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Anti-hepatotoxic Potential of *Indigofera tinctoria* and its isolated Isothiocyanate compound 'ITC-1' against NPYR-CCl_4 Intoxicated Mice

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Abstract

Isothiocyanate derivative from hydroethanolic extract of *Indigofera tinctoria* (HEIT) was previously isolated, purified and characterized as 1-[1,2-Diisothiocyanato-2-(3-isothiocyanato-2,2-dimethyl-propylsulfanyl)-ethoxy]-3-isothiocyanato-2,2-dimethyl-propane ($C_{16}H_{22}N_4OS_5$; m/z 446.70; ITC-1). To elucidate hepatoprotective efficacy, liver toxicity was induced in mice via intoxication of N-Nitrosopyrrolidine (NPYR) followed by CCl_4 for 50 days. Both low and high doses of crude HEIT were given orally to NPYR treated mice for 21 days. Silymarin was also administered to compare the results. After completion of post treatment, various hepatic toxicity markers were evaluated. Results showed that both doses of HEIT and ITC-1 have successfully normalized the levels of AST, ALT (*P<0.001* vs. NPYR treated group), ALP and bilirubin (*P<0.01*). ITC-1 has showed better remedial response against liver toxicity in comparison to Silymarin. Thus, we concluded that both I. tinctoria and 'ITC-1' have future remedial aspect in diminution of hepatic toxicity.

Keywords: AST, Bilirubin, Hepatic Toxicity, *Indigofera tinctoria*, Isothiocyanate Compounds, Silymarin

1. Introduction

Liver, an important organ involved in elimination of toxic compounds from body[1]. Several drugs, carcinogens etc. devastate its normal process that leads the accumulation of toxic substances in body. Srivastava and Shivanandappa[2] reported that chronic hepatic diseases have significant capability to convert into more severe liver problems like cirrhosis, fibrosis, and hepatocellular carcinoma. N-Nitrosopyrrolidine (NPYR), a well-known hepatocarcinogen is chemically formed in various nitrate containing foods, waste materials of rubber industry, drinking water, cured meats etc[3]. Its metabolism lead to the formation of several hepatic DNA adducts that cause mutations[4]. Since, numbers of beneficial hepatoprotective drugs are available in the market but still hepatologists believe to develop more potent herbal drug that would be cheaper and target specific with no side effect landmark.

Indigofera tinctoria (Family:Fabaceae), is distributed throughout South East Africa, tropical Africa as well as tropical America and India[5]. This plant has been reported to possess a wide range of therapeutic properties[6,7]. Phytoconstituents like flavonoids, isoflavones, organosulfur compounds esp. Isothiocyanates (ITC), terpenoids; sterols etc. are known to possess potent therapeutic properties. Isothiocyanate compounds (ITCs) possess –N=C=S- group and are week electrophiles. Some naturally occurring ITCs like PEITC (Phenethyl isothiocyanate), BITC (Benzyl isothiocyante) have been isolated from various vegetables like cabbage and are known to possess anticancerous activity[8].An isothiocyanate compound was

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already isolated and purified in our lab from hydroethanolic extract of *I. tinctoria* and the structure was elucidated by various spectral techniques as 1-[1,2-Diisothiocyanato-2-(3-isothiocyanato-2,2-dimethyl-propylsulfanyl)-ethoxy]-3-isothiocyanato-2,2-dimethyl-propane.

Thus, keeping all the criterion in mind, the present study was designed to elaborate the antihepatotoxic role of an isolated isothiocyanate compound and hydroethanolic extract of *I. tinctoria* against NPYR-CCl_4 intoxicated mice.

2. Material and Methods

2.1 Experimental Plant: Authentication and Procurement

Shaded dried *Indigofera tinctoria* plant sample was procured from S.M. Heena Industries, Jaipur, Rajasthan, India. It was collected by local farmers of Tindivanam, Villupuram, Tamil Nadu. The voucher specimen was authenticated and submitted at Herbarium division of 'Department of Bioscience and Biotechnology', Banasthali University with the authentication number 'BURI-13515'.

2.2 Plant Extraction

Dried plant material was minced, sieved and extracted with Soxhlet using 80% ethanol after defatted with petroleum ether. This extract was filtered and dried under reduced pressure in a rotary evaporator (HeidolphInkarp Instruments Pvt. Ltd., Germany) to afford 25.825 g/100g plant powder. This residue was designated as hydroethanolic extract of *I. tinctoria* (HEIT).

2.3 Isolation, Purification and Characterization of Bioactive Compound from *I. tinctoria*

Positive phytochemical analysis of HEIT for organosulfur compounds[9], followed the elution of bioactive fraction through column chromatography by gradient elution of *n*-hexane, chloroform and methanol solvents. Positive active fraction was eluted with solvent system 'chloroform: methanol' (90:10) with yield of 192 mg/5 g of HEIT and was green in color. HPLC analysis of this fraction showed the precence of various compounds which was further separated by HPLC followed the conformation of single sulphur containg compound (ITC-1). HPLC (Dionex-Ultimate 3000 HPLC) was configured as quaternary gradient pump, variable wavelength UV detector (Ultimate 3000 RS), Genetix G Chrom Chromatography, Ultra P silica RP C18 HS, Standard column, size 5 µm, 250 mm × 4.6 mm. Gradient elution system was (95% water at 0 min; 80% at 3 min; 50% at 10 min; 50% at 20 min; 80% at 25 min; 95% at 30 min; 95% at 35 min) with Water:Acetonitrile (ACN). Isolated compound was then lyophilized (Henil Science Industrial Pvt. Ltd, Korea; Model: Clean Vac 8 Vaccum Freeze dryer) to get the pure compound with the yield of 22 mg and 97% purity. Structural elucidation of ITC-1 was done by using various spectral techniques i.e. LC-MS (Agilent Technologies, California; Protein Digest method, Q-TOF B.05.00 (B5042.0) version, 6200 series TOF/6500 series), 1H-NMR (BRUKER Advance III 500 MHz) and FT-IR (Fourier transform-Infrared spectroscopy; Bruker, OPUS, Germany). The isolated compound that obtained after purification was light pale yellow in color, liquid in nature, stable at room temperature and completely soluble in water. The structure of ITC-1 was elucidated as 1-[1,2-Diisothiocyanato-2-(3-isothiocyanato-2,2-dimethyl-propylsulfanyl)-ethoxy]-3-isothiocyanato-2,2-dimethyl-propane ($C_{16}H_{22}N_4OS_5$; *m/e* = 446.17) (Figure 1).[10] Presence of –N=C=S- group in the isolated compound indicates it as isothiocyanate derivative.

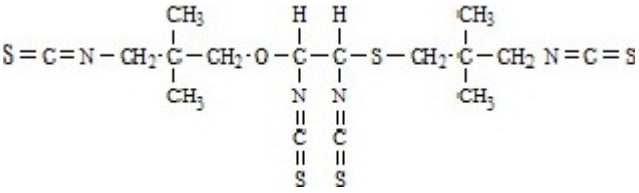


Figure 1. Possible proposed structure of ITC-1 on the basis of FT-IR, LC-MS and 1H-NMR having IUPAC name 1-[1,2-Diisothiocyanato-2-(3-isothiocyanato-2,2-dimethyl-propylsulfanyl)-ethoxy]-3isothiocyanato-2,2-dimethyl-propane.

2.4 Animal Maintenance and Ethical Clearance

Male Swiss albino mice (weigh 20-30 g) were obtained from 'Haryana Agricultural University', Hissar, India for experimental purpose. Ethical approval was obtained

from 'Institutional Animal Ethical Committee' (IAEC) before carrying out the experiments (BT/67-2.5.2012). All animals were housed in polypropylene cages under well maintained temperature (25±3˚C) with 12 h alternating light and dark cycle. Mice were provided nutritionally adequate pelleted chow diet (Ashirwad Pvt. Ltd., India) and drinking water *adlibitum* throughout the study.

2.5 Experimental Regimen

Total 36 mice were divided into six groups (n = 6). The experimental regimen of present study was tabulated in Table 1. NPYR was administered intraperitoneally on 1st day of experiment once whereas first dose of CCl_4 was administered subcutaneously on 14th day and then continued for next 5 doses (total 6 doses) once in a week. Plant doses and silymarin were administered orally whereas ITC-1 was administered intraperitoneally in to mice for 21 days regularly and were started on 50th day of experiment after 2 h of administration of last CCl_4 dose. The designed study was post treatment and total duration was of 70 days. Dose for NPYR-CCl_4 was decided on the basis of published report[11] and also on the basis of laboratory observations. Plant doses were also decided on the basis of previous published report[12] and modified according to the experimental observations conducted in our own laboratory.

The dose of Silymarin was selected on the basis of literature[13]. After complete treatment, mice were fasted overnight and then on next day, blood was collected by heart puncture to isolate serum. After this, livers were excised immediately and kept in 0.9% prechilled saline. Different homogenates of liver samples were prepared according to parameters that described later.

2.6 Evaluation of Hepatic Toxicity Markers

In vivo antiheptaotoxic potential of crude hydroethanol extract of *I. tinctoria* and its isolated compound "ITC-1" was studied by measuring various hepatic toxicity markers.

AST (Aspartate aminotransferase) and ALT (Alanine aminotransferase) activities in liver homogenates (10% in Sodium phosphate buffer, pH 7.4) of various treated groups were done according to Reitman and Frankel[14]. AST and ALT activities were calculated by using regression equation of standard sodium pyruvate and expressed as micromole of pyruvate formed/min/mg protein.

ALP (Alkaline Phosphatase) activity in liver homogenate of various treated groups was measured by the method of Sadashivam and Manickam[15]. To accomplish this, 10% w/v tissue homogenate was prepared from homogenized liver tissues in 50 mmol/L Glycine-NaOH buffer; pH 10.4. Specific activity of ALP was expressed as mmoles *p*-nitrophenol released/min/mg protein and calculated by using regression equation of standard curve of *p*-nitrophenol.

Total and Direct Bilirubin (TB and DB) in serum samples of all treated groups were evaluated by using kit (Reactivos GPL, Barcelona, Spain-Bilirubin T&D-DMSO) and expressed in mg/dl.

2.7 Statistical Analysis

All experiments were carried out in triplicates and results were expressed in Mean±S.D. Data was subjected to statistical analysis using 'One way analysis of Variance' (ANOVA) and groups were compared with PostHoc Tukey's multiple comparison test. Data was considered as significant at $P<0.05$; $P<0.01$ and $P<0.001$.

Table 1. Grouping and treatment schedule of mice in experimental regimen

Group number	*N = 6* in each group	Treatments
Group 1	Control vehicle only	Olive oil
Group 2	N-nitrsopyrrolidine (NPYR) + CCl_4	120 mg/kg b.w. (I.P. dose) and 3 ml/kg b.w. (Subcutaneous) respectively
Group 3	NPYR-CCl_4 + HEIT-L	Hydroethanol extract of *I. tinctoria;* low dose; 100 mg/kg b.w. (oral administration)
Group 4	NPYR-CCl_4 + HEIT-H	Hydroethanol extract of *I. tinctoria;* high dose; 300 mg/kg b.w.; (oral administration)
Group 5	NPYR-CCl_4 + Silymarin	Silymarin; 25 mg/kg b.w. (oral administration)
Group 6	NPYR-CCl_4 + ITC-1	Isolated compound 'ITC-1'; 15 mg/kg b.w. (I.P. dose)

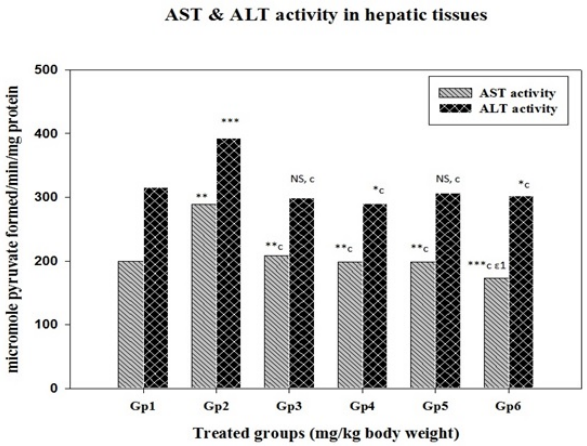


Figure 2. Effect of HEIT and its isolated ITC-1 on AST and ALT enzymatic activities in NPYR treated hepatic tissues (Values are in mean ± S.D. (n = 6). Significant differences in data are shown as *P*<0.05, P**<0.01, P***<0.001* v/s control group; *aP<0.05, bP<0.01, cP<0.001* v/s NPYR treated group; $^{\varepsilon 1}P<0.05$, $^{\varepsilon 2}P<0.01$, $^{\varepsilon 3}P<0.001$ vs. Standard group; NS (not significant).

Gp1 = Control; Gp2 = NPYR-CCl_4 treated; Gp3 = HEIT-L (Hydroethanolic extract of *I. tinctoria*; Low dose) treated; Gp4: HEIT-H (Hydroethanolic extract of *I. tinctoria*; High dose) treated; Gp5: Silymarin treated; Gp6 = ITC-1 treated

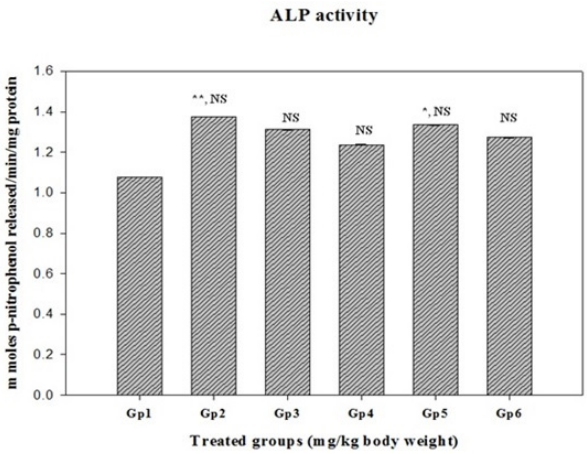


Figure 3. Effect of HEIT and its isolated ITC-1 on ALP enzymatic activity in NPYR treated hepatic tissues (Values are in mean ± S.D. (n = 6). Significant differences in data are shown as *P*<0.05, P**<0.01, P***<0.001* vs. control group; *aP<0.05, bP<0.01, cP<0.001* vs. NPYR treated group; $^{\varepsilon 1}P<0.05$, $^{\varepsilon 2}P<0.01$, $^{\varepsilon 3}P<0.001$ vs. Standard group; NS (not significant).

Gp1 = Control; Gp2 = NPYR-CCl_4 treated; Gp3 = HEIT-L (Hydroethanolic extract of *I. tinctoria*; Low dose) treated; Gp4: HEIT-H (Hydroethanolic extract of *I. tinctoria*; High dose) treated; Gp5: Silymarin treated; Gp6 = ITC-1 treated.

3. Results

Hepatoprotective activity of crude HEIT and its isolated 'ITC-1' was evaluated on the basis of various biochemical assays. AST and ALT activities in liver samples of all treated groups were calculated by using regression equation y = 0.776x + 0.035; $r2$ = 0.984 and y = 0.792x + 0.015; $r2$ = 0.868 respectively that were obtained by standard calibration curves of sodium pyruvate. Both AST and ALT activities were significantly (P<0.001) increased after NPYR intoxication (group 2) in comparison to healthy mice (group 1). HEIT at both doses i.e. 100 and 300 mg/kg b.w. and ITC-1 showed significant therapeutic potential to restore AST and ALT activities in hepatic tissues. Silymarin also showed significant protective effect (P<0.05) as equal to crude HEIT. ITC-1 possesses more efficacies to alter AST and ALT activities than Silymarin (Figure 2).

ALP activity in treated groups was calculated by using regression equation y = 0.025x – 0.019; $r2$ = 0.995, obtained by standard calibration curve of p-nitrophenol (pNP). Results stated that ALP activity was significantly increased (P<0.01) in NPYR intoxicated group when

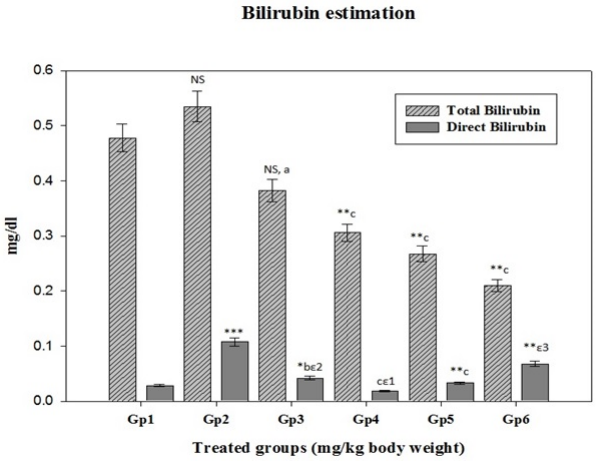


Figure 4. Effect of HEIT and its isolated ITC-1 on Total bilirubin and Direct bilirubin levels in NPYR treated hepatic tissues (Values are in mean ± S.D. (n = 6). Significant differences in data are shown as *P*<0.05, P**<0.01, P***<0.001* vs. control group; *aP<0.05, bP<0.01, cP<0.001* vs. NPYR treated group; $^{\varepsilon 1}P<0.05$, $^{\varepsilon 2}P<0.01$, $^{\varepsilon 3}P<0.001$ vs. Standard group; NS (not significant).

Gp1 = Control; Gp2 = NPYR-CCl_4 treated; Gp3 = HEIT-L (Hydroethanolic extract of *I. tinctoria*; Low dose) treated; Gp4: HEIT-H (Hydroethanolic extract of *I. tinctoria*; High dose) treated; Gp5: Silymarin treated; Gp6 = ITC-1 treated.

compared to control. High dose of HEIT showed more potential to normalize ALP level in comparison to others. ITC-1 also has more potency to reduce ALP level non-significantly when compared with silymarin treated group. Silymarin has showed very little effect in regulating ALP level (Figure 3).

Bilirubin estimation has great importance in hepatic toxicity measurements. Results stated that NPYR intoxication caused increase in the content of both total and direct bilirubin (TB; $P<0.001$ and DB; $P>0.05$) in blood serum (group 2). Both doses of HEIT and its isolated compound tried to normalize the level of TB and DB significantly in comparison to group 2 ($P<0.01$; $P<0.001$). Silymarin also showed significant reduction in TB and DB ($P<0.001$) thus showed better protective activity in comparison to ITC−1 ($P<0.001$). It was also noticed that crude HEIT (high dose) response was better than silymarin suggesting the good protective activity (Figure 4).

4. Discussion

N-Nitrosopyrrolidine (NPYR), an important hepatocarcinogenis capable to produce various short lived free Radicals/Reactive Oxygen Species (ROS) by liver microsomes[16]. During metabolism, NPYR produces various intermediate compounds i.e. crotonaldehyde,3-hydroxybutanal, paraldol and acetaldehyde that are responsible for various DNA adducts formation via react with dGuo and/or DNA[4]. CCl_4 works as an inducer of hepatotoxicity and is biotransformed by CYP450 enzymes in the endoplasmic reticulum into tricholromethyl free radicals that further produce trichloromethyl peroxyl radical after reacting with lipids and proteins in the presence of oxygen. It initiates lipid peroxidation reaction that causes disruption of plasma membrane and cell death[11,17]. Liver damage caused by NPYR-CCl_4 causes various changes both at tissue level and serum level via ROS generation.

AST (Aspartate aminotransferase) is primarily found in liver, heart, skeletal muscle and kidney whereas ALT (Alanine aminotransferase) is primarily found in liver and kidney. ALT is cytoplasmic while AST is both cytoplasmic and mitochondrial[18]. Increased amount of ALT and AST are the important and common cause of liver toxicity[19] and this statement is significantly correlated with the observed result. ALT activity is much higher than AST activity in most hepatotoxic condition as seen after NPYR-CCl_4 intoxication in mice. The reason behind this might be the half-life of ALT which is more than AST and also due to diminished hepatocyte cytosolic and mitochondrial AST activity[20]. Elevated level of aminotransferases pointed the severity of advanced hepatic toxic condition. HEIT, ITC-1 and Silymarin treatments showed a good potential to reduce these enzymatic activities that showed progressive decrement in hepatotoxic condition in mice.

ALP (Alkaline phosphatase) has remarkable importance in diagnosis of hepatic damage which is basically involved in metabolic transportation across cell membrane, protein synthesis, secretary activities and glycozen metabolism[21].NPYR administration in mice caused significant elevation in ALP activity that showed the hepatic toxicity and this statement is verified by a report given by Sahu[22]. He proposed that elevation in ALP is directly associated with various liver related problems including cancer. Malfunctioned ALP activity might be due to obliteration in secretary activity or in metabolite transportation, or may be due to modified synthesis of certain enzymes in toxic condition. HEIT has potent efficacy to normalize the ALP activity. ITC-1 and Silymarin both have efficiency to hold the activity at a particular level thus will be helpful to control the situation to become more drastic.

Bilirubin, a waste product that formed from the breakdown of hemoglobin, is the principle pigment of bile. A significant increase in bilirubin was clearly observed after NPYR intoxication in mice which enticed due to hepatic portal destruction. Drastic elevation in Direct Bilirubin (DB) may lead to hepatic jaundice and obstructive jaundice that may occur due to the defective conjugation of bilirubin by liver and anatomical destruction of bile ductules[23,24]. Singh et al.[25] reported the potent antioxidant and scavenging activity of hydroethanolic extract of *I. tinctoria* that might be responsible for their therapeutic activity because ROS are also responsible hepatic damage that generated by NPYR-CCl_4 intoxication[16].

5. Conclusion

Concluding the aforementioned studies, *in vivo* study of crude hydroethanolic extract of *I. tinctoria* and its isolated 'ITC-1' stongly indicated its potential antihepatotoxic activity. Toxicity can lead to several types of severe health issues. This study reveals the possible path of cure related to the liver toxicity via both crude extract and ITC-1, thus can be used in medical field.

6. Acknowledgements

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7. Conflict of Interest

Authors declare that they have no conflict of interest.

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