Study of Hepatoprotective Effect Produced by the Fruits of *Phyllanthus emblicus* and Silymarin against Cisplatin Induced Hepatotoxicity

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

Original Research Article

ABSTRACT

Cisplatin is a cytotoxic drug which induced the hepatotoxicity in the albino mice when intra-peritoneally administered at the dose of 10 mg/kg. Administration of cisplatin raised the level of LFT's enzymes and also reduced the level of antioxidant enzymes in the liver of the mice. Administration of *Phyllanthus emblica* extract and silymarin remarkably showed the hepatoprotective effect in the rat. Administration of *P. emblica* and silymarin decreased the level of ALT, AST, and ALP along with increasing the level of Total protein content. It also increased the level of antioxidant enzymes in the liver of rat showing its hepatoprotective activity.

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1. INTRODUCTION

Liver is a vital organ which play an important role in many body functions including; metabolism, bile secretion and detoxification and excretion of many compounds and drugs [1]. Exposure of liver to toxins and some drugs can cause the liver toxicities which can further cause cellular destruction and liver damage. Some drugs which are mainly involved in causing liver damage or hepatotoxicity are; INH, methyldopa, cisplatin, metformin human insulin and some antihypertensive agents.

For centuries plants have been used to treat various diseases in human, the popularity of plants to cure diseases is because of less unwanted effects than synthetic drugs. Some clinical trials have evidenced the hepatoprotective effect of some traditional herbs [2]. *Phyllanthus emblica* Linn, also known as *Emblica officinalis*. In Urdu it is called amla. It belongs to Family: *Euphorbiacea*. This is indigenous plant of India but also found in China, Srilanka, Malaysia, East Asia, Pakistan and Uzbekistan.

P. emblica is a natural plant used in folk medicine to treat a variety of ailments. Its fruit, seed, leaves, root, bark and flowers are used in herbal medicines. Traditionally, it was thought to have cooling, astringent and drying properties, and was used to stimulate hair growth by nourishing the hair and scalp. The high tannin content of *P. emblica* was also used as a dye in inks, shampoos and hair oils.

After *in vitro* tests, it was discovered that *P. emblica* has antiviral and antimicrobial properties. It is thought to contain very high amounts of Vitamin C, or Ascorbic Acid, a powerful antioxidant known to reduce wrinkles, suppress pigmentation, and help skin retain its natural moisture. However, studies have shown that the overall antioxidant strength of amla may derive instead from its high density of tannins and other polyphenols. The fruit also contains flavonoids, kaempferol, ellagic acid and gallic acid.

Phyllanthus emblica is considered safe and natural, and is rated a low hazard, 97% safe ingredient by the Cosmetics Database. No negative side effects or studies reporting warnings were found, although it may be drying to skin and hair. The active chemical constituents of *Phyllanthus emblicus* are proteins, carbohydrates, calcium, phosphorus, vitamin c nicotinic acid, carotene, D-glucose, trigallayl glucose, D-fructose, riboflavin, myoinositol, darabinosyl, lrhamnosyl, G-glycosyl, D-xylosyl, dmanosyl, D-galactosyl, mucic, Phyllambic acid, phellembin, punigluconin, embicol, emblicanin-A, emblicanin-B, fatty acid, procyanidin, 3-O-gallated prodelphindin, leucodelphinidine, ellagic acid, gallic acid, 3 ethylgallic acid, methyl gallate, 1-O-galloyl-beta-D-glucose, 3,6-di-O-galloyl-Dglucose, chebulagic acid, chebulinic acid, tannins, oleanolic acid, lupeol and pectin [3,4].

The plant has been reported to have number of pharmacological activities such as anticancer [5], antipyretic, analgesic, anti-inflammatory [6], aphrodisiac, antidiabetic, antimicrobial [7], gastro-protective [8], hepatoprotective [9], cardio-protective [10], nephroprotective [11] hypolipidemic [12], antioxidant, antitussive, dermoprotective [13], analgesic, antimutagenic, memory enhancing [14] and snake venom neutralizing effect [15].

Silybum marinum, commonly known as milk thistle belongs to Asteraceae family, is the rich source of silymarin. This plant widely used in the treatment of various diseases caused by oxidative stress. It is found in the regions of Europe, Asia, South and North America [16]. The plant grows in rocky soil and has large purple colored flowers. Silymarin possesses a mixture of four flavonolignan isomers such as silydianin, silybinin, silychristin and isosilybin and toxifolin (flavonoid) [17].

The most active component of these isomers is silybinin having hepatoprotective activity. Silymarin has potential to treat hepatic toxicity due to its antioxidant nature, role in protein synthesis and stabilization of membrabe phospholipids. Silymarin is also reported to have immunomodulatory, antilipid, anti-inflammatory and liver regenerating properties [18].

In this study we are comparing the effect of *Phyllanthus embilica*, silymarin, and *of Phyllanthus embilica*+ silymarin on serum parameters in cisplatin induced hepatic damage in rat.

1.1 Aims and Objectives

Objective of this study is to evaluate the hepatoprotective effect of *Phyllanthus emblica* and Silymarin against the toxic effects of cisplatin on rat liver.

2. MATERIALS AND METHODS

2.1 Chemicals

Cisplatin, 50 mg/100 ml, was purchased from Shaukat Khanam Cancer Hospital & Research Centre, Lahore, Pakistan.

2.2 Preparation of Extract

Fresh fruits of *P. emblica* were purchased from the market. *P. emblica* fruits were shade dried and then powdered mechanically, passing through sieves. The grinded powder was soaked into 70% methanol for three days. After three days the material was filtered through the muslin cloth and then Whatman No.1 filter paper. The procedure was repeated three times. The whole filtrate was pooled and evaporated in the rotary evaporator until it became thick blackish paste. The extract was collected and stored. Dilutions were made in distilled water for the purpose of experimentation.

The standardized silymarin extract was purchased from Sigma Aldrich Corporation. Dimethyl sulphoxide was used as solvent for *P. emblica* and ethanol was used for silymarin. All the samples were given by oral route.

2.3 Animals and Experimental Protocol

Experiment was conducted and evaluated in Al-Qassim University, Kingdom of Saudi Arabia after IRB approval. Albino rats of either sex weighing 200-250 g were used in the experiment. All of the animals were kept in animal house at 25°C with 12 hours light-dark cycle. Animals were divided into 5 groups with 6 rats in each group. Chow and water were provided.

Group A= served as negative control and were administered vehicle only.
Group B= cisplatin 10 mg/kg intraperitoneally (positive control)
Group C= 70% methanolic extract of *P. emblica* 200 mg/kg orally for 14 days.
Group D= Silymarin 200 mg/kg per oral for 14 days
Group E= *P. emblica* + Silymarin (100 mg/kg each) for 14 days

On 15th day, cisplatin, 10 mg/kg, was injected i.p. in group C, D and E, animals were sacrificed and liver was isolated after blood collection by cardiac puncture. Serum was separated after centrifugation at 3000 rpm for 10 min. Organs were preserved in the phosphate buffer saline (10 mM stock) and their homogenates were prepared which were further utilized for the assessment of biochemical markers and tests.

2.4 Preparation of Homogenates

Tissue homogenate were prepared in phosphate buffer saline (pH 7.4). After crushing, the mixture was centrifuged at 4000 rpm for 15 minutes. Supernatant was separated and stored at -20°C for biochemical analysis.

2.5 Biochemical Analysis

2.5.1 Estimation of glutathione

Glutathione level was estimated using Moron et al. [19] method.

2.5.1.1 Chemicals

50% Trichloro acetic acid (TCA), 0.02 M Ethylene diamine tetraacetic acid (EDTA), 0.15 M Tris HCl, 6 mM Dithio-bis 2 nitrobenzoic acid)/ Ellman's reagent and distilled water were used for the GSH estimation.

2.5.1.2 Principle

Liver GSH was estimated according to the method of Moron et al. [19]. GSH reacts with Ellman's reagent (5, 5-dithio bis Nitrobenzoic acid or DTNB) to produce a chromophore Thio Nitrobenzoic acid (TNB) that give maximal absorbance at 412 nm. Absorbance value can give the estimation of enzyme value.

2.5.1.3 Procedure

0.1 ml of tissue homogenate was taken in test tube, 2.4 ml of 0.02M EDTA was added in each test tube and was kept in ice bath for 10 minutes. Then 2.0 ml of distilled water and 0.5 ml of TCA were added in each test tube and again kept in ice bath for 15 minutes. The mixture was centrifuged at 3000-3500 rpm for 10 minutes. The supernatant (1 ml) was separated and added 2 ml of 0.15 M Tris-HCl and 0.05 ml of DTNB and then mixed thoroughly on vortex. Absorbance was measured at 412 nm within 2-3 of the last step. Absorbance was taken against reagent blank, which was prepared in the same manner but without using tissue homogenates. And standard solution was prepared by using GSH in place of tissue homogenates. The

standard curve of GSH was plotted for 40-200 µg concentration of standard. The absorbance was compared with standard curve generated by known GSH. Level of GSH in tissue homogenates was measured using linear regression equation. The conc. of GSH was measured in µg/g tissue.

2.5.2 Estimation of Catalase (CAT)

Catalase activity was assayed using Aebi, 1974 method [20].

2.5.2.1 Chemicals

Phosphate buffer (pH 7), hydrogen peroxide (2 mmol/l).

2.5.2.2 Principle

Catalase enzyme degrades hydrogen peroxide (H_2O_2) into oxygen and water. Ultraviolet absorption of H_2O_2 can be measured at 240 nm. In the presence of catalase, absorption decreases due to degradation of H_2O_2.

2.5.2.3 Procedure

0.1 ml of tissue homogenate, 1.0 ml freshly prepared hydrogen peroxide and 1.9ml phosphate buffer were taken in cuvette. Standard and blank were similarly prepared using CAT in place of tissue homogenate and without tissue homogenate respectively. Absorption was measured at 240 nm against blank. Using different conc. of CAT, a standard curve was generated and absorption was compared with standard curve. Specific activity of CAT was expressed in unit/g of tissue. Tissue activity of CAT was measured using linear regression equation.

2.5.3 Estimation of Malondialdehyde (MDA)

MDA was measured following Ohkawa et al. [21] method.

2.5.3.1 Chemicals

Thiobarbituric acid (TBA), Sodium dodecyl sulphate (SDS), n-butanil, acetic acid and distilled water were used in this assay.

2.5.3.2 Principle

Malondialdehyde (MDA) is the end product of lipid peroxidation. They are produced as a result of breakdown of polyunsaturated fatty acids. This compound is a reactive aldehyde and is one of the many reactive electrophile species that cause toxic stress in cells and form covalent protein adducts referred to as advanced lipoxidation end- products (ALE), in analogy to advanced glycation end-products (AGE). The production of this aldehyde is used as a biomarker to measure the level of oxidative stress in an organism.

Malondialdehyde reacts with deoxyadenosine and deoxyguanosine in DNA, forming DNA adducts.

MDA reacts with TBA to produce pink colored end product having maximum absorption at 532 nm.

2.5.3.3 Procedure

Lipid peroxidation was estimated calorimetrically by measuring Thiobarbituric acid reactive substances (TBARS). To 0.2 ml of tissue homogenate, 0.2 ml of 8.1% Sodium dodecyl sulfate (SDS), 1.5 ml of 20% acetic acid and 1.5 ml thiobarbituric acid (0.8%) and 0.6 ml of distilled water were added and vortexed. The solution was incubated in water bath at 95°C for 1 hour. After that mixture was cooled and 5 ml of pyridine butanol mixture (1:15 v/v) and 1 ml distilled water were added and centrifuged for 10 minutes at 3000 rpm. The upper organic layer was taken and its optical density was measured at 532 nm against blank. The standard solution was prepared using 10-100 nmol concentration. Level of MDA in the reaction was calculated using linear regression equation. The levels of lipid peroxides were expressed as nmol of Thiobarbituric acid reactive substances (TBARS)/g protein.

2.5.4 Estimation of superoxide dismutase (SOD)

SOD activity was determined using Kakkar et al. [22] method.

2.5.4.1 Chemicals

Phenazine methosulphate, Nitro blue tetrazolium (NBT), reduced (NADH), n-butanol, Trichloro acetic acid (TCA), sodium pyrophosphate buffer, glacial acetic acid.

2.5.4.2 Principle

This assay is based on the formation of formazan resulting from the reaction of NADH, Phenazine

methosulphate and nitro blue tetrazolium. A blue colored formazan is developed by the reduction of NBT during this reaction. SOD inhibits the reduction of NBT. The color is extracted into butanol and measured at 560 nm.

2.5.4.3 Procedure

To 0.2 ml of tissue homogenate, 1.2 ml of sodium pyrophosphate buffer (pH 8.3, 0.025 M), 0.1 ml of Phenazine methosulphate (186 µmol/l), 0.3 ml of nitro blue tetrazolium (300 µmol/l) and 0.2 ml of NADH (750 µmol/l) were added. Reaction was started after of NADH. After incubation at 30℃ for 90 sec, the reaction was stopped by the addition of 0.1 ml of glacial acetic acid. The reaction mixture was stirred vigorously with 4.0 ml of n-butanol. The mixture was allowed to stand for 10 min; centrifuged and n-butanol layer was separated. The color intensity of choromogen in butanol layer was measured at 560nm reagent blank. Standard curve was constructed for SOD using 10-100 µl concentration. Tissue activity if SOD was measured using linear regression equation. Concentration of SOD was expressed in unit/gram of liver tissue.

2.5.5 Estimation of LFT's

Liver function tests including AST, ALT, ALP and TP were estimated by using commercially available Bio Merux and Randox kits.

2.6 Statistical Analysis

Values were expressed as mean ± SD. When compared with toxicant control group. One way (ANOVA) analysis of variance was used followed by Dunnetts test to determine the difference between groups in terms of all studied parameters using SPSS computer program. Differences were considered significant when value of *P* is less than 0.05.

3. RESULTS

To evaluate the liver function of rat before and after the treatment strategies alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP) and total protein (TP) tests were performed. As per Table 1, the level of liver enzymes was elevated and total protein (TP) was decreased after the administration of Cisplatin (10 mg /kg) in group B. The level of these enzymes was decreased and protein content was increased in group C, D and E treated with *Phyllanthus emblica* (200 mg/kg), silymarin (200 mg/kg) and *Phyllanthus emblica* + silymarin (100 mg each). Maximum decrease in ALT, AST and ALP was observed in group E treated with *Phyllanthus emblica*+ silymarin (100 mg /kg each).

As per Table 2, s level of antioxidant enzymes (GSH, CAT and SOD) was decreased after the administration of Cisplatin (10 mg/kg), while MDA level was increased due to the hepatotoxic effects of Cisplatin on liver. Level of antioxidant enzymes was significantly increased and MDA level was decreased in group C, D and E. maximum antioxidant effect was observed in animal groups treated with *Phyllanthus emblica*+ silymarin (100 mg each).

4. DISCUSSION

Cisplatin is a cytotoxic drug used against various types of cancers. This drug may also cause much toxicity inside the different organs of body including heart, kidneys and liver [23,24]. In the present study application of cisplatin at 10 mg/kg induced the hepatotoxicity in the mice which was supported by biochemical findings i.e. increase in the ALT, AST, ALP and total protein contents (TP) and decrease in the antioxidant enzymes (SOD, Catalase, GSH and MDA). Various studies have also supported that treatment of rats with cisplatin induced changes in the

Table 1. Effect of *Phyllanthus emblicus* and silymarin on liver function tests (n=6)

Groups	ALT (IU/L)	AST (IU/L)	ALP (IU/L)	TP (mg/dL)
A	26.81±1.20	29.9±3.01	83.1±8.2	6.2±0.65
B	117.0±7.67	103.2±2.0	136.9±1.53	2.7±0.97
C	63.0±5.72	54.3±4.09	111.6±7.13	6.02±0.49
	P=0.01	*P*=0.004	*P*=0.059	*P*=0.05
D	44.7±1.25	39.1±1.07	106.41±6.29	5.50±0.03
	P=0.006	*P*=0.001	*P*=0.02	*P*=0.05
E	38.32±5.93	32.1±2.61	99.7±6.29	3.60±0.61
	P=0.008	*P*=0.001	*P*=0.015	*P*=0.38

Table 2. Effect of *Phyllanthus emblicus* and silymarin on oxidative stress markers in liver

Groups	GSH (µg/dl)	CAT (µg/dl)	SOD (µg/dl)	MDA (µg/dl)
A	6.6±0.05	28.9±0.24	26.8±1.28	34.3±2.93
B	2.8±0.09	15.6±0.53	18.9±1.03	94.0±2.18
C	4.4±0.09*	18.3±0.86	20.7±0.59	76.0±4.08
	P=0.003	P=0.06	P=0.223	P=0.008
D	5.9±0.97*	21.3±0.17	21.1±1.71	43.2±1.76
	P=0.046	P=0.005	P=0.26	P=0.002
E	6.11±1.04*	24.9±1.45	23.5±0.12	39.2±2.30
	P=0.05	P=0.014	P=0.024	P=0.002

ALT and AST by damaging hepatocytes. Cisplatin induced oxidative stress also contribute to damage the liver cells. Some studies have shown that repeated administration of cisplatin at high dose reduces the level of antioxidant enzymes. Cisplatin administration also elevated the level of MDA, which caused the hepatic cell damage. In the present study treatment of rats with *P. emblica* extract and silymarin prevented the hepatic cell damage by increasing the liver function test parameters and by also remarkably decreasing the MDA level and increasing the level of antioxidant enzymes i.e. catalase, superoxide dismutase and glutathione. Maximum antioxidant effect was observed in animals treated with both *P.emblica* and silymarin (100 mg/kg each). The healing of hepatic cells is due to the tissue regeneration property of flavonoids and bioactive tannoids present in both silymarin and *P. emblica*.

5. CONCLUSION

The given data suggests that cisplatin which is cytotoxic drug causes the hepatotoxicity and as well weakens and decreases the level of antioxidant enzymes in the liver of rats and hence increases the oxidative stress. From the present study it was also concluded that administration of *P. emblica* extract and silymarin individually after the administration of cisplatin remarkably reduced the level of ROS by increasing the level of oxidative enzymes inside the liver of the rats. *P. emblica* and silymarin also improved the liver function tests showing hepatoprotective effect. But on combining *Phyllanthus emblica* and silymarin a synergistic hepatoprotective effect was observed. Hence it is concluded that co-administration of *P. emblica* and silymarin together can be used as supportive adjuvant therapy which reduces the hepatotoxic effects of the cisplatin.

CONSENT

It is not applicable.

ETHICAL APPROVAL

Authors hereby declare that this research work was carried out by using albino mice after the necessary approval taken from the Ethical Research Committee in the College of Pharmacy, Qassim University during the year 2015-2016.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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