Molecular Mechanism of Formalin-Induced Toxicity and Its Management

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Abstract: The use of formalin (40% formaldehyde) for the preservation of food in an illegal way becoming a serious health issue in developing countries including Bangladesh. We investigated the Formalin (FA)-induced organ toxicity in Swiss albino mice. FA induction caused the significant elevation of the liver enzyme, SGOT and SGPT; the MDA levels in the liver and brain. Among the fractions of methanol extract of L. globosus, ethyl acetate (EA) fraction significantly reduced the elevated biochemical parameters (FA vs FA + EA fraction, µKa/L); SGOT (78.4 ± 0.3 vs 14.3 ± 0.9), SGPT (100.5 ± 5.2 vs 14.6 ± 0.7), MDA in liver (10.9 ± 0.2 vs 5.6 ± 0.1) and MDA in brain (16.9 ± 0.2 vs 6.3 ± 0.2). Morphological analyses also supported the beneficial effect of EA fraction in FA-induced liver toxicity. FA induction caused the phosphorylation of JNK, member of mitogen activated protein kinase (MAPK) in both the liver and brain, which were completely abolished by the treatment of EA fraction of L. globosus. Chemical analyses showed that the EA fraction exhibited antioxidant and free radical scavenging properties. The protective effect of the EA fraction on the FA-induced toxicity by the modulation of oxidative inflammatory pathway by its antioxidant and free radical scavenging activity.

Keywords: Loranthus globosus, SGOT and SGPT, Antioxidant and Free Radical Scavenging, JNK Phosphorylation, Formalin

1. Introduction

Formaldehyde is one of the common environmental agents found in tobacco smoke, paint, diesel, gasoline exhaust, and medical and industrial products (Flyvholm and Andersen 1993). It has been considered to be potentially carcinogenic that makes it a subject of major environmental concern (Heck, Casanova et al. 1990). In Bangladesh, FA is used as an illegal practice to preserve fish, fruits and vegetables which is dangerously affecting the health of local peoples. FA is an extremely reactive chemical, and reacts with monoamines or amides to form methylene bridges and produces covalently cross-linked complexes with proteins and DNA (Saito, Nishio et al. 2005). In addition to DNA-protein cross-links, it has been reported that FA modulates the cellular glutathione (GSH) status and generates oxidative free radicals (Teng, Beard et al. 2001, Saito, Nishio et al. 2005). Some studies have linked chronic FA exposure not only to cancer incidence, but also to teratogenicity, and to a variety of neurodegenerative and vascular disorders (Kilburn 1994, Sakanashi, Rogers et al. 1996, Yu, Wright et al. 2003).

When ingested, FA is rapidly metabolized and removed from the liver. The major metabolic enzymes, particularly NAD-dependent aldehyde dehydrogenase and GSH-dependent formaldehyde hydrogenase are involved in the metabolism of FA, which has been detected in human liver and red blood cells and in a number of animal tissues, such as respiratory and olfactory epithelium in the rat (Teng, Beard et
Various exogenous agents including FA can induce the generation of reactive oxygen species (ROS)(Riley 1994). An excess generation of reactive ROS in cells is known to damage DNA, lipids and proteins, resulting in a number of untoward pathophysiological effects such as mutagenesis, malignant transformation, cell death etc.(Wiseman and Halliwell 1996, Berlett and Stadtman 1997, Thannickal and Fanburg 2000). At the initial stage, to eliminate deleterious ROS from the body, cells utilize various enzymatic and non-enzymatic antioxidants. However, due to excessive oxidative stress, the body’s endogenous antioxidant source become exhausted which necessitates the supply of exogenous antioxidants.

In the present study, we investigated the antioxidant compound(s) in the methanolic extracts and its various fractions of *Loranthus globosus* and evaluated their protective effects against FA-induced organ injury in mouse model. We have analyzed serum glutamate oxaloacetate transaminase (SGOT) and serum glutamate pyruvate transaminase (SGPT) levels as biomarkers for liver injury, malondialdehyde (MDA) as biomarker for lipid peroxidation in liver and brain. The signaling mechanisms of FA-induced oxidative damage and the protective effects of *L. globosus* were also investigated.

2. Materials and Methods

2.1. Reagents

Dexamethasone was gifted from the Chemico pharmaceuticals Ltd., Rajshahi, Bangladesh. 0.9% NaCl solution (Beximco Infusion Lab., Dhaka, Bangladesh), SGOT and SGPT (AMP Medizintechnik GmbH; Austria), gallic acid standard, trichloro acetic acid, thiobarbituric acid, MDA standard and n-butanol (GE Health care, Buckinghamshire, UK) α, α-diphenyl-β-picrylhydrazyl (DPPH), sodium phosphate, ammonium molybdate, ascorbic acid and methanol (Sigma Aldrich, St. Louis, USA), anti-c-Jun N-terminal kinase (JNK), anti-phospho-JNK and anti-β actin (Cell Signaling Technology, Inc., Massachusetts, USA) were obtained from the sources noted. All employed chemicals and solvents were of analytical grade.

2.2. Plant Materials

The barks of *L. globosus* were collected from Pabna, Bangladesh and were taxonomically identified by Dr. Md. Anisuzzaman, Associate Professor, Department of Botany, University of Rajshahi, Bangladesh and the voucher specimen has been preserved there. The cleaned stem barks were dried under sunshine and subsequently in oven at 50°C temperatures for complete dryness.

2.3. Extraction and Fractionation of Plant Materials

The dried and pulverized plant material was cold extracted by methanol as described previously (Khan, Islam et al. 2010) and the methanolic extract (ME) was successively partitioned with petroleum ether (PE), chloroform (CF) and ethyl acetate (EA) using modified Kupchan partitioning method (BC, R et al. 1993). The resultant fractions were then evaporated by roto-dryer at low temperature (40-50°C) to dryness. The fractions were preserved at -20°C until use. Dimethyl sulfoxide was used as a solvent for the preparation of dose of various fractions.

2.4. Chemical Analysis of the Methanol Extract and Its Fractions

2.4.1. Determination of Total Phenolic (TP) Content

The total phenolic (TP) content of the crude methanolic extract (ME) of *L. globosus* and its various fractions (PE, CF, EA) were determined by Folin-Ciocalteu Reagent (FCR) according to the method of Kumar *et al.* (Kumar, Ganesan *et al.* 2007) with slight modification. Briefly, the solution of each extract (0.5 ml, 1mg/ml) was diluted to 10 ml with distilled water in a volumetric flask. FCR (1 ml) was added and mixed thoroughly, and then sodium carbonate solution (3 ml, 2%) was added. After 2h incubation at room temperature, absorbance was measured at 760 nm. The total phenolic content was determined by comparison with the standard calibration curve of gallic acid, and results are presented as mg of gallic acid equivalents (mg of GAE) per gram dry weight of extracts. All tests were conducted in triplicate.

2.4.2. Determination of Total Flavonoid (TF) Content

The total flavonoid content of each extract was estimated by Zhishen *et al.* (Zhishen, Mengcheng *et al.* 1999). Briefly, 0.5 ml (1 mg/ml) of each sample was mixed with 2 ml of distilled water and subsequently with 0.15 ml of NaNO2 solution (15%). After incubation for 6 min, 0.15 ml of AlCl3 solution (10%) was added and allowed to stand for another 6 min. Then 2 ml of NaOH solution (4%) was added to the mixture and adjusted the final volume to 5 ml by distilled water. The mixture was then mixed thoroughly and allowed to stand for another 15 min. The absorbance of the final solution was determined at 510 nm. The total flavonoid content was determined by comparison with the standard calibration curve of gallic acid, and results are presented as mg of gallic acid equivalents (mg of GAE) per gram dry weight of extracts.

2.5. In Vitro Antioxidant Assay

2.5.1. DPPH Radical Scavenging Assay

The plant extracts were tested for the scavenging effect on DPPH radical according to the method of Pan *et al.* (Pan, Wang *et al.* 2008). Accordingly, 0.2 ml of extract solution in ethanol (95%) at different concentrations (1, 2, 4, 8, 16, 32 and 64 µg/ml, respectively) was added to 8 ml of 0.004% (w/v) stock solution of DPPH in ethanol (95%). The scavenging activity on the DPPH radical was determined by measuring the absorbance at 517 nm until the reaction reached the steady state, using a UV-visible spectrophotometer (Shimadzu, Tokyo, Japan). Ascorbic acid was used as a positive control. The DPPH radical scavenging activity (%) was calculated using the following formula:
S% = [(A\text{control} - A_{\text{sample}})/A_{\text{control}}] \times 100

2.5.2. Total Antioxidant Activity Assay

The total antioxidant activity of the methanolic extract and its various fractions of *L. globosus* were assessed by the phosphomolybdenum method as described previously (Prieto, Pineda et al. 1999). Briefly, 0.5 ml sample solution of each fraction was mixed with 3 ml of phosphomolybdenum solution comprising: 0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate. The mixture was then incubated at 95ºC for 90 min followed by cooling at room temperature. The absorbance of the supernatant was measured at 695 nm against blank. Ascorbic acid was used as a positive control.

2.6. Induction of FA-Induced Organ Injury in Mouse Model

Male *Swiss albino* mice weighing about 25 - 30gm were used for the FA-induced animal model study. To measure FA-induced toxicity, we adopted several routes of administration as follows:

i. Oral administration of 4% FA mixing with fruits daily for one month.

ii. Oral administration of 4% FA with drinking water daily for one month.

iii. Intraperitoneal (IP) administration of 4% FA (0.1ml/day) for seven days.

iv. Subcutaneous (SC) administration of 4% FA (0.1ml/day) through hind paws for seven days.

2.7. Analysis of Various Biochemical Parameters

After completion of treatment (FA, drug, plant extract), mice were sacrificed and approximately 2-3 ml of blood was collected directly from heart by syringes, centrifuged at 4000rpm for 30minutes to collect supernatants. Serum GOT and GPT levels were measured by UV-visible spectrophotometric method using commercial reagent diagnostic kits (AMP Medizintechnik GmbH; Austria) according to the manufacturer’s protocol. Briefly, the amount of oxaloacetate and pyruvate formed by each of the two assays were measured by means of the 2, 4 -dinitrophenylhydrazone of pyruvic acid, the color of which was read at 520 nm by spectrophotometer. The intensity of color was proportional to the amount of enzyme in each sample.

2.8. Analysis of Malondialdehyde (MDA)

The MDA levels on the tissues were determined by the method of Draper and Hadley based on the reaction of MDA with thiobarbituric acid (TBA) at 95ºC (Draper and Hadley 1990). The brain and liver tissues were homogenized separately on ice in MDA lysis buffer and centrifuged at 13,000×g for 10 min to collect the supernatant. In the TBA test reaction, MDA and TBA react to form a pink pigment with absorption maximum at 532 nm. The reaction was performed at pH 2-3 at 95ºC for 15 min. The supernatant was mixed with 2.5 volumes of 10% (w/v) trichloroacetic acid to precipitate the protein. The precipitate was pelleted by centrifugation and supernatant was reacted with 0.67% TBA in a boiling water bath for 15 min. After cooling, the absorbance was read at 532nm. Arbitrary values obtained were compared with a series of standard solutions (1, 1, 3, 3-tetramethoxyxpropane). Results are expressed as nmol per milligram of tissue.

2.9. Immunoblotting

The liver and brain samples from the respective animal groups were disected and placed immediately into ice-cold phosphate-buffered saline (PBS). The collected tissues were homogenized by sonicating with an ultrasonic homogenizer (VP-050, Taitec Corp., Koshigaya, Japan) followed by lysis with RIPA buffer (50 mM Tris-HCl, 1% NP-40, 0.25% Na-deoxycholate, and 150mM NaCl, 1 mM Na2VO4 and NaF) containing protease inhibitors (1 µg/ml each of EDTA and phenylmethylsulfonyl fluoride). Cell lysates were centrifuged at 12,000×g for 15 min at 4ºC. The supernatant was collected and boiled with SDS sample buffer (12 mM Tris-HCl, 10% glycerol, 10% sodium dodecyl sulfate and 0.1% mercaptoethanol and 0.1% bromophenol blue, pH 6.8) for 5 min at 100ºC. Proteins were separated by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were probed with appropriate concentrations of primary antibody. The immunoreactive proteins were detected by horseradish-peroxidase-labeled secondary antibody with Amersham ECL advance Western blotting Detection Kit (GE healthcare).

2.10. Histopathological Study

After seven days of observation, the animals were killed by cervical dislocation. The liver was carefully excised, rinsed in cold sucrose solution, and blotted in dry filter paper. The specimen was fixed with 10% buffered-formaline and dehydrated in ascending order of ethanol and embedded in paraffin. The blocks were sectioned with the help of rotation microtome at 6-micron thickness. The sections were subjected to Hematoxylin and Eosin staining procedures and the histological examination was done with the aid of the high power microscope. The histological outline of each photomicrograph was conducted through a stereological grid in order to access the population of the cells in each organ. The permanent photomicrographs of each slide were recorded with a Kodak Digital Camera for subsequent histological analysis.

2.11. Statistical Analysis

Data were expressed as mean ± standard error of mean (SEM). Statistical comparisons were performed by one-way analysis of variance (ANOVA), followed by Scheffe’s post-hoc test or students paired or unpaired t-test where appropriate. Results were considered to be significant when *p* values were less than 0.05 (*p* <0.05). Statistical calculations and the graphs were prepared using Graph Pad Prism version...
5.00 for Windows (Graph Pad Software, San Diego, CA, USA, www.graphpad.com).

3. Results

3.1. Chemical Analysis of Total Phenol and Flavonoid Contents of the Crude Methanol Extract (ME) and Its Fractions of L. globosus

Table 1. Determination of total phenol and flavonoid contents in the crude methanol (ME) extract and its various fractions of L. globosus.

| Sample    | Total phenol GAE/gm of dried extracts | Total flavonoid GAE/gm of dried extracts |
|-----------|--------------------------------------|-----------------------------------------|
| ME extracts | 73.07 ± 0.08                        | 198.43 ± 5.19                           |
| PE fractions | 14.03 ± 0.03                        | 102.50 ± 11.3                           |
| EA fractions | 114.84 ± 0.20                       | 276.90 ± 16.34                          |
| CF fractions | 31.84 ± 0.04                        | 53.21 ± 6.09                            |

The phenolic and flavonoid compounds are considered as potential antioxidants and free radical scavengers. Here, we investigated the contents of total phenol (TP) and total flavonoid (TF) compounds in the various fractions (PE, CF and EA) of methanolic extracts of L. globosus. The results are shown in Table 1. The highest amount of TP and TF contents were found in EA fractions (114.84 ± 0.20 and 276.90 ± 16.34 GAE per gram, respectively).

3.2. In Vitro Antioxidant Activity Analysis of the Crude Methanol Extract (ME) and Its Fractions of L. globosus

The Fig. 1A shows that there was significant scavenging of DPPH free radicals on various fractions of L. globosus. Maximum scavenging of 95.6 ± 2.5% was observed by EA fractions in a similar extent to that of standard ascorbic acid (94.3 ± 4.2%), followed by CF (62.0 ± 1.2%) and PEF (61.5 ± 2.4%), respectively.

Similarly, the Fig. 1B shows that the EA fractions have the highest total antioxidant activity. The order of total antioxidant activity in various fractions of L. globosus were EA>PE>CF. The results are concordant with the contents of total phenolic and total flavanoid in various fractions.

Fig. 1. In vitro antioxidant activity of the methanol extract and fractions of L. globosus: (A) DPPH radical scavenging activity of crude methanol extracts (ME) and its various fraction of L. globosus. (B) Total antioxidant activity of the crude methanol extract (ME) and its various fractions of L. globosus. Ascorbic acid was used as standard scavenger of the oxidant as well as standard antioxidant agent.

3.3. Time Course of FA-Induced Liver Injury

Fig. 2. Time course of FA-induced elevation of liver enzymes (SGOT and SGPT) in various routes of administration. FA was supplied by (A) intraperitoneal injection; (B) hind paw injection; (C) oral route with drinking water and (D) oral route with fruits. * indicates significantly different (p<0.05) from time control after treatment with FA.
In order to estimate the FA-induced liver injury, we measured SGPT and SGOT levels at various time periods upon FA treatment for different routes of administration. The intrapitoneal (IP) route of administration shows approximately 10-fold maximum increase of both the liver enzymes (Fig. 2A) on 6th day of administration as compared to control. In the case of oral routes, similar increase of liver enzymes were achieved at longer duration (15-30 days) as shown in Fig. 2C and 2D. Elevated level of both the enzymes was least when injected in the hind paw (Fig. 2B). Because of the difficulty in dose adjustment along with administration in oral route and less sensitivity to hind paw, we chose IP administration for 7-day FA treatment period in our experimental model.

3.4. Ethyl Acetate (EA) Fraction of L. globosus Reduces the FA-Induced Elevated SGOT and SGPT Levels in a Dose-Dependent Manner

Since the EA fraction of L. globosus possesses the maximum amount of antioxidant compounds (TP and TF), we investigated the effect of this fraction on the FA-induced elevated SGOT and SGPT levels. Single IP injection of EA fraction reduced the FA-induced elevation of SGOT and SGPT levels in a dose dependent manner (Fig. 3). EA fraction at 12-mg/kg-body weight reduced the elevated SGOT and SGPT levels completely. 12-mg/kg-body weight of EA fraction of L. globosus were used for further experiments unless mentioned.

3.5. Ethyl Acetate (EA) Fraction of L. globosus Suppress the FA-Induced Lipid Peroxidation

Malondialdehyde (MDA) is one of the most important biomarker of lipid peroxidation, which is generated due to the excessive load of free radicals. In the present study, we found that IP administration of FA caused the elevation of serum and brain MDA levels which were suppressed significantly by the co-administration of single IP injection of EA fraction of L. globosus in a similar extent to that of standard anti-inflammatory drug, dexamethasone. The results are shown in Fig. 4A and 4B.

3.6. Histopathology of Liver Tissues after FA-Induced Damage

Liver tissues were collected from the control and treatment group of mice were fixed and stained with Hematoxylin and Eosin as described in methods. The specimens were then visualized under microscope with 40× magnifications. As shown in Fig. 5B, there was swelling of liver tissues with marked fatty degeneration with FA treatment. Areas of focal necrosis were also observed in the liver of the FA-induced mice as compared to the control (Fig.5A). The histopathological degeneration was repaired considerably by the supplementation of EA fraction of L. globosus as shown in Fig. 5D.
3.7. Ethyl Acetate (EA) Fraction of L. globosus Suppress the FA-Induced Phosphorylation of c-Jun N-Terminal Kinase (JNK)

The c-Jun N-terminal kinase (JNK) pathway is important in modulating cellular responses to inflammation and oxidative stress. Since FA is an inflammatory mediator, we estimated the phosphorylation levels of JNK in serum and brain after 7 day of FA treatment. There was approximately 10-fold significant increase of serum (Fig. 6A) and brain (Fig. 6B) phospho-JNK levels above the baseline. Co-administration of EA fraction of L. globosus completely abolished the FA-induced phospho-JNK levels both in liver and brain, suggesting the potential anti-inflammatory and anti-oxidant effects of the fraction.

![Histopathological studies of FA-induced liver damage with or without the treatment of EA fraction of L. globosus and Dexamethasone. The representative photographs shown are (A) normal control (B) FA-induced control (C) FA-induced mice with the supplementation of anti-inflammatory compound, dexamethasone and (D) FA-induced mice with the supplementation of EA fraction of L. globosus. Scale bars, 50 µm.](image)

![FA-induced activation of JNK in mouse liver and brain. (A) FA-induced Phosphorylation of JNK in mouse liver tissue. (B) FA-induced Phosphorylation of JNK in mouse brain tissue. Samples were collected from liver and brain tissues, 7 days after FA induction and were lysed, separated in SDS-PAGE, blotted and probed with anti-phospho-JNK, anti-JNK and anti-β actin. (n=3).](image)
4. Discussion

The serum enzyme levels are direct measure of hepatic injury and they show the status of the liver. The elevation of enzymes induced by FA causes hepatotoxicity which may be due to its metabolite, a free radical that binds to lipoprotein and leads to peroxidation of lipids of endoplasmic reticulum. The disturbance in the transport function of the hepatocytes as a result of hepatic injury causes the leakage of enzymes from cells due to altered permeability of membrane (Zimmerman and Seeff 1970). The increased serum GOT and GPT level is evidence that these enzymes play an important role in the development of acute and chronic inflammation (Anderson, Bocklehurst et al. 1971).

In this study, we found that EA fractions of L. globosus significantly suppressed the FA-induced elevated SGOT and SGPT levels (Fig. 3). Most of the anti-inflammatory drugs exert their beneficial effect by inhibiting either release of these enzymes or by stabilizing lysosomal membrane which is one of the major events responsible for the inflammatory process (Nair, Ravishankar et al. 1988). Thus it can be assumed that EA fractions of L. globosus might be acting by either inhibiting the enzymes or stabilizing the membrane.

The elevated level of MDA, which may be due to the free radicals, is responsible for damaging cell membranes and further intensifies inflammatory damage (Telang, Chatterjee et al. 1990). The inflammatory tissue damages could be due to the liberation of reactive oxygen species from phagocytes that invades the inflammatory sites (Conner and Grisham 1996).

In the present study, we found that the concentration of MDA in brain and liver tissues was found to be higher in FA-induced mice, which were reduced significantly by treatment with the EA fractions of L. globosus (Fig. 4). The increased MDA levels in the serum and brain also comprise with the previous report by Teng et al. (Teng, Beard et al. 2001).

We demonstrated that EA fractions of methanolic extracts of L. globosus exhibited potential antioxidant and free radical scavenging capacity (Fig. 1) due to the presence of flavonoids and phenolic compounds (Table 1). Hence, we assumed that the protective effect of EA fraction could be the result of direct free radical scavenging properties (Gurel, Coskun et al. 2005) or by reacting with membrane phospholipid bilayers to break the chain reaction initiated by ROS (Verma and Nair 2001). Histological analyses also supported the protective effects of EA fractions of L. globosus (Fig. 5).

Finally, we discovered the molecular mechanism of the ameliorative effects of EA fraction of L. globosus against organ toxicity by investigating the intracellular signaling events. Our results clearly shows that FA induction caused the activation of oxidative stress responsive JNK pathway, a member of the mitogen-activated protein kinase (MAPK) family in the brain and liver, which were completely suppressed by the supplementation of EA fractions of L. globosus. All isoforms of JNK are constitutively expressed in the liver and brain, which plays a key role in cell death and hepatotoxicity. JNK activation has been well recognized in both rodent and human liver diseases (Malhi, Bronk et al. 2006, Puri, Mirshahi et al. 2008, Wang, Ausman et al. 2008).

5. Conclusion

FA induction caused the elevation of liver biomarkers; SGOT and SGPT and lipid peroxidation biomarker, MDA in liver and brain tissues, which were suppressed towards normal levels by the supplementation of the EA fractions of L. globosus presumably via the suppression of oxidative stress responsive JNK pathway by its antioxidant and free radical scavenging activity. Although more detailed mechanisms need to be further investigated, the present work provides a potential strategy for treating liver and brain damage. Therefore, the modulation of JNK pathway could be a good target in the treatment of liver and brain toxicity.

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Abbreviations

FA, formalin; SGOT, serum glutamate oxalate transaminase; SGPT, serum glutamate pyruvate transaminase; MDA, malondialdehyde; EA, ethyl acetate; ME, methanol extract; PE, petroleum ether; CF, chloroform; MAPK, mitogen activated protein kinase; DPPH, α, α-diphenyl-β-picrylhydrazyl.

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