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Drug Induced Hepatotoxicity: Effect of Polyherbal Formulation

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ABSTRACT

To evaluate effect of Polyherbal Formulation (PHF) on drug induced hepatotoxicity in rats and assessment of any possibility of co administration of PHF along with such hepatotoxic drug. Hepatotoxicity in rat was induced by Methotrexate (1mg/kg p.o./week for 3 weeks) and protective effect of PHF (0.25ml/kg/p.o. and 0.5ml/kg/p.o. either along with drug or followed by inducing hepatotoxicity) was measured by estimating marker enzymes for liver function like Aspartate aminotransferase, Alanine aminotransferase, Alkaline phosphatase and γ glutamic transpeptidase. Oxidative stress markers like lipid peroxidation, reduced glutathione, super oxide dismutase and catalase. Protein profile likes Total bilirubin, direct bilirubin, Total albumin and Total protein. Histopathological study was carried out to confirm hepatotoxicity. Methotrexate induced hepatotoxicity characterized by significant (P<0.001) increase in marker enzymes for liver function and oxidative stress. Protein profile significantly depleted. Administration of PHF either along with Methotrexate or followed by inducing hepatotoxicity significantly improved (P<0.001) the level of marker enzymes for liver function, oxidative stress and depleted proteins profile. The study suggests protective role of PHF in drug induced hepatotoxicity and it can be utilized to treat the hepatotoxicity with long-term clinically useful drugs.

Keywords: Hepatotoxicity; Methotrexate; Oxidative stress; Polyherbal formulation; Protein profile.

INTRODUCTION

Drug-induced Hepatotoxicity (DIH) account for 9.5% of all suspected adverse drug reaction (1). Injury may be a direct toxic effect or immunological reaction to either of the drug or an active metabolite formed by bioactivation (2). It is reported that 62% of withdrawn drugs having toxic metabolite (3). Although, with the exception of rare cases, DIH subsides after cessation of treatment with the drug, this represents an important diagnostic and therapeutic challenge for physicians.

Methotrexate major activity results from binding to the enzyme dihydrofolic reductase, thus preventing conversion of folic acid into its active form, folinic acid. This in turn blocks the synthesis of nucleic acids, certain amino acids and indirectly proteins. This might lead to damage of organelles and plasma membranes of hepatic parenchymal cells interfering with their function and allowing leakage of enzymes. The hepatic malfunction is probably due to a direct toxic action of the methotrexate, since most reaction is dose dependent (4). Methotrexate causes repression of enzymes involved in lipid peroxidation (acyl-CoA dehydrogenase, medium chain enoyl CoA hydratase and very long chain acyl-CoA synthatase) were associated with microvesicular lipidosis which leads to oxidative stress mediated liver necrosis, fibrosis and bile duct hyperplasia (4).

Polyherbal formulation contains plant extract of Himsra, Kasani, Kakamachi, Arjuna, Kashmarda, Birranjshipa, Jharuka and processsed in 50mg herbs each in powder form of Bhringraja, Punarnava, Guduch, Daruharidra, Mulaka, Amalaki, Chitraka, Vidanga, Haritaki, and Parpata. Each having reported hepatoprotective, liver tonic, antioxidant and anti-inflammatory activity.

The main objective of present study was to assess hepatoprotective effect of Polyherbal formulation in drug induced hepatotoxicity and assessment of any possibility of co administration along with such hepatotoxic drug.

MATERIALS AND METHODS

Polyherbal formulation

Polyherbal Formulation (PHF) was procured as a gift sample from M/S Apostle Remedies, Vadodara.

Animals

Healthy adult male Wistar rats weighing 150–200g were used. Rats were housed in polypropylene cages, maintained under standardized condition (12-h light/dark cycle, 24°C, 35 to 60% humidity) and provided free access to palleted CHAKKAN diet (Nav Maharashtra Oil Mills Pvt. Ltd., Pune) and purified drinking water ad libitium. All experiments and protocols described in present study were approved by the Institutional Animal Ethics Committee (IAEC) of M. S. University, Baroda and with permission from Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India.

Drug Induced Hepatotoxicity

Methotrexate a clinically useful drug was selected for inducing hepatotoxicity in rats. Drug induced hepatotoxicity in rats was produced by orally administration of methotrexate (1mg/kg/week) for 3 weeks (4). Silymarin was used as reference standard.

Experimental protocol

Animals were divided into seven groups, each having 6 rats and treated accordingly. Group: 1 - rats received normal standard diet for 3 weeks, Group: 2 - rats received PHF (0.25ml/kg/p.o.) alone for 3 weeks, Group: 3- rats received methotrexate, Group: 4 - rats received methotrexate along with PHF (0.25ml/kg/p.o.) for 3 weeks, Group: 5 - rats received methotrexate along with PHF (0.5ml/kg/p.o.) for 3 weeks, Group: 6 - rats received methotrexate for 3 weeks followed by PHF (0.25ml/kg/p.o.) for 1 week. Group: 7 - rats received methotrexate along with Silymarin (SLM) (20 mg/kg/p.o.) for 3 weeks.

Collection of serum

Blood samples were withdrawn from retro-orbital plexus under light ether anesthesia without any anticoagulant and allowed for 10 minutes to clot at room temperature. It was centrifuged at 2500 rpm for 20 minutes. The serum obtained was kept at 4°C until estimation of liver function. All the animals were euthanasiously sacrificed after blood collection with spinal dislocation method and liver removed for study of oxidative stress markers and histopathology.

Estimation of liver function

Estimation of marker enzymes for liver function like Aspartate aminotransferase (AST), Alanine aminotransferase (ALT) and Alkaline phosphatase (ALP) were done by using kit, Span Diagnostic Ltd, India and γ glutamic transpeptidase (γGTP) was done by using kit, Dade Behring Ltd., UK. Estimation of Lactate dehydrogenase (LDH) was done by using kit, Enzopak-Reckon diagnostics. Protein profile likes Total bilirubin, direct bilirubin; Total albumin and Total protein were done by using kit, Span Diagnostic Ltd, India.

Estimation of oxidative stress markers

Liver kept in cold conditions (precooled in inverted Petridish on ice). It was cross chopped with surgical scalpel into fine slices in chilled 0.25 M sucrose, quickly blotted on a filter paper. The tissue was minced and homogenized in 10 mM Tris-HCl buffer, pH 7.4 (10%w/v) with 25 strokes of tight teflon pestle of glass homogenizer at a speed of 2500 rpm. The clear supernatant was used for oxidative stress markers assays like lipid peroxidation (5), Reduced Glutathione (6), Super oxide dismutase (7) and Catalase (8).

Histopathological study

Liver was collected after blotting free of blood and tissue fluids, it was kept in 5% formalin. 5–15µm thick section was serially cut on a leitz microtome in horizontal plane and mounted on glass slide with the help of egg albumin in glycerine solution (50% v/v). They were then stained with 10% hematoxylin for 3–5 minutes and placing in running water intensified the staining. The hematoxylin stained sections were stained with 10% eosin for 2 minutes. The sections were observed and desired areas were photographed in an Olympus photomicroscope. The sections were viewed under 40X magnifications.

Statistical analysis

All the values are expressed as mean ± S.E.M. Statistical significance between more than two groups was tested using one-way ANOVA followed by the Bonferroni multiple comparisons test using computer based fitting program (Prism, Graphpad 3.). Differences were considered to be statistically significant when $p < 0.05$.

RESULTS

Effect Polyherbal formulation on Marker Enzymes of Liver Function

Serum levels of ALT, AST, γGTP, ALP and LDH were significantly (P<0.001) increased after treatment with methotrexate compared to control. PHF (0.25ml/kg) perse has no effect on ALT, AST, γGTP, ALP and LDH but PHF (0.25ml/kg and 0.5 ml/kg) when administrated along with or after treatment with methotrexate produced significant (P<0.001) decreased in the levels of ALT, AST, γGTP, ALP and LDH. Silymarin treatment also produced significant (P<0.001) decreased in the levels these enzymes but was less than that of PHF. Correspondingly, there was significantly (P<0.001) increased in the ratio of ALT/AST after methotrexate, which was significantly (P<0.001) decreased after treatment with PHF and Silymarin (Table: 1).

Effect Polyherbal formulation on Protein Profile

Methotrexate administration caused significant (P<0.001) increase in serum Total Bilirubin and significant (P<0.001) decrease in Direct Bilirubin, Total Protein and Albumin compared to control. There was no effect of PHF (0.25ml/kg) alone on serum Total Bilirubin, Direct Bilirubin, Total Protein and Albumin. PHF (0.25ml/kg and 0.5ml/kg) along with methotrexate, PHF (0.25ml/kg) followed by methotrexate and Silymarin showed significant (P<0.001) decreased in Total Bilirubin and increased in Direct Bilirubin. PHF (0.25ml/kg and 0.5ml/kg) along with methotrexate, Silymarin and PHF (0.25ml/kg) followed by methotrexate treatment were found to caused significant (P<0.001, P<0.05 respectively) increase in Total Protein and Albumin compared to methotrexate control. Correspondingly the changes were observed in the ratio of Albumin/ Globulin (Table: 2).

Table 1: Effect Polyherbal formulation on Marker Enzymes of Liver Function

Groups	ALT (IU/L)	AST (IU/L)	ALT/AST	γGTP (IU/L)	ALP (IU/L)	LDH (IU/L)
CONTROL	35.17 ± 2.613	40.00 ± 1.414	0.8800 ± 0.028	40.83 ± 1.014	152.5 ± 1.478	345.3 ± 4.702
PHF (0.25ml/kg) alone	32.50 ± 2.262	37.33 ± 1.476	0.7767 ± 0.097	39.00 ± 0.9661	151.0 ± 2.221	340.0 ± 3.347
MTX (1mg/kg p.o./week)	167.2 ± 3.400^{+++}	84.50 ± 1.688^{+++}	1.982 ± 0.064^{+++}	83.33 ± 1.20^{+++}	294.5 ± 3.433^{+++}	534.8 ±3.351^{+++}
MTX+ PHF(0.25ml/kg)	40.50 ± 2.291***	48.83 ± 1.721***	0.8350 ± 0.037***	49.33 ±1.606***	159.7 ± 2.860***	360.2 ± 2.960***
MTX+ PHF (0.5ml/kg)	37.17 ± 2.701***	41.00 ± 2.793***	0.9250 ± 0.084***	45.33 ± 2.996***	155.0 ± 3.276***	355.0 ± 4.155***
MTX followed by PHF (0.25ml/kg)	42.17 ± 2.638***	43.17 ± 2.386***	0.9800 ± 0.030***	46.17 ± 2.786***	160.2 ± 2.892***	368.2 ± 3.790***
MTX + SLM	44.00 ± 1.390***	55.00 ± 2.352***	0.8017 ± 0.012***	53.00 ± 0.966***	163.0 ±1.528***	375.0 ± 2.280***

Values are expressed as mean ± SEM of 6 animals in each group.
***p<0.001,
+++p<0.001,
+compared with control,
*compared with methotrexate control (One way ANOVA followed by Bonfferoni's multiple comparison tests).

Table 2: Effect Polyherbal formulation on Protein Profile

Groups	Total Bilirubin (mg/dl)	Direct Bilirubin (mg/dl)	Total Protein (mg/dl)	Albumin (mg/dl)	Globulin (mg/dl)	A:G	Indirect Bilirubin (mg/dl)
CONTROL	0.7033 ± 0.008	0.3017 ± 0.0060	7.497 ± 0.116	4.995 ± 0.058	2.502 ± 0.146	2.037 ± 0.128	0.4050 ± 0.014
PHF (0.25ml/kg) alone	0.6900 ± 0.023	0.3100 ± 0.0093	7.330 ± 0.075	4.900 ±0.096	2.430 ± 0.104	2.043 ± 0.126	0.3817 ± 0.024
MTX (1mg/kg p.o./week)	1.788 ± 0.054^{+++}	0.1300 ± 0.007^{+++}	6.417 ± 0.16^{+++}	3.392 ± 0.177^{+++}	3.025 ± 0.297	1.082 ± 0.138^{+}	1.583 ± 0.017^{+++}
MTX+PHF (0.25ml/kg)	0.7400 ± 0.013***	0.3300 ± 0.013***	7.050 ± 0.076**	4.935 ± 0.014***	2.115 ± 0.087*	2.325 ± 0.112***	0.4117 ± 0.011***
MTX+PHF (0.5ml/kg)	0.7200 ± 0.031***	0.3100 ± 0.020***	7.117 ± 0.107**	4.833 ± 0.261***	2.283 ± 0.273	2.363 ± 0.427***	0.4100 ± 0.018***
MTX followed by PHF (0.25ml/kg)	0.7617 ± 0.027***	0.3117 ± 0.016***	6.483 ±0.151	4.067 ± 0.111*	2.417 ± 0.047	1.680 ± 0.029	0.4500 ± 0.012***
MTX + SLM	0.7500 ± 0.015***	0.3083 ± 0.009***	7.132 ±0.031**	4.840 ± 0.019***	2.293 ± 0.042	2.113 ±0.046**	0.4400 ± 0.024***

Values are expressed as mean ± SEM of 6 animals in each group.
***p<0.001
+++p<0.001,
++p<0.001
**p<0.001
+,* p<0.001.
+compared with control,
*compared with methotrexate control (One way ANOVA followed by Bonfferoni's multiple comparison tests).

Effect Polyherbal formulation on Oxidative stress markers

Methotrexate significantly (P<0.001) increased lipid peroxidation and caused significant (P<0.001) decrease in reduced glutathione (GSH), Super oxide dismutase (SOD), Catalase (CAT) compared to control. There was no significant effect of PHF (0.25ml/kg) alone on lipid peroxidation, GSH, SOD and CAT. PHF (0.25ml/kg and 0.5ml/kg) along with methotrexate and PHF (0.25ml/kg) followed by methotrexate was found to caused significant (P<0.001) decrease in lipid peroxidation and significant (P<0.001) increase in GSH, SOD and CAT as compared to methotrexate control. Silymarin along with Methotrexate produced effect similar to PHF (Table: 3).

Effect of Polyherbal formulation on Histopathological changes

Liver section of control rats revealed the normal hepatic hexagonal lobules and normal morphology. Liver tissue of methotrexate treated rats showed vacuolation, degeneration of hepatocyte, mild inflammation and piecemeal necrosis. PHF and Silymarin treatment improved structural integrity of liver cells (figure 1).

Table 3: Effect Polyherbal formulation on Oxidative stress markers

Groups	MDA (Nmole/gm of tissue)	GSH (µmole/gm of tissue)	SOD (Unit/gm of tissue)	CAT (µmole H_2O_2 consumed/min/ gm of tissue)
CONTROL	5.102 ± 0.094	106.0 ± 1.633	43.67 ± 1.282	295.0 ±1.592
PHF (0.25ml/kg)	5.100 ± 0.093	104.0 ± 2.266	44.00 ± 1.673	308.7 ±16.87
MTX	20.50 ± 0.885^{+++}	44.00 ± 1.673^{+++}	21.67 ± 1.726^{+++}	95.83 ± 4.037^{+++}
MTX+ PHF (0.25ml/kg)	9.000 ± 0.577***	95.67 ± 2.667***	39.83 ± 1.470***	284.3 ± 4.341***
MTX+ PHF (0.5ml/kg)	5.917 ± 0.289***	104.2 ± 3.016***	44.17 ± 1.641***	325.2 ± 18.37***
MTX followed by PHF (0.25ml/kg)	5.117 ± 0.270***	90.17 ± 2.023***	38.17 ± 0.9458***	280.7 ± 3.138***
MTX + SLM	6.900 ± 0.118***	89.17 ± 2.315***	29.50 ± 2.078***	260.0 ± 2.221***

Values are expressed as mean ± SEM of 6 animals in each group.

****p<0.001*

+++p<0.001,

+compared with control,

**compared with methotrexate control (One way ANOVA followed by Bonfferoni's multiple comparison tests).*

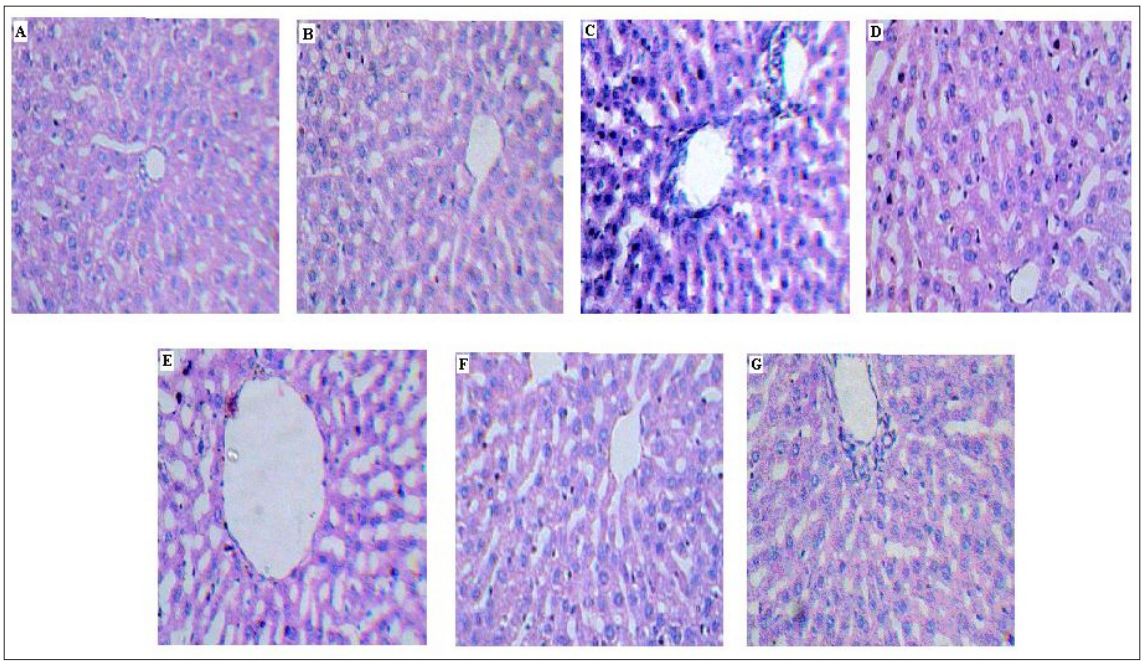

Figure 1: Hematoxylin and eosin-stained sections of rat liver (magnification X 100): (A) Control rat; (B) Polyherbal formulation (0.25 ml/kg) treated rat; (C) Methotrexate (1 mg/kg) treated rat; (D) Methotrexate with Polyherbal formulation (0.25ml/kg) treated rat; (E) Methotrexate with Polyherbal formulation (0.5ml/kg) treated rat; (F) Methotrexate followed by Polyherbal formulation (0.25ml/kg) treated rat; (G) Methotrexate with silymarin (20 mg/kg) treated rat.

DISCUSSION

ALT and AST are enzymes produced within the cells of the liver, as the cells are damaged, leaks into the bloodstream leading to a rise in the serum levels. ALP is an enzyme, which is associated with the biliary tract, and is elevated; biliary tract damage and inflammation should be considered. It is used often times to confirm that the alkaline phosphatase is of the hepatic etiology by γGTP. Mild to moderate elevation of ALT, AST, ALP (1–3 times) are usually seen in drug toxicity (9, 10, 11).

Elevated serum ALT, AST, ALT/AST, γGTP, ALP, LDH and CPK levels in methotrexate treated animals compared to control animals is attributed as damage to the structural integrity of liver (12), and presumptive markers of drug induced necrotic lesions in the hepatocyte. This decrease in elevated serum ALT, AST, ALT/AST, γGTP, ALP and LDH levels in PHF along with or followed by methotrexate treated animals in part may be due to the protective effect of PHF on liver cells following restoration of liver cell membrane permeability (13).

This protective effect indicates reduction in enzymes present in the extra cellular milieu as liver cell. Protective effect of component of PHF has also been observed in several experimental studies (14, 15, 16). It can be stated that PHF contains *Tamarix gallica*, and crude herbal extracts of *Capparis spinosa, Cichorium intybus, Solanum nigrum, T. arjuna*, and *A. millefolium*, these medicinal herbs alone or in combination can influence in restoration of the cellular functions and structural integrity of liver.

Methotrexate treated animals significantly increased Total Bilirubin and decreased in Direct Bilirubin, Total Protein and Albumin reflects liver cell damage or bile duct damage and synthetic function of the liver has been markedly diminished, indicated drug induced hepatotoxicity (17).

PHF along with and followed by methotrexate significantly normalized Total Bilirubin, Total Protein, Direct Bilirubin and Albumin indicating liver curative effect. The curative and hepatoprotective effect *Cassia occidentalis, Cichorium intybus* and *Solanum nigrum* of PHF were observed against chemically induced liver damage in experimental animals (18). The diuretic effect of *T. arjuna* and anti-inflammatory and anti-immunotoxicity effect of *Cichorium intybus* have been shown in clinical and experimental studies (19, 20).

In present study increased in lipid peroxidation and depletion of antioxidant enzymes such as GSH, SOD and CAT in methotrexate treated animals compared to control animals indicated generation of oxidative stress. Methotrexate causes repression of enzymes involved in lipid peroxidation (acyl-CoA dehydrogenase, medium chain enoyl CoA hydratase and very long chain acyl-CoA synthatase) were associated with microvesicular lipidosis which leads to oxidative stress mediated liver necrosis, fibrosis and bile duct hyperplasia (4).

PHF along with and followed by methotrexate significantly reduced lipid peroxidation and increased antioxidant enzymes such as GSH, SOD and CAT as compared to methotrexate treated animals indicated modification of oxidative stress by PHF. The protection of liver cells against toxic materials including drugs, lipid peroxidation and free radical injury may decrease inflammation (21). Immune dysfunction is component of liver disease and thus immunomodulation by herbal therapy prevent oxidative stress, inflammation and strengthens the detoxifying power of liver cell (22). The anti-oxidative property of esculetin and p-methoxybenzoic acid the main constituent of *Cichorium intybus* and *Capparis spinosa*, respectively, have been reported in chemically induced hepatotoxicity in experimental animals (23, 24). *Achillea millefolium*, another component of PHF contains flavonoids and terpenoids with anti-oxidative and anti-inflammatory properties (25, 26). Furthermore anti-oxidative property of flavonoid content of *Tamarix gallica* and inhibitory effect *Solanum nigrum* crude extracts on free radical-mediated DNA damage increase the hepatoprotective effect of PHF (27). In addition, the antioxidative, anti-lipoproxidative and increase in glutathione content of the liver cells was observed with arjunolic acid and flavonoids present in *T. arjuna*(28).

Although there is insufficient information to establish the mechanism of action of PHF protection, this could be due to its anti-inflammatory, anti-oxidative, immunomodulating as well as restorative effects.

CONCLUSION

The study suggest protective role of Polyherbal Formulation in drug induced hepatotoxicity and this effect may be due to its anti-inflammatory, anti-oxidative, immunomodulating as well as restorative effects. Polyherbal Formulation can be utilized to treat the hepatotoxicity with long term clinically useful drugs, which are at the risk of developing hepatotoxicity.

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