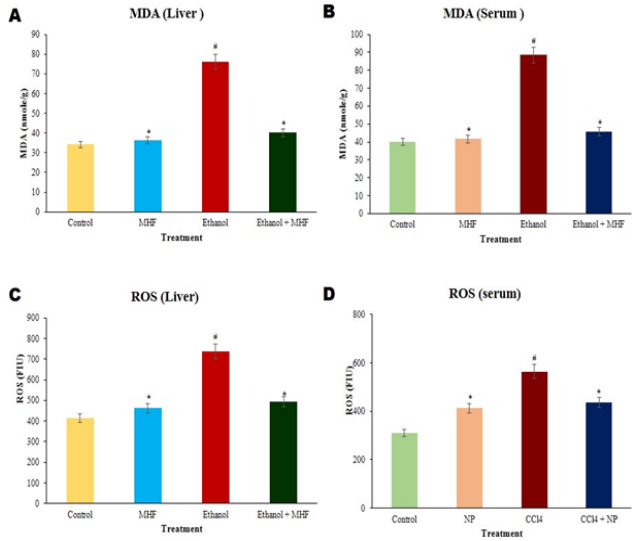

Figure 2: Effect of multi herbal formulation (MHF) against ethanol induced oxidative stress in mice. A) MDA content in serum B) MDA content in liver C) Level of ROS in serum D) Level of ROS in liver. Values are expressed as Mean ± SD (n = 10 per group). *Significantly different from control # (p<0.001) and significantly different from Ethanol * (p<0.001) using analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison Test.

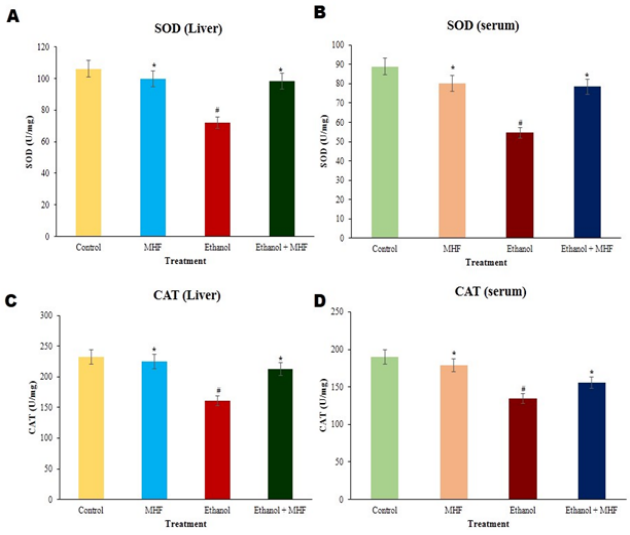

Figure 3: Effect of multi herbal formulation (MHF) against ethanol induced oxidative stress in mice. A) SOD content in serum B) SOD content in liver C) Level of CAT in serum D) Level of CAT in liver. Values are expressed as Mean ± SD (n = 10 per group). *Significantly different from control # (p<0.001) and significantly different from Ethanol * (p<0.001) using analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison Test.

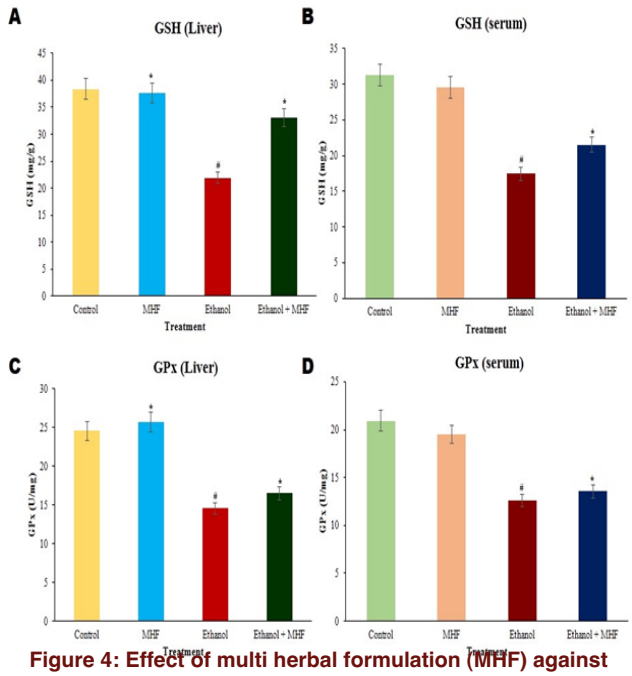

Figure 4: Effect of multi herbal formulation (MHF) against ethanol induced oxidative stress in mice. A) GSH content in serum B) GSH content in liver C) Level of GPx in serum D) Level of GPx in liver. Values are expressed as Mean ± SD (n = 10 per group). *Significantly different from control # ($p<0.001$) and significantly different from Ethanol * ($p<0.001$) using analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison Test.

DISCUSSION

In the past decades, considerable evidence has established the role of oxidative stress in the pathogenesis of liver complications. Indeed, several studies have reported that ethanol induced liver damage contribute to the accumulation of ROS and antioxidants deficiency (e.g., SOD and GSH) in both experimental animals and patients. This study showed that multi herbal formulation (MHF) increased serum and liver antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) and glutathione peroxidase (GPx) and decreased lipid peroxidation in ethanol induced experimental mice. The current work showed a significant increase in the level of lipid peroxidation (LPO) in serum and liver tissue after 60 days of oral administration of ethanol (50% v/v). LPO is an auto catalytic process leading to oxidative degradation of lipids causing demolition of cell membranes and subsequently cell damage.[29] It is mainly generated by the effect of various reactive oxygen species (ROS) such as hydrogen peroxide, superoxide's and hydroxyl radical.[30] LPO is a chain reaction initiated by the hydrogen abstraction from the side chain of polyunsaturated fatty acids resulting in cell membranes deterioration.[31] Decomposition of latest compounds lead to production of several products particularly malondialdehyde (MDA).[32] This tending to diminish the fluidity of cell membrane which plays a vital role in cell functioning. The elevated level of MDA observed in the current work, which is an indicator of LPO, denotes cell membrane damage in serum and liver of ethanol treated animals. Treatment with multi herbal formulation (MHF) significantly inhibited increasing of serum and liver lipid peroxidation in comparison with the ethanol treated animals.

Glutathione is a non-enzymatic cellular antioxidant which plays a crucial role in scavenging damaging free radicals. GSH can function as a co-substrate for peroxide detoxification by glutathione peroxidases.[33] GSH also catalyses' the reduction of hydrogen peroxide to water.[34] Depletion of cellular glutathione content may be one of the reasons for the increase in cell vulnerability to oxidative stress.[35-37] In this study decline in GSH level in this investigation could be caused by ethanol intoxication. Treatment with multi herbal formulation (MHF) significantly inhibited decreasing of serum and liver glutathione in comparison with the ethanol treated animals.

Study showed that there was a significant decrease in SOD and CAT activities in liver and renal tissues following oral administration of ethanol. SOD is an enzyme that repairs cells and decreases their damage through conversion of endogenous cytotoxic superoxide radicals to hydrogen peroxide and ordinary molecular oxygen, which have harmful effects on proteins and polyunsaturated fatty acids.[38] CAT is an important enzyme in protecting the cell from oxidative damage by catalyzing the decomposition of hydrogen peroxide to water and oxygen.[32] In the presence of insufficient activity of CAT to decompose hydrogen peroxide, more of it could be converted to toxic hydroxyl radicals that might contribute to oxidative stress after intoxication with ethanol. The significantly suppressed catalase activities after intoxicated with ethanol were recovered by the administration of multi herbal formulation (MHF). The endogenous scavenger, SOD, which removes the superoxide anion radicals by converting them into hydrogen peroxide (H_2O_2) and O_2, was significantly increased in the MHF experimental groups. The observed increase in liver SOD enzyme activity after administration of the multi herbal formulation (MFH) may be a consequence of oxidative activation of enzyme protein or increased of their synthesis. Therefore, the increase in the activity of SOD in liver tissues of treated mice might indicate a reduce accumulation of superoxide anion radical with oxidative stress, contributing decrease liver toxicity.[39]

GPx has a role in defending cells against oxidative stress and this in turn involves GSH as a cofactor. GPx catalyzes the oxidation of GSH to GSSG at the cost of H_2O_2. Decreased GPx activity was observed in the alcohol exposure group. This reduced activity may be involved in either free radical-dependent inactivation of enzyme or depletion of its co-substrate (i.e., GSH) or NADPH on ethanol treatment. Administration of multi herbal formulation (MHF) significant increased reduced GPx level activity after alcohol exposure.

CONCLUSION

In summary, the exposure of male Swiss albino mice to alcohol revealed signs of toxicity that were evidenced by a reduction in antioxidant defense system. Moreover, the activities of SOD, CAT, GPx and the concentration of MDA and GSH in the liver and kidney clearly indicate that our developed multi herbal formulation (MHF) is able to inhibit the oxidative stress during the co-exposure with ethanol, but its effect depends on the dose and time of exposure.

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CONFLICT OF INTEREST

Authors disclose no conflicts of interest for publication of the manuscript.

ABBREVIATIONS

SOD: Superoxide dismutase; **CAT:** Catalase; **GSH:** Glutathione; **GPx:** Glutathione peroxidase; **MDA:** Malondialdehyde; **ROS:** Reactive Oxygen Species; **GST:** Glutathione, S-transferase; **DNA:** deoxyribonucleic acid; **ALD:** Alcoholic liver disease; **MHF:** Multi herbal formulation; **TBA:** Thiobarbituric acid; **IAEC:** Institutional Animal Ethics Committee; **CPCSEA:** Committee for the Purpose of Control and Supervision of Experiments on Animals; **EDTA:** Edetate disodium; **DCF-DA:** Dichlrofluorescein diacetate; **NADPH:** Nicotinamide adenine dinucleotide phosphate.

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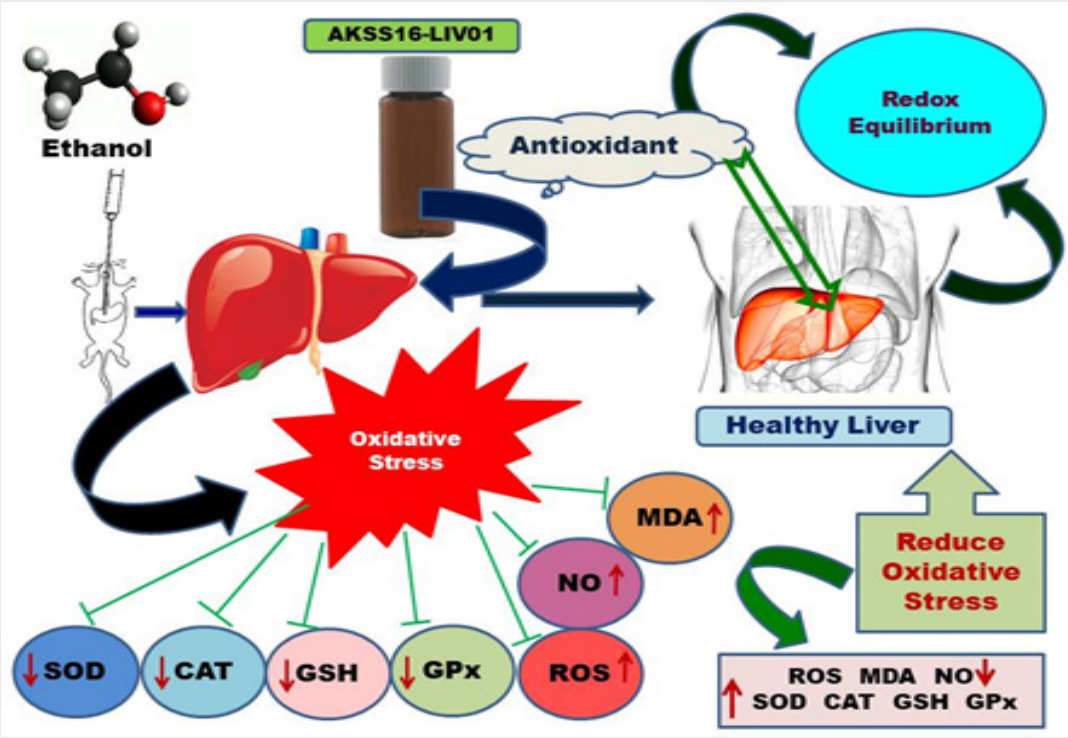
PICTORIAL ABSTRACT



SUMMARY

Life nearly in all its aspects is intricately linked to the intracellular redox homeostasis and its modulation. Intracellular redox potential is dependent on an intricate balance between the intrinsic and extrinsic reactive oxygen species (ROS). Worldwide, alcoholic liver disease (ALD) is a major cause of illness and mortality. Prolonged and heavy alcohol intake, is one of the leading health problems after cancer and cardiovascular diseases. Regular intake of alcohol impaired the cellular redox balance which produces oxidative stress and disrupts cells integrity. Liver is the main target organ of alcohol that inhibits normal hepatic function creates lots of cellular complications. Scientist from all over the world are engaged for a safe and symptomatic medication which mitigates ethanol induced liver dysfunctions. We developed a novel multi herbal composition containing six medicinal herbs and three medicinal spices. Dose dependent application of the herbal composite gradually inhibits ethanol induced hepatic abnormalities. Our *in vivo* experimental results depict that administration of this novel formulation upon mice normalized the hepatic oxidative stress caused by ethanol. So, the formulation maintained redox equilibrium and supplied antioxidants towards the cell which maintains cellular homeostasis.

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