Research Article

Protective Effects of *Picrorhizakurroa* Against Fumonisin B1 Induced Hepatotoxicity in Mice

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ABSTRACT

Objectives: The aim of the present study is to evaluate hepatoprotective effect of *Picrorhizakurroaextracts* (PKE) against Fumonisin b1 (Fb1) induced hepatotoxicity.

Methods: Hepatoprotective analysis was done by estimating serum biochemical parameters Alanine aminotransferase (ALT) and aspartate aminotransferase (AST), antioxidant enzyme status lipid peroxidations, ROS generation, and protein carbonyls were also estimated to know the oxidative damage. Further protein expressions were confirmed by western blotting.

Results: The mice treated with 100 and 200 mg/kg b.wt PKE, showed a significant reduction in serum AST and ALT to 175.41 ± 7.82, 68.52±1.57 and 126.64 ± 1.72, 57.41±1.28 units/ml of serum respectively. The decrease in antioxidant levels with Fb1 was significantly restored with 100 and 200 mg/kg by wt PKE. The lipid peroxidation products with Fb1 treatment was also reduced with PKE supplementation. The Fb1 induced ROS was reduced with 60 and 140% by 100 and 200 mg/kg/ by wt PKE administration. The PKE restored CAT and GPx enzyme/protein levels and further down regulated caspase 8 and caspase 3 expression.

Conclusion: The present observations suggested that the treatment with PKE extract enhance the recovery from Fb1 induced hepatic damage due to its antioxidant and hepatoprotective property.

Key Words: Fumonisin b1, Picrorhizakurroa, Antioxidants, Oxidative stress, Apoptosis

INTRODUCTION

Fumonisins are group of mycotoxins mainly produced from *Fusariumverticillioides, Fusariummoniliforme* is a common contaminants of corn and corn products throughout the world. The most potent and abundant of the class of fumonisins is fumonisin b1 (Fb1) which causes specific toxicological effects in humans and animals [1,2]. Fumonisin b1 causes equine leukoencephalomalacia in horse and porcine pulmonary edema in swine [3,4],and it has been reported as hepatotoxic, nephrotoxic, and carcinogenic in animals and human [5]. *In vivo* studies demonstrated that fumonisin b1 also induced apoptosis and oxidative stress in kidney and liver [6,7].

Fb1 are known to affect sphingolipid metabolism by inhibiting the enzyme ceramide synthase. Inhibition of ceramide synthase causes apoptosis, accumulation of sphingoid bases (sphinganine and sphingosine) and decreases the ceramide [8,9]. Fb1 has been shown to disrupt lipid biosynthesis *in vitro* and *in vivo* in rat liver with changes occurring in the major cellular membrane phospholipids fractions and their fatty acid (FA) content by inducing the oxidative radicals such as superoxide anion and nitric oxide which are responsible for oxidative stress in the tissue [10]. Hence Fb1 is more toxic and abundant fumonisins causes liver damage in many animals [11].

*Picrorhizakurroa*Royle ex Benth (*P.kurroa*) commonly called kutki a well-known Ayurvedic herb. Generally it has been used to treat liver toxicity and upper respiratory tract, and also to treat dyspepsia, scorpion sting and chronic diarrhea [12,13,14]. The *P.kurroa* contains chemical constituents like Apocynin, picrorhizoside, picroside-I, cinnamic acid, verminosidepicrogentioside, piscrocin, cucurbitacin, pikuroside, vanillic acid, picrosecoside –I [12,15,16].

P. kurroa has many medicinal benefits such as anti-allergic, anti-neoplastic immunomodulatory and anti-anaphylactic activities [12,17,18] The *P.kurroa* has been recently reported to inhibit arthritis through inhibition of pro-inflammatory cytokines and angiogenesis and which have protective effects against 2-acetylaminofluorene-induced hepatotoxicity in Wistar rats and non alcoholic fatty liver disease [19,20,21]. The active component Picroliv against Leishmaniadonovani infections in Mesocricetusauratus and hepatoprotective compound in mice [22,23,24]. The flavonoid apocynin is one of the active component of *P. kurroa* and has been noted to attenuate Parkinson's, ischemia-reperfusion and hypoxia by arresting NADH oxidase expression during oxidative stress. [12,25,26,27].Hence the present study is to investigate the *P. kurroa* protective effects against Fb1 induced hepatotoxicity.

MATERIALS AND METHODS

Chemicals and reagents
Fumonisin b1, 2, 7-dichloro-dihydrofluorescein diacetate (Sigma Bangalore), 2-nitrobenzoicacid (DTNB) Hi-Media (Bangalore, India). The other chemicals used were of high purity grade and were procured from Merck (Bangalore, India).

Plant material
P. kurroa root material was purchased from the local market and identified by Dr. K. Madhava Chetty, Botanist, Department of Botany, Sri Venkateswara University, Tirupati, India. A voucher specimen (Herbarium Accession Number 801) was deposited in the herbarium, Department of Botany, S.V. University, Tirupati, India.

Preparation of 70 % ethanolic fraction of *P.kurroa* (PKE)
P. kurroa roots were washed thoroughly, shade dried and finely powdered. The 100g root powder was macerated with 70% ethanol in a shaker for 2 days. The extract was filtered using micropore membrane and concentrated using flash evaporator followed by lyophilization to remove the residual water. The yield of the extract was recorded as 25g.

Experimental design
Forty eight male Balb/c mice weighing 25g were selected from the stock colony, Defence Food Research Laboratory, Mysore, India. The animals were housed in acrylic fibre cages, temperature (25 ± 2^0C) and maintained in 12 h light/dark cycle. The mice were fed with a commercial pellet diet (Sri Venkateswara Enterprises, Bangalore, India) and water was provided *ad libitum*. The animal studies were conducted according to the guidelines from institute animal ethical committee for the purpose of the control and supervision of

experiments on animals NO: 28/IAEC/CPCSEA. (I) Control group (II) PKE 200 mg/kg b/wt (body weight) (III) Fb1 2.25mg/kg b/wt (IV) Fb1 2.25mg/kg b/wt + PKE 50mg/kg b/wt (V) Fb1 2.25mg/kg b/wt + PKE 100mg/kg b/wt (VI) Fb1 2.25mg/kg b/wt + PKE 200mg/kg b/wt. PKE was pretreated for 7 days oral administration. Fb1 2.25mg/kg b/wt was injected subcutaneously for 3 days. Controls mice were fed orally with an equal amount of saline. After the experiment mice were sacrificed, serum and liver tissues were collected for analysis. Bhandari et al., 2001 [28].

Serum biochemistry

Estimation of ALT and AST
The serum biochemical markers such as ALT and AST were determined according to the kit suppliers protocols (Cat no. 11409003 and 11408002 Canada).

Tissue biochemical parameters

Estimation of hepatic antioxidant enzymes
Liver tissues were homogenized in 50mM phosphate buffer saline (pH 7.4). The hepatic antioxidant enzymes such as SOD, GPx and GR were determined according to the protocols of kit supplier (Randox, Cat no. SD. 125, RS 504 and GR 2368, Canada). DTNB method was used to determine Glutathione content [29]. CAT enzyme content was estimated manually by spectrophotometric method [30]and the results were expressed in U/g tissue. Protein contents were determined by Bradford method (1976) [31] and the results were expressed as U/mg of protein.

Estimation of hepatic lipid peroxidation
Hepatic lipid peroxidation was determined by measuring the malondialdehyde according to Buege and Aust (1978) [32]. Liver tissues were homogenized in 2mL of phosphate buffer (pH 7.0). TCA (10%), 0.5mL and 2mL of TBA mixture were added to tissue homogenate (0.5mL). The TBA mixture contained TBA (0.35%), SDS (0.2%), $FeCl_3$ (0.05mM) and BHT in glycine-HCl buffer (100mM, pH 3.6). The above reaction mixture was boiled at 100^0C for 30 min. The mixture was centrifuged at 8000 rpm (Revolutions per minute) for 10min and the absorbance was measured at 532 nm and the results were expressed as moles/mg of protein.

Estimation of ROS
Fluorescent probe 2', 7'- dichloro-dihydrofluoresceindiacetate was used to detect ROS generation in liver tissue by Wang and Joseph (1999) [33] method. To the liver fractions 40 μl of 1.25 mM 2', 7'- dichlorfluorescein-diacetate and 142 μl methanol was added and the samples were incubated for 15 min at 37^0C. The fluorescence was measured at 485 nm excitation and 525 nm emission using Hidex plate chameleon TM 144 V (Finland).

Western blot analysis

The oxidative and apoptosis marker expression was analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting. The liver sample was homogenized with lysis buffer, pH 7.4 with protease inhibitor cocktail and total protein levels were estimated by the method of Bradford (1976) [31] The liver homogenate 50µg of proteins were separated on SDS-PAGE and transferred onto a nitrocellulose membrane using an electro blotting apparatus (Cleaver Scientific Ltd, UK). After transfer, the membranes were probed with primary antibodies against GAPDH (sc-5286) GPx (sc-22146), CAT (sc-34280), caspase 8 (sc-81657), caspase 3(sc-52746),(Santa Cruz Biotechnology, Santa Cruz, CA, USA, sigma) at 1:1,000 dilution and incubated at room temperature for 3 h. The membranes were then washed in TBST followed by incubation for 2 h at room temperature in dark with horseradish peroxidase (HRP) conjugated rabbit anti-goat, goat anti-mouse and goat anti-rabbit secondary antibodies (DAKO, Denmark) at 1:10,000 dilutions. The membranes were washed again and developed using a chemiluminescence detection system (ProteoQwest1, Sigma). Developed membranes were exposed to X-ray film and the developed band intensity was captured. NIH image J software was used to measure the western blot band intensity.

Statistical analysis

The results were analyzed by one-way ANOVA followed by a Tukey's HSD- post hoc test. Significance was set at 0.05 and all comparisons were made against the control group.

RESULTS

P.kurroa regulates ALT and AST

The hepatic function marker namely alanine aminotransferase (ALT), aspartate aminotransferase (AST) were estimated to diagnose the liver health. These enzymes were elevated in toxic or stress conditions due to the extracellular release particularly in liver. In the present study observed an increase in ALT and AST (p < 0.05) (Table. 1). However *P. kurroa* supplemented mice showed a dose dependent decrease in ALT and AST contents (p < 0.05) which indicates its protective effects against liver damage.

Protective effect of P. kurroa against oxidative stress

Antioxidant enzymes namely CAT, GPx, GR and GSH gets decreased when the liver undergo toxic condition induced by Fb1. In the present study observed decrease in antioxidant enzyme content in Fb1 challenged mice (*p*< 0.05). However a significant increase (*p*< 0.05) in antioxidant enzymes was observed with 100 and 200 mg/kg by wt *P. kurroa* supple-

mentation. Whereas SOD was not affected by Fb1 treatment. (Table 2).

P. kurroa inhibits lipid peroxidation

Oxidative degradation of lipids caused by free radicals was measured by estimating malondialdehye content. The Fb1 treated liver showed the increase of malondialdehyde formation (*P*< 0.05), whereas *P. kurroa* supplemented mice decreased lipid peroxidation with 100 and 200mg/kg by wt PKE treatment (*P*< 0.05) which suggests that *P. kurroa* exerts protective effects against free radicals mediated lipid damage (Table 2).

P. kurroa inhibits ROS generation

The reactive oxygen species such as hydroxyl radical (HO$^•$), superoxide anion ($^•O_2^-$), and hydroxyl anion (HO$^-$) trigger the cellular damage by oxidizing the biomolecules therefore estimation of ROS is essential to study the oxidative stress. The ROS estimation was carried out using fluorescent probe DCFH$_{2DA}$. Here we observed 264% increases (p < 0.05) in fluorescence in Fb1 treated liver homogenates. The pretreatment of PKE decreased 92 to 165% (p < 0.05) of ROS generation respectively (Fig. 1(A)).

Protective effects of *P. kurroa* on oxidative stress and apoptotic biomarker

The effect of *P. kurroa* against Fb1 stress was further evaluated by western blotting. The key enzymes of oxidative stress such as GPx, CAT and GAPDH and apoptotic enzymes caspase 3 and caspase 8 was evaluated (Fig. 2). The antioxidant biomarkers GPx and CAT were down-regulated with Fb1 treatment and caspase 8, caspase 3 were increased in the expression. Whereas *P.kurroa* pretreatment significantly restored the GPx, CAT expression and down regulated the apoptotic enzymes caspase3, caspase 8 protein expression.

DISCUSSION

Fumonisin b1 are secondary metabolites of fungi which are associated with many disorders in animals and humans and also responsible for apoptosis and oxidative stress. Protective effect of herbal extracts against fumonisin b1 induced toxicity has fewer reports. El-Adawi et al. (2011) [34] studied protective effects of milk thistle and grape seed extracts on fumonisin B1 induced hepato- and nephro-toxicity in rats and Sozmen et al. (2014) [35] reported protective effects of sylimarin against fumonisin B1-induced hepatotoxicity in mice.*P. kurroa* is a well-known ayurvedic herb and shown hepatoprotective activity in diverse models of liver toxicity. Hence the present study investigated the hepatoprotective effects induced by fumonisin b1 induced toxicity.

AST and ALT are enzymes that catalyze the transfer of α-amino groups from aspartate and alanine to the α-keto group of ketoglutaric acid to generate oxalacetic and pyruvic acids respectively when the liver is in toxic or stress condition, therefore serum concentrations of aminotransferases level increase. Several reports show that plants and phytochemicals attenuate the aminotransferases level [36]. The pretreatment of animals with the extracts *P.kurroa* resulted to a significant and dose dependent decrease in the levels of ALT and AST.

Enzymes such as superoxide dismutases (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione (GSH) have been proposed as biomarkers of oxidative stress and toxic effect of tissue. CAT is well known antioxidant enzyme which detoxifies the hydrogen peroxide without electron donor and GPx also metabolize H_2O_2 to non-toxic products [37]. The GSH plays a fundamental role in cellular metabolism which provides the defence against reactive oxygen species and other toxic radicals and the GR is responsible for generation of GSH. In the present study we observed a decrease concentration of these antioxidant enzymes and the pretreatment of *P. kurroa* showed a recovery in the activity of these enzymes several reports also demonstrated that supplementation of herbal extracts can increases that antioxidant level induced by other toxic radicals.

The lipid peroxidation involves the mechanism formation or propagation of lipid radicals by up taking the oxygen and rearrangement of the double bonds in unsaturated lipids and the eventual destruction of membrane lipids and final products breaks downs including ketones, alkanes, aldehydes and ethers [38]. This lipid peroxidation was measured by malondialdehyde in the present study the malondialdehyde level was increased in the Fb1 treated group and it was minimized by *P. kurroa* pretreated group.

Western blot is used to separate and identify proteins separated based on molecular weight. The house keeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) used as reference proteins to normalize the target protein during the protein targeting. Therefore, to accurately compare western blotting signals, one must compensate for these non-sample-related variations in signal intensity so GAPDH increasing expression shows that the house keeping proteins are subject to change in many biological conditions, such as toxic condition and some diseases [39].

The antioxidant marker proteins CAT and GPx already demonstrated that in biochemical parameters the enzyme activity decreased in oxidative stress condition. In the western blotting also the expression of GPx and CAT down regulated with Fb1 whereas pretreatment of *P. kurroa* significantly restored the enzyme. Increasing the antioxidant able to accelerate the removal of the reactive oxygen species so which may

the reason for increasing the GPx, CAT in the present study.

Fb1 treatment activates enzyme Caspase-8, Caspase-8 is signalling molecule involved in TNF tumor necrosis factor-alpha apoptotic pathway plays a pivotal role in Fb1-induced apoptosis [26]. In the present study we observed up regulation of Caspase-8 and Caspase-3 whereas *P. kurroa* pretreated with 200mg down regulates the expression of apoptotic enzymes. *P. kurroa* seems to be a promising herb to protect Fb1 induced hepatotoxicity. The extract was found to prevent lipid oxidation and also to increase the activity of the antioxidant enzymes apart from down regulating the caspases. Hence with the protein expression studies *P. kurroa* showed the protective effect against Fb1 induced hepatotoxicity.

CONCLUSION

Fb1 associated with hepatotoxic effect and induction of oxidative stress. Modulating roles against ROS mediated damage for cell survival by nutrition supplement is therefore of interest. Present study evaluated the modulating properties of *P. kurroa* against oxidative stress and apoptosis mediated by Fb1. There inhibitory effects might be due to enriched active ingredients present in *P. kurroa*. Further molecular and analytical studies are necessary to understand the concealed mechanism of action of the *P. kurroa* extract and also for the usage as therapeutic agent.

ACKNOWLEDGEMENT

Abbreviations
Fb1- Fumonisin b1
PKE - *Picrorhizakurroa* extracts
ALT - Alanine aminotransferase
AST - Aspartate aminotransferase
ROS – Reactive oxygen species
DTNB - 2-nitrobenzoicacid
CAT - Catalase
GPx - Glutathione peroxidase
GR - Glutathione reductase
SOD - Superoxide dismutases
GSH - Glutathione
TCA - Trichloroacetic acid

TBA - Thiobarbituric acid
MDA - Malondialdehyde

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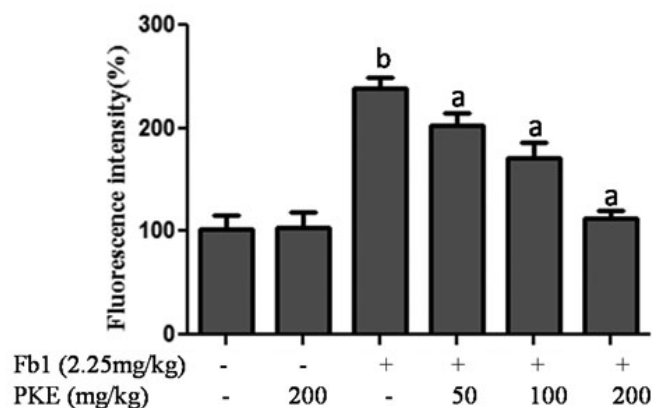
Figure 1: Effect of PKE on hepatic ROS generation. Mice were treated with different doses of PKE followed by Fb1 and the liver ROS generation was measured using spectrofluorimeter (n= 8, P < 0.05, significantly different from the control group and homogenous sub groups share common letter (a= *P*< 0.05 versus Fb1 treated group and b= *P*< 0.05 versus control group)).
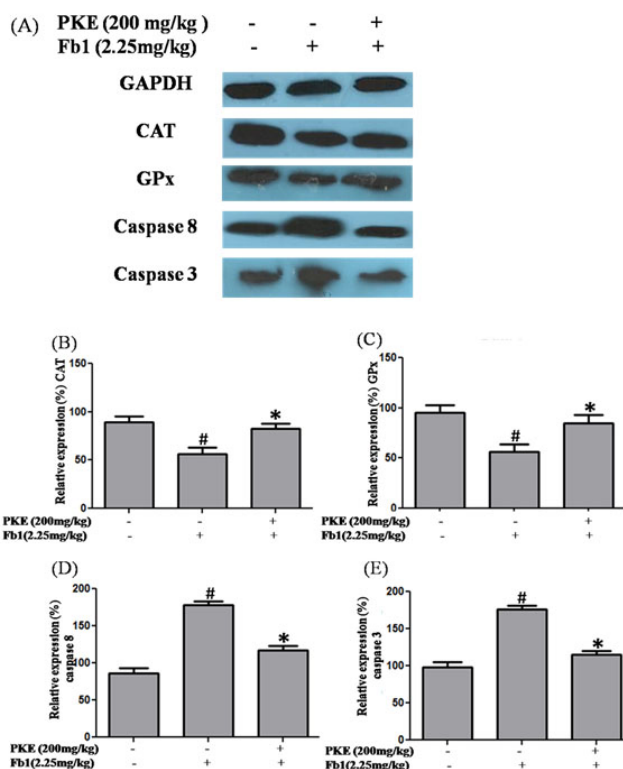

Figure 2: The protective effect of pre-treatment of PKE on Fb1 induced expression of oxidative stress marker proteins CAT, GPx and apoptosis marker proteins caspases-8, caspase-3 analysed by western blotting (B,C,D,E). The band intensity is calculated by Image-J software. The data are represented as mean ± SD of three independent experiments. #*P*<0.05 versus control group, *P*<0.05 versus Fb1 treated group.

Table 1: Effect of PKE on serum AST, ALT level. Mice were treated with different doses of PKE followed by Fb1 and the serum content of AST, ALT was measured (n = 8, P < 0.05, significantly different from the control group and homogenous sub groups share common letter (a= *P*< 0.05 versus Fb1 treated group and b= *P*< 0.05 versus control group)).

Groups (mg/kg bwt)	AST (IU/L)	ALT (IU/L)
Control	65.32 ± 4.25	53.85 ± 2.67
PKE 200 mg	67.21±2.01	55.96±1.52
Fb1 2.25 mg	175.41 ± 7.82[b]	126.64 ± 1.72[b]
Fb1 2.25mg + PKE50mg	145.79 ±8.53[a]	102.29 ± 1.77[a]
Fb1 2.25 mg+ PKE100mg	106.32±6.11[a]	96.93±2.38[a]
Fb1 2.25 mg+ PKE200mg	68.52±1.57[a]	57.41±1.28[a]

Table 2: Effect of PKE on hepatic antioxidant markers such as GPx, GR, GSH and lipid peroxidation. Mice were treated with different doses of PKE followed by Fb1 and the liver GPx GR, GSH and lipid peroxidation were measured (n = 8, P < 0.05, significantly different from the control group and homogenous sub groups share common letter. (a= *P*< 0.05 versus Fb1 treated group and b= *P*< 0.05 versus control group)).

Groups (mg/kg bwt)	SOD (U/mg protein)	CAT (U/mg protein)	GPx (U/mg protein	GR (U/mg protein)	GSH (µM/mg protein)	MDA (M/mg protein)
Control	1.75±0.62	23.51±1.22	25.55±1.32	32.16±1.33	10.36±0.51	30.12±1.2
PKE 200 mg	1.70± 1.01	21.21±2.01	23.07±1.93	31.86±1.87	8.36±0.64	29.43±0.64
Fb1 2.25mg	1.68±0.23[b]	10.23±0.5[b]	15.17±0.8[b]	18.11±1.11[b]	4.15±0.16[b]	50.77±2.4[b]
Fb1 2.25mg + PKE50mg	1.59± 0.55	16.22±0.71[a]	18.11±1.2[a]	24.44±1.31[a]	6.91±0.31[a]	32.81±1.7[a]
Fb1 2.25mg+ PKE100mg	1.60 ±0.41	19.12±1.2[a]	20.91±1.3[a]	28.51±1.42[a]	7.2±0.41[a]	34.91±1.8[a]
Fb1 2.25mg+ PKE200mg	1.63 ±1.26	22.87±0.84[a]	23.04±0.98[a]	32.13±0.54[a]	9.67±0.42[a]	29.68±1.27[a]