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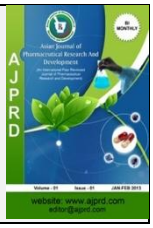

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Research Article

Modulation of immune mechanisms during hepatoprotective effects of Dawa-Ul-Kurkum, a Unani polyherbal preparation in experimental model of paracetamol induced liver damage in rats

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A B S T R A C T

Objective: Iimmunomodulatory effects of Dawa-Ul-Kurkum, a Unani polyherbal preparation and the possible mechanisms in experimental model of paracetamol induced liver damage in rats.

Materials and methods: The drug Dawa-Ul- Kurkum has been prepared and provided by CRIUM, Hyderabad. Silymarin was purchased from Sigma-Aldrich (USA) and paracetamol (marketed by Cipla LTD) purchased from general pharmacy shop. Biochemical kits were purchased from ERBA Diagonostic Mannheim Gmbh. Serum alanine aminotransferase (ALT), serum aspartate aminotransferase (AST) and serum alkaline phosphatase (ALP) were estimated by Kinetic method of International Federation of Clinical Chemistry (IFCC), serum bilirubin and total protein were estimated by End Point assay as per the instruction of the Kit Manufacture's manual.Immunoglobulin and cytokine levels were assessed by ELISA kit manual method. Delayed type hypersensitivity reaction by Institoris et al method. Lipid peroxidation is measured spectrophotometrically as 2-thiobarbituric acid-reactive substance (TBARS), Glutathione (GSH) levels were estimated by the method of Ellman and NOx concentrations were determined by using the Griess reaction as described previously by Tracey et al.

Results: Liver damage was induced in Wistar rats by administration of paracetamol and the effects of various drug treatments were assessed on morphological, biochemical, immunoglobulin, cytokine level and histological markers of liver toxicity. In the vehicle treated experimental group, administration of paracetamol induced significant derangements in liver function as evidenced by increased levels of SGOT, SGPT, alkaline phosphatase bilirubin and decrease in total protein, and reductions in body weight and increased liver weights as compared to controls. Histopathological examination showed Periportal necrosis with haemorrhages in experimental control. Pretreatment with Dawa-Ul-Kurkum (DK, 250 and 500 mg/kg) and hydroalcoholic extract of DK (HA, 500 and 1000 mg/kg) showed protective effects against the paracetamol induced biochemical, immunoglobulin, cytokine, delayed type hypersensitivity reaction and histopathological derangements of liver function following paracetamol. **Conclusion:** Both DK and its 50% hydro-alcoholic extract were found to be effective against paracetamol induced liver damage as they significantly prevented the hepatotoxic damage induced in rats.

Keywords: Hepatotoxicity, Paracetamol, Dawa-Ul-Kurkum, Histopathology

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INTRODUCTION

The liver is an essential organ and its strategic location and multidimensional functions support almost every other organ in the body[1], which act a pivotal role in regulating various physiological processes in the body. It is involved in various crucial functions such as metabolism, secretion and storage. It has prominent capacity to detoxicate toxic substances and synthesize functional ones[2]. Paracetamol

is widely used an analgesic and antipyretic but when it is taken in higher doses leads to acute liver damage[3]. Mostly paracetamol is metabolized in liver to excretable glucuronide and sulphate conjugates[4, 5]. Though, the hepatotoxicity of paracetamol has been allocated to the formation of toxic metabolites when a part of paracetamol is activated by hepatic cytochrome P-450 to a highly reactive metabolite N-acetyl-P-benzoquinone imine (NAPQI) [6]. Paracetomol is a strong inducer of cytochrome P450. NAPQI exerts its toxicity primarily via its oxidative effect on cellular proteins. The inactivation of proteins leads to death of liver cells[7]. Initially NAPQI is detoxified by conjugation with reduced glutathione (GSH) to form mercapturic acid[8]. But, the rate of NAPQI development exceeds the rate of detoxification by GSH, it oxidizes tissue macromolecules like as lipid (SH group) of protein and changes the homeostasis of calcium after diminishing GSH.

Overdose of paracetamol is also known to be linked with inflammation, increase in inflammatory cytokines as well as the up-regulation of nitrogen oxide (NO) from serum, macrophages and hepatocytes[9]. The disturbance of prooxidant-antioxidant balance in tissues results in increased levels of reactive oxygen species (ROS) and oxidative damage of macromolecules[10]. It can lead to various pathological conditions in humans and animals, like hepatic and renal dysfunction, testicular damage, respiratory disorders, and cancer[11]. In other hand, there are various reports suggesting that paracetamol-mediated oxidative stress or hepatotoxicity is attenuated by use of naturally occurring antioxidants and/or free radical scavengers such as vitamins, medicinal plants and natural products[12, 9].

Conventional or synthetic drugs used in the treatment of hepatic diseases are inadequate and can have serious adverse effects. So there is a worldwide trend to go back to traditional medicinal plants. Many natural products of herbal origin are in use for the treatment of liver disorder[3]. Herbal drugs are emerging as strong alternatives or adjuncts to conventional modern medical therapy. The comparative lesser adverse effects of traditional combined with the regulatory issues arising out of the TRIPS agreement have generated a renewed interest in the traditional remedies. In recent years, complementary and alternative medicinal approach using medicinal plants for prevention and treatment of diseases is gaining popularity[13]. A huge number of medicinal plants are being used traditionally for immunomodulation and hepatoprotection and these effects need to be validated following modern scientific methodology. So that they can be a part of the main stream health care system for complex pathophysiological states. In Unani system of medicine, a polyherbal formulation Dawa-Ul-Kurkum is used in cases of liver dysfunction, anorexia, ascites and abdominal pain. The study has been designed to evaluate the hepatoprotective and immunomodulatory effects of Dawa-Ul-Kurkum and the possible mechanism in the experimental model of paracetamol induced liver damage. The polyherbal Unani preparation, Dawa-Ul-Kurkum is composed of 9 herbs namely Sunbul-ut-Teeb, Mur Makki, Saleekha, Qust, Shagufa-e-Izkhir, Darcheeni, Zafran, Sharab-e-musallas and Asal[14, 15].

MATERIALS AND METHODS

Drugs and Chemicals

The drug Dawa-Ul- Kurkum has been prepared and provided by CRIUM, Hyderabad. Silymarin waspurchased from Sigma-Aldrich (USA) and paracetamol (marketed by Cipla LTD) purchased from general pharmacy shop; other routine chemicals were procured from SRL, New Delhi. Biochemical kits were purchased from ERBA Diagonostic Mannheim Gmbh.

Animals

Inbred Wistar rats of either sex (180-250 g) were used for the study. Animals were taken from the state-of the-art Animal House of Vallabhbhai Patel Chest Institute, University of Delhi. Animals were housed at a constant temperature (25 ± 2°C) under standard laboratory conditions. The animals had free access to food and water throughout the experiment**.** Care of animals was taken as per guidelines of CPCSEA for use of animals in Scientific Research with approval of Institutional Animal Ethics committee (IAEC) (CPCSEA Registration number 170/GO/ReBi/S/99/CPCSEA).

The Investigational Drug

The standardized drug, Dawa-ul-Kurkum, was prepared and provided by Central Research Institute of Unani Medicine (CRIUM), Hyderabad, Ministry of AYUSH, Govt. of India with a batch no. 3-1/2018-19/CRIUM. This polyherbal is composed of Sunbul-ut-Teeb (Nardostachysjatamansi DC), Mur Makki (Commiphoramyrrha Nees), Saleekha (Cinnamomum tamala), Qust (Saussurealappa), Shagufa-eIzkhir (Cymbopogon shoenanthus), Darcheeni (Cinnamomumzylenicum bark), Zafran (Crocus sativa) with Sharab-e-musallas (Saussureacostus) and QandSafaid (Saccharum offininarum) Q.S. The formulation is well documented in standard Unani literature[16] and is certified to have been prepared as per traditional classical Unani text by CRIUM.

Experimental procedure

Paracetamol induced liver damage in rats

The study was approved by the Institutional animal Ethical Committee (IAEC) of V.P. Chest Institute, University of Delhi. The experimental model of liver damage was induced in wistar rats by administration of paracetamol (2g/kg, orally) daily for 14 days[17]. Animals were divided into seven groups. Group 1 served as healthy control receive water; Group 2 served as experimental control administered with paracetamol; Group 3 served as positive control and received Silymarin (50mg/kg, orally)[18] + paracetamol; Group 4 & 5 animals were administered Dawa-Ul-kurkum (DK) at dose (250 or 500 mg/kg, orally) respectively + paracetamol; Group 6 & 7 animals were administered with 50% hydro alcoholic extract of Dawa-Ul-Kurkum (HA) at dose (500 or 1000 mg/kg, orally) + paracetamol. The dose of Dawa-Ul-Kurkum was calculated from the human dose being

prescribed by the Unani physicians. All drugs were administered for 14 days. Paracetamol was administered (2g/kg, orally) daily for 14 days in all groups except group 1. On 15th day, animals were anesthetized and blood was collected by cardiac puncture, centrifuged and stored at -80ºC. After blood collection, animals were sacrificed and liver was collected for histopathological studies and estimation of biochemical and oxidative stress parameters. As per approval of the IAEC, total 35 animals were included in the experimental study. Animals were divided into total seven groups, in each group contains 5 rats / group.

Biochemical estimations

Serum alanine aminotransferase (ALT), serum aspartate aminotransferase (AST) and serum alkaline phosphatase (ALP) were estimated by Kinetic method ofInternational Federation of Clinical Chemistry (IFCC), serum bilirubin and total protein were estimated by End Point assay as per the instruction of the Kit Manufacture's manual.

Estimation of MDA levels

Malondialdehyde (MDA) the organic compound [$CH_2(CHO)_2$] is widely used as oxidative stress biomarker in biomedical research. Lipid peroxidation is measured spectrophotometrically as 2-thiobarbituric acid-reactive substance (TBARS) in supernatant of liver homogenate [8]. 0.1 ml of supernatant was mixed with 0.2 ml of sodium dodecyl sulfate (8.1 %), 1.5 ml of 20 % acetic acid and 1.5 ml of 2-thiobarbituric acid (0.8 %). The reaction mixture was finally made up to 4.0 ml with distilled water. After vortexing, samples were incubated for 1 h in 95° C and after cooling with tap water; 1.0 ml of distilled water and 5.0 ml of mixture of butanol–pyridine 15:1 (v/v) were added. The mixture was shaken for 10 min. and then centrifuged at 4000 rpm for 10 min. Butanol–pyridine layer is measured spectrophotometrically at 532 nm. TBARS values are expressed as MDA equivalents. 1, 1, 3, 3-tetramethoxypropane (TMP) was used as the standard[19].

Assay of reduced glutathione (GSH) levels

Glutathione (GSH) levels were estimated by the method of Ellman[20]. This assay is based on the enzymatic recycling procedure in which glutathione was sequentially oxidized by the DTNB and reduced by NADPH in the presence of glutathione reductase. For assay, an equal quantity of sample was mixed with 10% trichloroacetic acid and centrifuged to separate the proteins. To 0.1 ml of this supernatant, 2 ml of phosphate buffer (pH 8.4), 0.5 ml of 5'5-dithiobis (2-nitrobenzoic acid) and 0.4 ml of double distilled water was added. The mixture was vortexes and absorbance was read at 412 nm within 15 min. The concentration of 2-nitro-5-benzoic acid formation was measured and reduced glutathione is expressed as µmol/mg protein.

Nitrates and Nitrites (NOx) assay

NOx concentrations were determined by using the Griess reaction as described previously by Tracey et al.[21]. 6µl of sample/supernatant was mixed with 44µl of distilled water, 20µl of 310 mM phosphate buffer (pH 7.5) and 10µl each of 0.86 mM NADPH, 0.11 mM flavin adenine dinucleotide (FAD) and 10µl Nitrate reductase (1 U/ml) in individual wells of a 96-well plate. Plate was thereafter incubated for 1 h at room temperature in the dark. 200µl of Griess reagent [1:1 mixture of 1% sulfanilamide (1% solution with 5% orthophosphoric acid) and 0.1% N(1-naphthyl) ethylenediamine (NEDA) (1% solution with distilled water)] was added to each well and the plate was incubated for an additional 10 min at room temperature. Absorbance was measured at 540 nm using a microplate reader. Total protein was estimated by method of Lowry et al.[22]. Concentration of total nitrate and nitrite (NOx) in liver homogenates was calculated from the standard curve and expressed as nM/mg protein.

Cytokine and Immunoglobulin levels

Serum Interleukin (IL-4), Tumor Necrosis Factor (TNFα) and Interferon (IFNγ) were estimated as per the instruction of the Kit Manufacture's manual (Diaclone), serum Immunoglobulin E(IgE), Immunoglobulin G(IgG), Immunoglobulin M(IgM), Immunoglobulin A(IgA) and Interleukin (IL-13) were estimated as per the instruction of the Kit Manufacture's manual (QAYEE-BIO).

Delayed type hypersensitivity (DTH) reaction

DTH assay was carried out to assess the cell mediated immune response of CPS or CUS exposed rats. DTH reaction was estimated by the method as described by Institoris et al. with some minor challenge dose modification. Animals were immunized subcutaneously at the base of the tail by 1 mg KLH in 0.4 ml of antigen preparation (KLH was dissolved in equal volume of PBS and FCA to form antigen suspension for immunization) on day 0. After various treatments from day 0 to day 14, animals were challenged by injecting 100µg of KLH in 0.08 ml sterile PBS into the left hind paw and equal volume of PBS was administered in the right hind paw which served as control. Paw volume was measured at time 0 just before challenging and about 24 h after, using UGO basile plethysmometer (model no. 7140) and specific paw swelling (D%) was calculated as described by Siroki et al.[23].

$$D\% = \frac{(d24L - d0L) - (d24R - d0R)}{D0L} \times 100$$

D% = Specific paw swelling.
d24 = Paw volume 24 h after challenge (Time = 24 h).
d0 = Paw volume before challenge (Time = 0 h).
L and R = Left and right hind paw.

Statistical Analysis

The values were expressed as mean ± standard error of the mean. One-way analysis of variance (ANOVA) followed by appropriate post hoc test (Tukey test) were used for analysis. $P < 0.05$ was considered as statistically significant.

RESULTS

Effects of Dawa-Ul-Kurkum and its hydro-alcoholic extract on Liver Function test (LFT) in paracetamol induced liver damage in rats

In experimental control group, paracetamol (2g/kg, orally) given daily 14th days resulted in significant increase in serum levels of SGOT (P<0.05), SGPT (p<0.05), ALP (p>0.05), total bilirubin, direct bilirubin (p<0.05) and reduction in total protein as compared to control rats. This is suggestive of notable degree of hepatotoxicity and tissue injury in the rat liver and validated our model of paracetamol induced liver damage. In Group 4 and 5, treatment with Dawa-Ul-Kurkum at doses 250 and 500mg/kg respectively for 14 days significantly attenuated the effects of paracetamol and reduced level of serum SGOT (p < 0.05 at each dose), SGPT (p<0.05 at 500mg/kg dose), ALP (p<0.05), total bilirubin and direct bilirubin (p < 0.05 at each dose) and increased level of serum total protein (p < 0.05 at 250 mg/kg dose) as compared to that in Experimental control group (treated with paracetamol alone). Similarly, in Group 6 and 7 treatment with 50% hydro-alcoholic extract of Dawa-Ul-Kurkum (500 and 1000mg/kg) produced hepatoprotective effect as it reduced the levels of serum SGOT, SGPT and total bilirubin as compared to that in Experimental control. However, significant change was observed in the levels of ALP (p < 0.05 at 1000 mg/kg), direct bilirubin (p<0.05) and total protein (p < 0.05 at each dose). Pretreatment with silymarin also significant reduced the hepatotoxic effects of paracetamol and reduced the levels of serum SGOT (p<0.05), SGPT (p<0.005), ALP (p<0.05), Total bilirubin and Direct Bilirubin (p<0.05) as compared to that in Experimental control. The results of Dawa-Ul-Kurkum and its hydro-alcoholic extract are comparable to that of Silymarin. The results are shown in Table 1, 2 and Figure 1, 2.

Table 1: Effects of Dawa-Ul-Kurkum and its hydro-alcoholic extract on SGOT, SGPT and ALP in paracetamol induced hepatotoxicity in rats

Treatment	SGOT(IU/L)	SGPT (IU/L)	ALP(IU/L)
Control	105.0 ±18.68	42.45±5.298	94.05±7.677
Experimental control	205.4±6.076#	.25±14.64#	179.7±14.04##
Silymarin	115.4±8.642*	37.18±4.951**	98.71±11.38**
DK250	107.6±10.31**	48.94±7.274*	104.3±8.285*
DK500	117.4±14.58*	43.27±3.100*	99.40±11.45**
HA500	139.7±22.78	44.92±5.868*	104.4±10.25*
HA1000	132.6±18.89	55.07±14.26	106.7±10.14*

The values are expressed as mean ± SEM; DK-Dawa-Ul-kurkum; HA-Hydro-alcoholic extract of DK. All groups except control group were treated with paracetamol 2g/kg.# p<0.05 and ## p<0.01 when compared with control group; * p<0.05and** p<0.01 when compared with experimental control.

Table 2: Effects of Dawa-Ul-Kurkum and its hydro-alcoholic extract on total bilirubin, direct bilirubin and total protein in paracetamol induced liver damage in rats

Treatment	Total bilirubin (mg/dl)	Direct bilirubin (mg/dl)	Total protein (g/dl)
Control	1.353±0.4001	0.8600±0.1601	6.067±0.6903
Experimental control	3.523±0.4680	2.063±0.3417#	3.347±0.3446
Silymarin	1.594±0.3869	0.9380±0.1140*	6.316±0.6097*
DK250	1.624±0.5669	1.158±0.2878	6.116±0.5093
DK500	1.348±0.4029*	0.9680±0.1069*	5.476±0.4222
HA500	1.754±0.2762	1.274±0.2560	6.430±0.7564*
HA1000	1.308±0.3143*	1.072±0.1880	6.484±0.5817*

The values are expressed as mean ± SEM; DK-Dawa-Ul-kurkum; HA-Hydro-alcoholic extract of DK. All groups except control group were treated with paracetamol 2g/kg.# p<0.05 vs control group; * p<0.05 vs Experimental control.

Effects of Dawa-Ul-Kurkum and its hydro-alcoholic extract on body and liver weight in paracetamol induced liver damage in rats

The mean body weight was recorded in all groups at 0 and 15th day and liver weight was recorded on 15th day after various drug treatments. The results showed that dose of paracetamol (2g/kg) on 14th day caused significant reduction in the body weight (p < 0.01) but no significant change in the liver weight when compared to corresponding control rats. Interestingly, treatment with Dawa-Ul-Kurkum (250 and 500 mg/kg), 50% hydro-alcoholic extract of Dawa-Ul-Kurkum (500 and 1000mg/kg) and silymarin blocked the effects of paracetamol and resulted in significant increase in the body weight with no significant changes in the liver weight. The increase in body weight can be due to improvement in appetite which may have due to hepatoprotective effect of Dawa-Ul-Kurkum. The results are shown in Table 3.

Table 3: Effects of Dawa-Ul-Kurkum and its hydro-alcoholic extract on %change in body weight and liver index

Treatment	Initial body weight	Final body weight (g)	% change in body weight	Liver weight (g)	Liver index (%)
Control	168.3±43.26	186.0±51.79	9.516	6.600±1.501	3.54
Experimental contr	193.7±11.20	192.3±9.597	-0.728**	6.597±0.5401	3.43
Silymarin	210.6±8.17	212.8±5.295	1.033*	6.880±0.5161	3.23
DK 250	199.8±7.399	204.2±7.599	2.154*	6.320±0.3153	3.09
DK500	208.2±17.47	210.0±12.57	0.857*	6.220±0.3929	2.96
HA500	161.6±15.07	162.4±20.56	0.492*	5.700±0.8843	3.50
HA1000	187.0±13.57	188.2±17.18	0.637*	6.800±0.8025	3.61

Liver index was calculated as (liver weight/body weight×100%). **P<0.01 and *P<0.05 when compared with control group,

Effects of Dawa-Ul-Kurkum and its hydro-alcoholic extract on humoral immunity: Antibody Response and Immunoglobulin levels

In experimental control group, daily administration of paracetamol(2g/kg, p.o.) for 14 days resulted in significant increase in the levels of IgM, IgA, IgE, IL-13, Interferon-`γ TNF α, IL-4 (P<0.05 and P<0.01) and no significant change in IgG in serum as compared to that in control rats. This is suggestive of notable degree of humoral immunity: Antibody Response and Immunoglobulin levels, which could have contributed to tissue injury and hepatotoxicity in liver and validated our model of hepatotoxicity in rats. In Group 4 and 5, treatment with Dawa-Ul-Kurkum at doses 250 and 500mg/kg respectively for 14 days significantly attenuated the effects of paracetamol and reduced level of IgM, IgG IgA, IgE, IL-13, Interferon-`γ TNF α, and IL-4 significantly (P<0.05 and P<0.01) as compared to that in experimental control group (treated with paracetamol). Similarly, in Group 6 and 7 treatment with 50% hydro-alcoholic extract of Dawa-Ul-Kurkum (500 and 1000mg/kg) attenuated the effects of paracetamol and reduced the levels of IgM, IgG IgA, IgE, IL-13, Interferon-`γ TNF α, and IL-4(P<0.05 and P<0.01) significantly as compared to that in experimental control group. Pretreatment with silymarin also significantly reduced the levels of serumIgM, IgG IgA, IgE, IL-13, Interferon-`γ TNF α, and IL-4(P<0.05 and P<0.01) significantlyas compared to that in experimental controls which is suggestive of the hepatoprotective effects of the drug in this model. The results of Dawa-Ul-Kurkum and its hydro-alcoholic extract were comparable to that of Silymarin.These results are summarized in Table 4,5.

Table 4: Effects of Dawa-Ul-Kurkum and its hydro-alcoholic extract on immunoglobulin levels

Treatment	IgM (µg/ml)	IgG (µg/ml)	IgA (µg/ml)	IgE (µg/ml)
Control	0.0385 ± 0.0009	0.0101 ± 0.0006	0.0337 ± 0.0014	0.0634±0.0014
Experimental control	0.0496 ± 0.0006##	0.0147 ± 0.0006	0.0419± 0.0039##	0.0734±0.0005##
Silymarin	0.0412 ± 0.0005**	0.0103±0.0005*	0.0350± 0.0003**	0.0664 ±0.0015*
DK 250	0.0419 ± 0.0005*	0.0106 ± 0.0010	0.0354 ± 0.0003*	0.0688±0.00071
DK500	0.0407 ± 0.0008**	0.0102±0.0005*	0.0343± 0.0006**	0.0647±0.0015**
HA500	0.0417 ± 0.0005**	0.0107 ± 0.0008	0.0347± 0.0004**	0.0666±0.0016*
HA1000	0.0426 ± 0.0003*	0.0103± .0009*	0.0352± 0.0005**	0.0671±0.0008

##P<0.01 when compared with control group; **P<0.01 and *P<0.05 when compared with Experimental control group.

Table 5: Effects of Dawa-Ul-Kurkum and its hydro-alcoholic extract on Cytokine levels

Treatment	IL-13 (pg/ml)	Interferon-`γ (pg/ml)	TNF α (pg/ml)	IL-4 (pg/ml)
Control	0.0129 ± 0.0004	0.0219 ± 0.0005	0.0197 ± 0.0008	0.0720 ± 0.0049
Experimental control	0.0182 ± 0.0008##	0.0267 ± 0.0006##	0.0268 ± 0.0026#	0.1180 ± 0.0121#
Silymarin	0.0145 ± 0.0002**	0.0230 ± 0.0005**	0.0216 ± 0.0008	0.0792 ± 0.0034*
DK 250	0.0147 ± 0.0004**	0.0236 ± 0.0005**	0.0207 ± 0.0003*	0.0748 ± 0.0067*
DK500	0.0140 ± 0.0004**	0.0233 ± 0.0004**	0.0219 ± 0.0015	0.0816 ± 0.0080
HA500	0.0145 ± 0.0006**	0.0241 ± 0.0003*	0.0210 ± 0.0005*	0.0866 ± 0.0096
HA1000	0.0152 ± 0.0005*	0.0240 ± 0.0002*	0.0223 ± 0.0010	0.0778 ± 0.0078*

#=P<0.05 and ##P<0.01 when compared with control group; **P<0.01 and *P<0.05 vs Experimental control group.

Effects of Dawa-Ul-Kurkum and its hydro-alcoholic extract on Delayed type hypesensitivity response in paracetamol induced liver damage in rats

In experimental control group, daily administration of paracetamol (2g/kg, p.o.) for 14 days resulted in decrease in delayed type hypersensitivity response as compared to

that in control rats. In Group 4 and 5, treatment with Dawa-Ul-Kurkum at doses 250 and 500mg/kg respectively for 14 days significantly attenuated the effects of paracetamol and increased the delayed type hypersensitivity response as compared to that in experimental control group. Similarly, in Group 6 and 7 treatment with 50% hydro-alcoholic extract of Dawa-Ul-Kurkum (500 and 1000mg/kg) attenuated the effects of paracetamol and increased the delayed type hypersensitivity response as compared to that in experimental control group. Pretreatment with silymarin also increase the delayed type hypersensitivity response as compared to that in experimental controls which is suggestive of the hepatoprotective effects of the drug in this model. These results are summarized in Table 6.

Table 6: Effects of Dawa-Ul-Kurkum and its hydro-alcoholic extract on Delayed type hpersensitivity (DTH) response

Treatment	DTH% (Siroki et al)
Control	40.17 ± 4.28
Experimental control	15.18 ± 1.88#
Silymarin	21.37±7.87
DK250	18.75 ±4.01
DK500	22.21± 4.65*
HA500	34.50± 5.67**
HA1000	35.00 ± 14.43

#P<0.05 when compared with control group; **P<0.01 and *P<0.05 when compared with Experimental controls.

Effects of Dawa-Ul-Kurkum and its hydro-alcoholic extract on oxidative stress parameters in paracetamol induced liver 0damage in rats

In experimental control group, paracetamol (2g/kg, orally) given daily 14 days resulted in increase in stable metabolites of nitric oxide (NOx) and MDA (P<0.05) in supernatant of liver homogenates and significant reduction in GSH as compared to control rats. This is suggestive of notable degree of hepatotoxicity and tissue injury in the rat liver and corroborated to validate this model of hepatotoxicity. In Group 4 and 5, treatment with Dawa-Ul-Kurkum at doses 250 and 500mg/kg respectively for 14 days significantly attenuated the effects of paracetamol and reduced level of homogenate supernatant NOx ($p < 0.05$ at 500 mg/kg doses), MDA ($p < 0.05$ at 500 mg/kg doses) and significantly increased GSH ($p < 0.05$ at each dose) as compared to that in Experimental control group (treated with paracetamol). Similarly, in Group 6 and 7 treatment with 50% hydro-alcoholic extract of Dawa-Ul-Kurkum (500 and 1000mg/kg) produced hepatoprotective effect as it significantly reduced the levels of NOx in homogenate supernatant ($p < 0.05$ at dose 1000 mg/kg), MDA ($p < 0.05$ at dose 1000mg/kg) and non-significant increased GSH, as compared to that in Experimental control group. Pretreatment with silymarin also significantly reduced the hepatotoxic effects of paracetamol and reduced the levels of NOx ($p<0.05$), MDA ($p > 0.05$) and increased GSH ($p<0.05$) as compared to that in Experimental control group. The results of Dawa-Ul-Kurkum and its hydro-alcoholic extract are comparable to that of Silymarin[24]. The results are shown in Table 7.

Table 7: Effects of Dawa-Ul-Kurkum and its hydro-alcoholic extract on oxidative stress parameters

Treatment	NOx (nmol/mg) protein	MDA (nmol/mg)protein	GSH (µmol/mg)protein
Control	0.1943± 0.0325	0.2453± 0.03635	2.524± 0.07029
Experimental control	0.3163± 0.02483	0.4097± 0.02123#	1.445± 0.3211
Silymarin	0.2040±0.02492*	0.2612± 0.02098*	2.779± 0.1371*
DK250	0.2138± 0.02744	0.2760± 0.01447	2.666± 0.2198*
DK500	0.2036± 0.01371*	0.2544± 0.03728*	2.826± 0.3648*
HA 500	0.2096± 0.02048	0.2820± 0.02684	2.393±0.1548
HA 1000	0.1948± 0.01259*	0.2472± 0.03069*	2.243± 0.1612

($p<0.05$) vs control group; * ($p<0.05$) vs Experimental control. The data were analyzed using one way ANOVA followed by Tukey test. All groups except control group were treated with paracetamol 2g/kg.

DISCUSSION

Paracetamol is a non-steroidal anti-inflammatory drug and well known safest antipyretic drug. It is easily available when it's used in safest dose, when used in over dosage leads to hepatic damage[25-27].The pharmacological action of paracetamol on the liver is by covalent binding of its toxic metabolite(n-acetyl-p-benzoquinone-amine) to the sulfhydryl group of protein producing in cell necrosis and lipid peroxidation[28]. Results showed that paracetamol

induced increase in organs weight, change in animal growth and liver function, which is due to obstruction of secretion of hepatic triglyceride into plasma[29]. However, treatment with Dawa-Ul-kurkum, restored the altered organs weight and its percentage, i.e. liver index in paracetamol group.

During hepatic injury the function of hepatocytes gets blocked and result in the leakage of the plasma membrane due to over dosage of paracetamol[30],causingrisein serum hepatic enzyme levels. Liver enzymes such as SGOT, SGPT, AST, direct bilirubin, total bilirubin and total protein have still remained the gold standards for the assessment of liver injury[31]. Liver injury is always analogous with cellular necrosis which results to raise in tissue lipid per-oxidation and depletion in antioxidant reduced glutathione (GSH) level. The results shown that oxidant and antioxidant ratio are interrupted due to over dosage of paracetamol which causes hepatic injury and increase excess free radical generation. Numerous studies have been reported that paracetamol causes oxidative stress and alteration in endogenous antioxidant enzyme activities in rat[32]. Reduced glutathione (GSH) is a vital endogenous antioxidant which counterbalances free radical mediated damage. Reduced cellular GSH levels and capacity for GSH synthesis sensitize cells to radiation and to certain drugs[33].

Changes in serum immunoglobulin (Ig) levels arise routinely in hepatic disease and are frequently used as indication for different types of hepatic injury. Early work concludes that rise in serum concentrations of IgA, IgM and IgG was feature of cirrhosis, primary biliary cirrhosis, and chronic active hepatitis[34]. In hepatic disease, immunoglobulin outline have been used to evaluate the severity of injury[35-37],althoughthere is a lack of diagnostic specificity of any immunoglobulin composition in hepatic disease[38]. Despite this, immunoglobulin levels continue to be recommended as valuable indication in the diagnosis and prevention of diseases of the liver[39-43].

Another study marked that serum IgE levels are also hugely increased in so many patients with a variety of acute or chronic hepatic disorders. It was suggested that that the serum of patients with hepatic disease might curb a blocking factor which could produce a false rise in IgE levels. Such inhibitors have been reported in immunodeficient patients[44], and in patients with cancer[45].

The mechanism of elevated serum immunoglobulins in hepatic disease has been detailed investigated but remains mysterious. Theoretically, raised immunoglobulins may arise from increased immunoglobulin synthesis or reduced immunoglobulin catabolism. Elevated serum immunoglobulin in patients with hepatic disease might result from a depletion of the suppressor T-cell population[46].

The mechanism of drug induced liver injury (DILI) has been explained on the basis of cell stress, mitochondrial damage, and specific immune response. The liver as the organ that serves to detoxifycell experience prolonged stress. Stress on the cell can trigger an increase in inflammatory cytokines. As a result, the liver cells become more susceptible to apoptotic effects of TNF-α and IFN-γ. These effects can be inhibited by inhibitors of apoptosis proteins (IAPs) or Bcl-2[47]. IL-4 is one cytokine that has been linked to the development of immune-mediated DILI as well as its associated antibodies. An earlier study has clearly associated variant IL-4 alleles with the development of immune-mediated DILI fromdiclofenac[48].

The present results showed that concurrent administration of Dawa-Ul-Kurkum along with paracetamol significantly prevented the rise in the level of serum SGOT, SGPT, ALP, total bilirubin, direct bilirubin and no significant change in total protein, 50% Hydro-alcoholic extract of Dawa-Ul-Kurkum along with paracetamol significantly prevented SGPT, ALP, total bilirubin and increase in total protein but no significant change in SGOT and direct bilirubin. There is also significant change in percentage of body weight with both the doses.Also, significant changeswere found with both the doses of Dawa-Ul-Kurkum and 50% Hydro-alcoholic extractin immunoglobulin levels, cytokine levels anddelayed type hypersensitivity response. Further, measurement of oxidative stress parameters in liver homogenates showed protective effects of Unani polyherbal prepararion Dawa-Ul-Kurkum against raised levels of reactive oxygen and nitrogen species in response to paracetamol as seen by significantly lowered levels of MDA and NOx and elevated GSH levels. The effects with the DK were more consistent as compared to the HA extract on oxidative stress parameter.These results showed that both Dawa-Ul-Kurkum and the HA preparation modulate immune mechanisms during their hepatoprotective effects against the paracetamol induced liver damage. The protective effects may be mediated through maintenance/restoration of the oxidant-antioxidant homeostatic balance.

CONCLUSION

The present study demonstrated that paracetamol is potentially hepatotoxic to Wistar rats, when given for 14 days as proven by changes in markers of liver functions, immune functions, cytokine levels, oxidative stress and histopathological studies. Both DK and its 50% hydro-alcoholic extract were found to modulate immune functions during their protective effects against paracetamol induced liver damage in rats. Such translational studies using the reverse pharmacology approach could help in the integration of traditional and modern medicinal concepts in the greater interest of drug development and rational therapy.

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