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MORUS ALBA LEAVES AQUEOUS EXTRACT PREVENTS THE OXIDATIVE STRESS IN RATS DURING ACUTE LIVER FAILURE

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ABSTRACT

Acute liver failure (ALF) is the rapid onset of hepatic dysfunction in a patient without known pre-existing liver illness. ALF could result into multi-organ failure (MOF) by causing adverse effects on other organs including spleen, testis, kidney, and brain. Oxidative stress is believed to play central role in the development of MOF; however, effective treatment is not available. Present study was aimed to evaluate whether *Morus alba* aqueous extract (M.A) has therapeutic potential against oxidative stress in liver, in a thioacetamide (TAA) induced experimental rat model of ALF. In comparison to control groups, liver of rats with ALF showed significant decrease in the activity of antioxidant enzymes such as superoxide dismutase (SOD), catalase, and glutathione reductase (GR), which is followed by rise in lipid peroxidation (LPO) level and fall in total reduced glutathione (GSH) level. On the other hand, treatment of M.A normalised the LPO and GSH levels in liver by activating the most antioxidant enzymes. Findings of the present study suggested that oxidative stress causes adverse effect on TAA induced ALF. M.A has therapeutic role as an effective antioxidant in the treatment of ALF and oxidative damage occurs in liver due to ALF.

KEYWORDS: Acute Liver Failure, *Morus Alba* extract, Thioacetamide, Antioxidant enzymes, Oxidative stress, Reactive oxygen species, Free radicals.

INTRODUCTION

Liver failure or hepatic impairment is the incapability of liver to carry out its typical metabolic and synthetic function during normal frequently, liver failure develops gradually over many years. However, a rarer illness known as acute liver failure (ALF) manifests quickly (in as little as 48 h) and it can be challenging to recognise at first. The main precipitants include ammonia and free fatty acids, which are finally distributed in different tissues and organ where they may cause series of adverse effect on those tissues. ALF is a rare syndrome marked by rapid decline in normal liver function after an acute insult in patient (Dong *et al.,* 2019) leading to altered coagulation and mentation without a prior known chronic liver disease (Stravitz and Lee, 2019). The progression of the disease is accompanied by the emergence of a liver-related coagulopathy and clinically apparent altered level of consciousness brought on by hepatic encephalopathy (HE). The etiology of ALF include drug induced, metabolic, genetic, viral infections (hepatitis B, C, D, and E viruses), hemodynamic, oncologic injuries and autoimmune insults of liver (Squires *et al.,* 2018) and identifying the correct cause can be difficult. Patients with ALF develops a series of serious complications of multi-organ failure, haemorrhage, intracranial hypertension, infection, and leads to death of the patients (Rajajee *et al.,* 2017). Oxidative stress and its accompanying events play a critical role in the hepato-toxicity with different etiologies (Heidari *et al.,* 2018). Furthermore, it has been noted that TAA metabolites produce significant oxidative stress and the generation of mitochondrial dysfunction in the Thioacetamide (TAA) driven model of ALF (Jamshidzadeh *et al.,* 2017)). Generation of excessive production of reactive oxygen species (ROS) due to ALF induced by TAA can overwhelm the antioxidant defence system and harm cellular components such as proteins, lipids, and DNA which leads to disruption of cellular structure and function (Zargar *et al.,* 2017). Patients suffering from ALF are often treated in intensive care unit at the hospital where liver transplant can perform, if necessary. But in many cases, controlling complications with treatment to heal liver is advisable. In recent decades many potent synthetic agents and/or treatments havebeen developed; one of them is acetylcysteine, which reverse the poisoning of ALF caused by acetaminophen. Other includes controlling of the disease progression but not fully cure them. Scientist all over the world continue to research new treatments to cure ALF, especially those that can minimises the need of a liver transplant The aqueous extract of andrographolide also having significant effects on mental illness, neurological disorders and antimicrobial activity (Mittal *et al*., 2016). Moreover, it has also been reported that the aqueous extract of Morus Alba leaves significantly increased the

activity of antioxidant defence enzymes and exhibited free radicals-scavenging ability (Nsir *et al*., 2013). Previous report from our lab also stated that AP has significant effects on the Oxidative stress in various areas of the brain associated with HA. The aim of the present study was to look at how extract of *Morus Alba Leaves* protected the rat liver from oxidative damage brought on by ALF.

MATERIALS AND METHODS

Animals

Adult Sprague Dawley male albino rats (160g- 180g) were selected, maintained at standard conditions in animal house and fed with recommended diet as directed by the Institutional Animal Ethical Committee (IAEC). This study has been approved by IAEC, for the use of animal with Ref. No.208/GSMCOP/MA/S/09/CPCSEA.

Chemicals

Chemicals that used in this present study were of molecular and/or analytical grade purchased from SRL (India), Sigma.

Preparation of M.A and dosage

Morus Alba plant specimen was identified and authenticated at Botanical Survey of India, Pune. The extraction of the plant herb part was done following the method of Hawthorne *et al*. (1993), using hydro distillation process and filtration of the extract was done through NLT 100% 40 mesh screen. The rate of evaporation loss during drying of the extract was 1g/105^{O}C/2h. The main compound isolated from this extract quercetin. The extract was introduced in rats orally by a metal gavage needle at a fixed dosage concentration of 250 mg/ kg b.w. daily up to seven days prior to induction of ALF.

***Induction of ALF*:** ALF was induced by intraperitoneal injection of 300 mg thioacetamide (TAA) /kg b.w. prepared in 0.9% NaCl,once up to two days at an interval of 24 h.

Preparation of tissue extracts

Tissue extracts were prepared by homogenizing with 0.02M Tris–Cl (pH 7.4) buffer with protease inhibitors. The homogenate of the organs was centrifuged for 45 min at 35,000 g at 4^{0}C, thensupernatants obtained were collected and stored at -80^{0}C for use in different parameters.

Biochemical Estimations

Lipid peroxidation assay

Malondialdehyde (MDA), a stable product of lipid peroxidation was estimated using the method of Heath and Packer (1968) with some alteration. Where, 1 mole of MDA reacts with 2 moles of thiobarbutaric acid (TBA) in an acid solution and forms trimethionine (pink color) substance having maximum absorption at 620 nm. Briefly, 1.5 ml of 0.5% TBA was diluted in 20% TCA with 0.5 ml of tissue extract and it was incubated at 95°C for 25 min. Reaction was stopped by incubating the reaction mixture on ice. Thereafter, sample were centrifuge for 5 min (15000 g, 4°C) to clear the solution. Absorbance was recorded at a wavelength of 450 and 620 nm. OD620 values were subtracted from the MDA-TBA complex values at 532 nm. Then the level of MDA concentration was calculated by using the Lambert-Beer law. The lipid peroxidation levels were expressed as µM MDA/g wet wt.

GSH Level

Total thiol was estimated by the method of Sedlak and Lindsay (1968) with some alteration. 1.5 ml of 0.2 M Tris buffer, pH 8.2, and 0.1 ml of 0.01 M 5,5'- Dithio-bis (2-nitrobenzoic acid) (DTNB) were combined with 0.1 ml of tissue extracts. The final volume of mixture was diluted to 10 ml by adding methanol and incubated for 30 min. Then it was centrifuged at 3,000 rpm for 15 min andabsorbance was recorded at 412 nm. To calculate GSH (reduced glutathione) molar extinction coefficient of 13,100 was used and units as nmol/mg protein.

SOD

The activity of superoxide dismutase (SOD; EC: 1.15.1.1) was measured by using the standardized protocol of our lab. The reaction mixture containing 0.1 ml tissue extracts, 0.02 M sodium pyrophosphate buffer (pH 8.3), 30 µM nitroblue tetrazolium (NBT), and 6.2 µM phenazine methosulphate (PMS). Then the reaction was initiated by adding 50 µM NADH at 30^{O}C and stopped at 90 s by adding 2.0 ml glacial acetic acid to the reaction mixture. The reaction mixture was stirred and shaken with 4 ml of n-butanol, then it is allowed to stand for 10 min. Then the butanol layer was separated by centrifugation. Absorbance at 560 nm was measured using butanol as blank. The activity of SOD was expressed as one unit/mg of protein, and one unit represents the quantity of the enzyme that provided 50% inhibition of NBT degradation per minute.

Catalase

The activity of Catalase (EC: 1.11.1.6) was measured by using a reaction mixture of 0.01 M Potassium phosphate buffer (pH 7.0) and 0.1 ml tissue extract (Singh *et al*., 2008). Then the reaction was started by adding 0.8 M H_2O_2 and to stopped it after 60s, 2 ml of dichromate acetic acid reagent was added to the reaction mixture. Then the tubes containing reaction mixture were incubated in a boiling water bath for 10 min. Following that, the tubes were cooled to room temperature, and absorbance at 240 nm was measured. Catalase activity was quantified as µM of H_2O_2 consumed/min/mg protein after comparing the results to a reference plot (with a range of 10 - 160 µM of H_2O_2).

GR assay

Using Carlberg and Mannervik's method, glutathione reductase (GR; EC: 1.6.4.2) activity was assessed (Carlberg and Mannervik 1985).Where the

component of the reaction mixture of 1 ml are, 0.2 M sodium phosphate buffer (pH 7.0),0.2 mM EDTA, 1 mM oxidised glutathione (GSSG), and 0.2 mM NADPH. The process started when the tissue extract was added, and the oxidation of NADPH was observed as a 5 min drop in absorbance at 340 nm. The enzyme activity was expressed as units/mg protein, and the unit of the enzyme in this process was defined as a mole NADP/min/ at 30°C.

NOx Level

To estimate the nitric oxide level in sample, the level of NO_2 was measured by using Gries reagent following the method described earlier (Singh and Trigun, 2010). Separately, 100 µl of tissue fractions and 400 µl of 50 mM carbonate buffer were combined (pH 9.0). After centrifuging tubes, supernatant (400 µl) was collected and incubated with Griess reagent (200 µl 1% sulfanilamide prepared in 2.5% H_3PO_4 and 200 µl 0.1% N- napthylethylenediamine). Following the incubation, absorbance was measured at 545 nm in comparison to a blank that had all the components but no sample.

Statistical Analysis

All the experiments were conducted thrice using four replicas of each sample from each experimental group for all parameters used in this study. The data shown here as mean ± SD where n=3. Variance between groups were calculated through one-way analysis of variance (ANOVA) using Sigma plot 14.5 software. The minimum probability of $p<0.01$ and $p<0.05$ were taken as significant level between groups.

RESULTS AND DISCUSSION

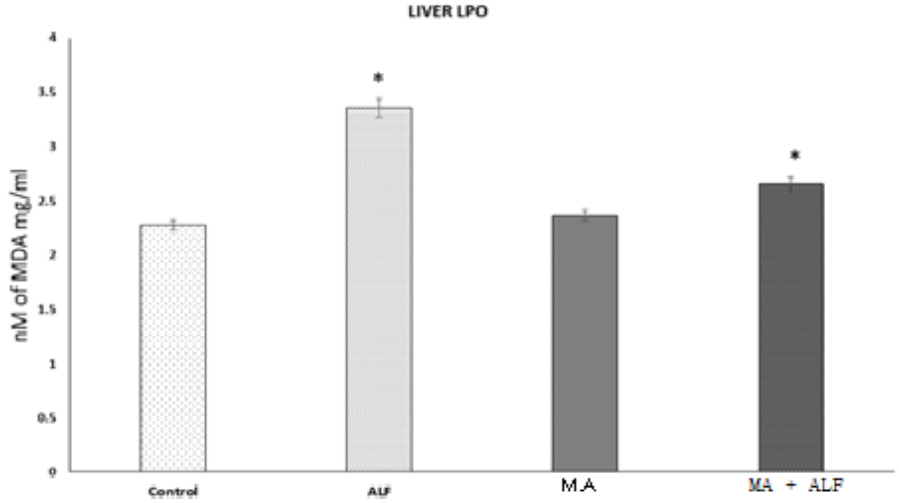

Fig. 1(a): Level of MDA; stable product of LPO significantly increased in liver of ALF group rats however, on treatment of M.A to the ALF treatment group significantly lower the level of MDA. Values represent mean ± SD where n = 3. Level of significance was determined by one-way ANOVA; *p<0.05.

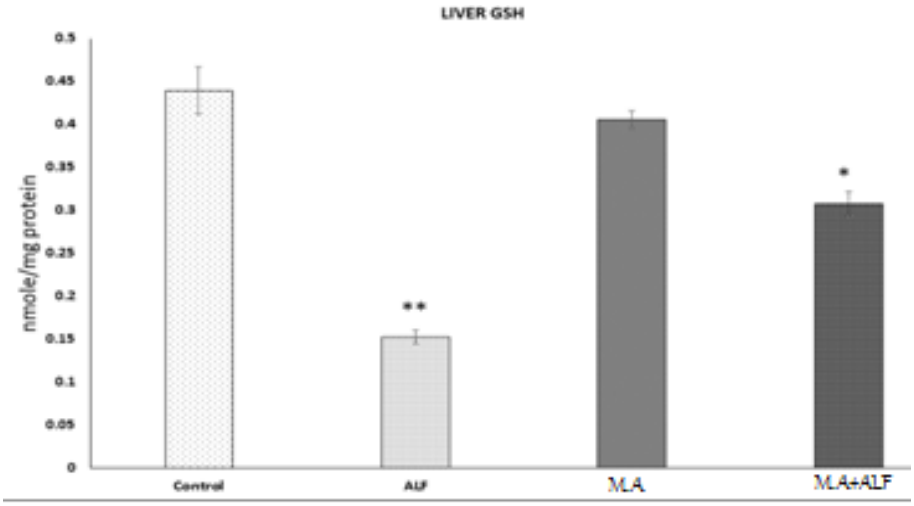

Fig. 1(b): Level of Glutathione; which is significantly decreased in liver of ALF group rats however, on treatment of M.A to the ALF treatment group significantly restore the level of Glutathione towards that of control group rats. Values represent mean ± SD where n = 3. Level of significance was measured by one-way ANOVA; *p<0.05 and **p < 0.01.

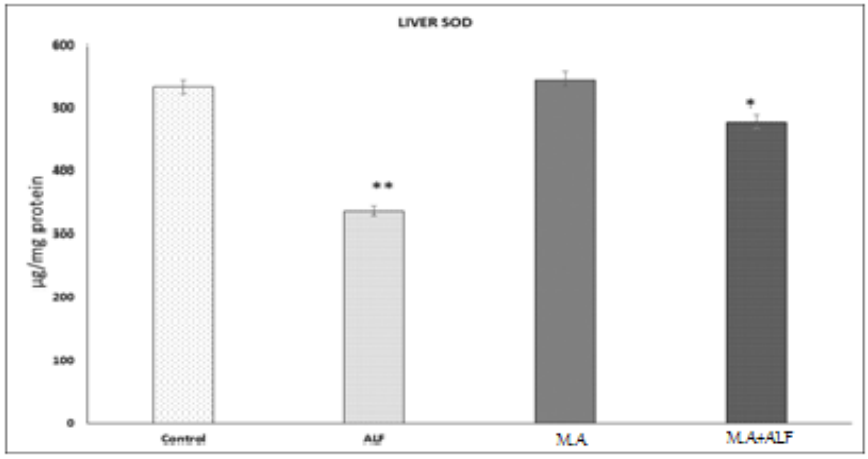

Fig. 1(c): Activity of Superoxide dismutase; which is significantly decreased in liver of ALF group rats however, on treatment of M.A to the ALF treatment group significantly restore the activity of SOD in liver of rats with ALF. Values represent mean ± SD where n = 3. Level of significance was measured by one-way ANOVA; *p<0.05 and **p < 0.01.

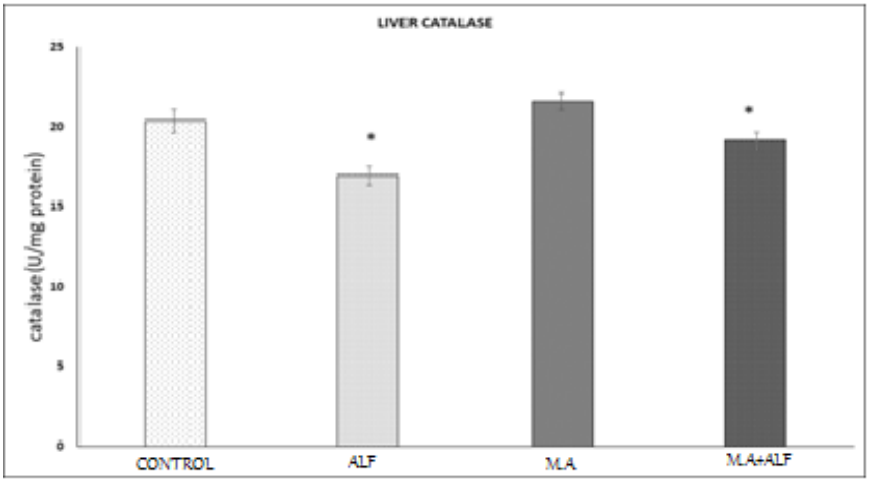

Fig. 1(d): Catalase activity in liver of ALF rat model; where catalase activity significantly decreased in case of ALF group rats. However, on treatment of M.A to the ALF treatment group significantly restore the activity of catalase in liver of rats with ALF. Values represent mean ± SD where n = 3. Level of significance was measured by one-way ANOVA; *p<0.05.

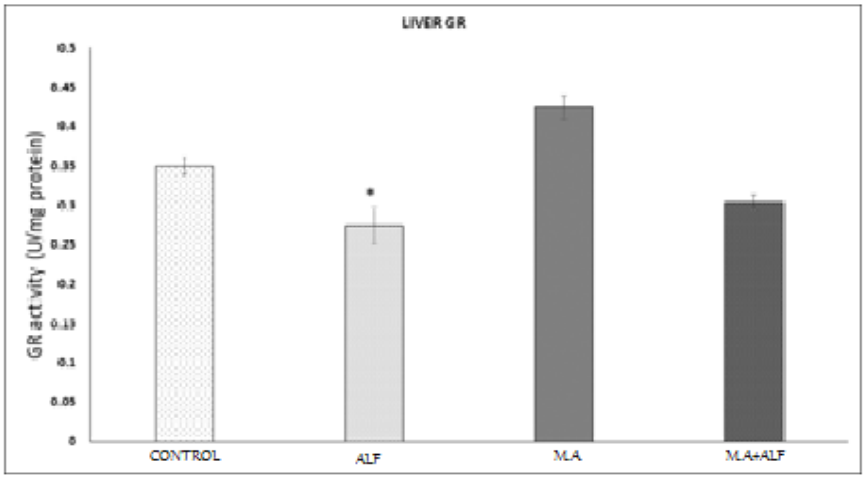

Fig. 1(e): Activity of Glutathione reductase in liver of ALF rat model; where GR activity significantly decreased in case of ALF group rats. However, on treatment of M.A to the ALF treatment group the activity of catalase seems to be static in liver of rats with ALF. Values represent mean ± SD where n = 3. Level of significance was measured by one-way ANOVA; *p<0.05.

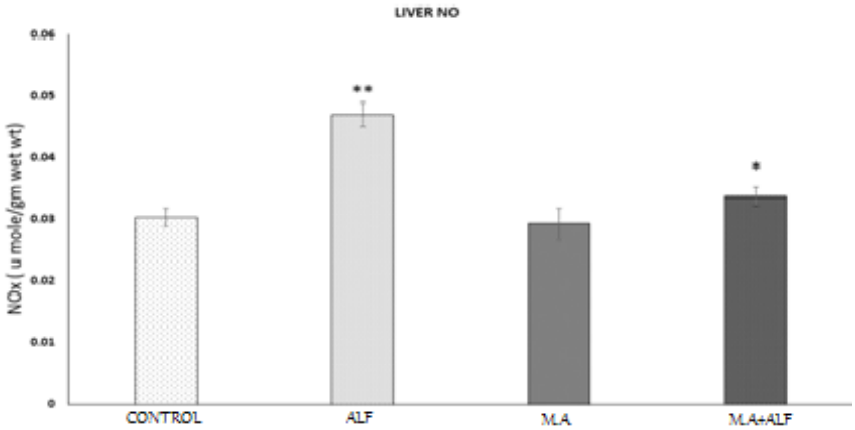

Fig. 1(f): Level of NOx in liver of ALF rat model; where NOx level significantly increased in case of ALF group rats. However, on treatment of M.A to the ALF treatment group the NOx level significantly decreased in liver of rats with ALF. Values represent here as mean ± SD where n = 3. Level of significance was measured by one-way ANOVA; *p<0.05 & **p<0.01.

In this study there was significant increase in the MDA level in liver, spleen, and testis of ALF induced rats as comparedto the control groups. However, in case of ALF group pre-treated with M.A significantly lower the MDA level in liver Glutathione (GSH) is a natural antioxidant present inside the organ and has a central role in ROS regulation. GPx is an enzyme which utilised theGSH and convert it into oxidised GSSG during free radical formation, and so, it is one of the other oxidative stress markers for analysing the oxidative stress in this present study. The hydrogen peroxide (H_2O_2) converts into H_2O and O_2 molecule with the help of GPx by utilizing the GSH. Then another enzyme GR play the role in converting back the GSSG into GSH so the total GSH/GSSG ration depends on the synchronous activity of GPx and GR. In the present study, the GSH level significantly decreased in ALF groups of liver. This may corelate with the oxidative stress caused by the liver failure and generation of free radicals in the spleen and testis of rats, which further leads to damage of these tissues. However, when the M.A is given to the rats of ALF group, the level of GSH was significantly increased in liver.

Likewise, the GR activity in the liver and testis of rats with ALF showed significant decline but interestingly there was increase in the activity in case of spleen. Treatment of M.A restored the GR activity in organ liver toward the control. The treatment of M.A to the ALF induced rats, restored the natural antioxidant GSH levels and GR activity to normal, which suggest the antioxidant nature of M.A. This finding clearly shows that M.A restore the GSH level in ALF groups on the liver of rats also increases the activity of GR by lowering production of ROS and free radicals due to oxidative damage in the liver, and liver failure mediated oxidative stress in spleen, and testis of rats with ALF. It has been stated that the leaf extracts of M.A upregulate the antioxidant status in the liver (Lv *et al*., 2019).

These findings of the present study may conclude that the bioactive component of the M.A helps in the protection of these organs against the oxidative damage caused by ALF.

The SOD, catalase and GPx, the antioxidant enzyme complex is considered to be the initial defence mechanism against the oxidative damage in the body due to ROS and free radicals generated during ALF in the liver. Both the enzyme SOD and catalase, scavenge free radicals and defend the cells against oxidative damage. First the action of antioxidant enzyme SOD reduces the oxidative damage in cells by converting H_2O_2 into water (Ighodaro and Akinloye, 2018). Further, the activity of catalase lowers the ROS by converting the H_2O_2 into water molecule. In the present study activity of both enzymes, SOD and catalase significantly reduced in the liver of ALF rats, which is also reported by Onuoha *et al.* (2022) as they have stated that the activity of SOD and catalase decreased in the ALF rat model. However, on treatment of M.A to the ALF rats, significantly restored the antioxidant status by lowering the liver failure induced oxidative stress in theses organs. Nitric oxide (NO) is a crucial molecule involved in signalling mechanism in immune system, and effect on intra- and intercellular physiological mechanism (Gantner *et al*., 2020). In the present study level of NO in liver in ALF rats was significantly high as compared to those of control rats.NO overproduction and helps in lowering the cytotoxicity and prevent cell damage. The observed result suggested that, the bioactive component of AP is lowering the oxidative damage caused by TAA induced liver failure by activating antioxidant enzymes and also lowers the NO level and prevents the cytotoxicity and inflammation caused by the hyperproduction of NO.

CONCLUSION

The present study indicated that TAA induced liver failure caused the liver injury and causes oxidative stress in liver. Oxidative stress induced through liver failure has adverse effect on different organs like spleen and

testis. The treatment of M.A in the ALF model actively ameliorates the oxidative damage in these organs of rats caused due to ALF by activating antioxidant enzymes complex. Hence, it can be suggested that the M.A could be a treatment for the patientswith ALF.

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