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Veena Sharma
 Department of Bioscience and
 Biotechnology Banasthali
 University, Jaipur Rajasthan,
 304022, India.

Aastha Agarwal
 Research Student, Department
 of Bioscience and Biotechnology
 Banasthali University, India.

Shatruhan Sharma
 Department of Bioscience and
 Biotechnology Banasthali
 University, Jaipur Rajasthan,
 304022, India.

Chemopreventive and antioxidant upshots of *Indigofera tinctoria* hydromethanolic extract in NPYR induced lung cancer in murine subjects

Veena Sharma, Aastha Agarwal, Shatruhan Sharma

Abstract

Indigofera tinctoria is one of the important plants of various traditional medicinal systems. We assessed the effect of hydromethanolic extract of *Indigofera tinctoria* on biochemical parameters, glutathione-metabolizing enzymes, lung specific markers and glycoproteins in *N*-nitrosopyrrolidine (NPYR) induced experimental lung toxicity in Swiss albino mice. Experimental subjects bearing lung cancer showed a significant increase in lipid peroxidation (LPO), superoxide dismutase (SOD) activity, total protein content (TPC) and total cholesterol content (TCC). Catalase (CAT) alleviated in NPYR intoxicated mice. Glutathione metabolizing enzymes decreased while lung specific markers-lactate dehydrogenase (LDH), alcohol dehydrogenase (ADH) and γ -glutamyl transpeptidase (GGT) and glycoprotein level increased in NPYR administered mice subjects. Confirmed by histological study, post treatment of low and high doses of *I. tinctoria* hydromethanolic (ITHML and ITHMH) extract normalizes the abnormal enzymatic and biochemical factors which signify antioxidant and anticancer activity of the plant.

Keywords: Lung cancer, chemoprevention, *Indigofera tinctoria*, oxidative stress.

1. Introduction

Epidemiological studies suggest that lung cancer is the second leading cause of death around the globe (Coll and Josserand, 2006)^[1]. Every year nearly 1, 70, 000 cases are diagnosed with lung cancer (Meuwissen and Berns, 2005)^[2]. Apart from many genetic and environmental reasons, the key cause of lung cancer is smoking which contains a number of nitrosamine compounds. Lung cancer malignancy is related to the oxidative stress. This hypoxic condition arises due to the depletion of oxygen from microenvironment of the cell (Valko *et al.*, 2006) which boost in the cancer cell proliferation and caused impinge on various cell mediated responses and its functioning. The severity of damage can be seen in lipid bilayer membranes, nucleic acids and various proteins which also include enzymes. A number of studies showed that genotoxicity and cytotoxicity transpired by these electrophilic entities caused DNA damage by the formation of DNA adducts. Mitogen activated protein kinases as c-Jun and extracellular signal regulated kinases get activated and promote cellular proliferation (McLean *et al.*, 2014) and alter expression of redox signaling pathways (Puig-Vilanova *et al.*, 2015)^[3]. *N*-nitroso Pyrrolidine (NPYR), a cyclic nitrosamine is a well known carcinogen present in tobacco and tobacco smoke (Tricker *et al.*, 1991)^[4]. This yellow colored, odorless compound formed both cyclic and open chain DNA adducts (Chung and Hecht, 1983; Wang and Hecht, 1997)^[5, 8]. Number of these adducts are sometimes higher in RNA than in DNA. A prolonged presence of adducts in genetic material leads to miscoding during replication and transcription and induced mutations in p53, k-ras genes which play a critical role in lung carcinogenesis (Hecht, 1999)^[6]. A number of chemopreventive drugs such as paclitaxel, cisplatin and other therapies are not so much effective, these conformist therapies are costly and impart a number of side effectives and lower the chances of patient's survival. In spite of vast research all over the world, there is a need to unearth an alternate, cost-effective cure which cause lesser side effects and thus ensure the permanence of the human subjects.

Medicinal plants as *Indigofera tinctoria* can be a promising source in filling the voids which armamentarium therapies made. Due to the presence of various phytoconstituents having antioxidant and chemopreventive properties, the plant is documented in Indian traditional medicinal system as a potent source to ameliorate various human ailments (Saraswathi *et al.*, 2012)^[7].

The study was demeanor in order to spotlight biochemical changes, oxidative stress parameters, glutathione-metabolizing enzymes, lung toxicity biomarker assays, glycoprotein levels in NPYR induced toxicity and to evaluate the antioxidant and chemopreventive perspectives of hydromethanolic extract of *Indigofera tinctoria*.

Correspondence:
Veena Sharma
 Department of Bioscience and
 Biotechnology Banasthali
 University, Jaipur Rajasthan,
 304022, India.

2. Material and Methods

2.1 Chemicals: NPYR, Paclitaxel, Ehrlich's reagent were purchased from Sigma Aldrich, India. Thio Barbituric acid (TBA), riboflavin, Nitro Blue Tetrazolium (NBT), 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), reduced glutathione (GSH), 1-chloro 2,4-dinitrobenzene (CDNB) were procured from HiMedia. All other chemicals were of analytical grade and obtained from Qualigens, CDH and Merck.

2.2 Experimental plant: Aerial parts of *Indigofera tinctoria* were procured from HM Heena Industries, Rajasthan (India). The collected plant material was washed, shade dried, milled to obtain a coarse powder and subject to soxhlet extraction using aqueous methanol (80%, v/v). The extract was collected, dried and stored in china dish for dosing purpose.

2.3 Animals: Healthy adult male Swiss albino mice (25-30 g) provided by Lala Lajpat Rai University of Veterinary and Animal Science, Hisar, India were used as experimental subjects during study. The animals were housed in poly propylene cages and a balanced environmental provision was maintained in animal house. Mice were given a standard pellet diet and had access to drinking water *ad libitum*.

2.4 Ethics avowal: The study was approved by the Institutional Animal Ethics Committee, Banasthali University. All experimental assays were performed following standard measures.

2.5 Experimental Design: Thirty animals, weighing 25 to 35 g were indiscriminately divided into five groups having six mice in each. Group 1 served as control having healthy subjects and was given olive oil. Rest 24 male mice were used for the preparation of lung toxicity model, for which they were given a single shot of NPYR (120 mg/kg of body weight, i.p.) on first day of the start of the experiment. After two weeks, CCl₄ (3 ml/kg of body weight, s.c.) was administered weekly for 6 weeks. These mice were then divided into remaining 4 groups according to the treatment they were given for next 21 days as below:

Group 2: NPYR treated group (NPYR)

Group 3: NPYR + *I. tinctoria* hydromethanolic extract low dose, 100 mg/kg of body weight, by oral gavage (ITHML)

Group 4: NPYR + *I. tinctoria* hydromethanolic extract high dose, 300 mg/kg of body weight, by oral gavage (ITHMH)

Group 5: NPYR + Standard Paclitaxel, 2 mg/kg of body weight, i.p. (PTX)

At the end of the experimental duration, animals were sacrificed by cervical dislocation under ether euthanasia. Lung tissue was excised instantly, cleaned, weighed and subject to homogenization for various assays.

2.6 Biochemical parameters: For biochemical analysis, lung tissue homogenates were subjected to lipid peroxidation (LPO), superoxide dismutase (SOD), catalase (CAT), total protein content (TPC), total cholesterol content (TCC).

2.7 Glutathione Metabolizing parameters: Glutathione peroxidase (GPx), reduced glutathione content (GSH), glutathione-s-transferase (GST) and glucose-6-phosphate dehydrogenase (G6PD) were assayed using standardized protocols.

2.8 Lung toxicity markers: Lung cytoplasmic parameters: Lactate dehydrogenase (LDH) activity was estimated

following the method described by Bergmeyer. (1983)^[18] and alcohol dehydrogenase (ADH) by Vallee and Hoche. (1955)^[19]. Clara cells marker enzyme gamma glutamyl transpeptidase (GGT) was done in accordance to the method by Orłowski & Meister. (1973)^[20].

2.9 Glycoprotein level estimation: Hexose level by Niebes. (1972)^[21], hexosamine by Wagner. (1979)^[22] and sialic acid estimation was done by method depicted by Warren. (1945) on lung tissue homogenates.

2.10 Statistical Analysis: Data is expressed as Mean ± SE of the average of the readings from three independent experimental subjects and subjected to statistically analyze by one way analysis of variance or ANOVA follow by Tukey's t-test using SPSS software. A probability of p<0.01 was considered to be significant.

3. Results

Biochemical status of experimental subjects in each group is tabularized in table 1. The levels of lipid peroxidation, SOD, total protein and total cholesterol contents mounted up to a significant level in NPYR treated animals in comparison to control mice, while the low dose and high dose treatments of hydromethanolic extract of *I. tinctoria* and group V which was given paclitaxel as standard normalize the amount of these biochemical parameters.

CAT activity was found to be decreased in NPYR administered male mice. The values of catalase were restored in groups ITHML, ITHMH and PTX.

Table 2 shows the results of glutathione metabolizing enzymes. All these enzymes, namely GPx, GSH, GST and G6PD were found to be significantly increased by the action of hydromethanolic extract of *Indigofera tinctoria* in both low dose and high dose groups which were noticeably depleted in the NPYR treated group. The worldwide known chemotherapeutic drug, paclitaxel also restored the abnormally increased values of all these enzymes significantly.

Lung cytoplasmic parameters (LDH and ADH) and Clara cell marker enzyme, GGT showed augmentation in NPYR treated subjects when compared to the normal healthy subjects. Treatment of low dose of *I. tinctoria* normalized the values up to considerable amount. The treatment of high dose of plant and paclitaxel significantly alleviated the levels of LDH and ADH in comparison to NPYR treated mice. The values are depicted in table 3.

Level of glycoproteins, hexose, hexosamine and sialic acid content are described in table 4. In group of NPYR treated animals, the levels of these parameters significantly boost up when compared to the values of animals of control group. The post treatment of plant doses and PTX, both low and high, normalize these heightened values.

4. Discussion

Endogenous antioxidant defense system of the body plays a vital role in combating stress generate through the administration of carcinogens like NPYR. The first cellular indication of oxidative stress is peroxidation of lipid membranes. NPYR being a toxic compound causes origination of hydroxyl, hydroperoxyl and superoxides radicals by the peroxidation of lipid molecules present in the plasma membranes of lung cells (Abbassy *et al.*, 2014)^[24]. These oxidants are highly reactive oxygen metabolites and generate MDA (malondialdehyde). In the persisting environment of stress, MDA form immunogenic adducts with protein and

DNA by cross linking. MDA-induced adducts play a vital role in promoting carcinogenesis (Ayala *et al.*, 2014) [25] *In vivo* lipid peroxidation also gives complex products namely aldehydes and hydroperoxides. Hydroperoxides give rise to hydrocarbons like ethane and pentane by a process called β -scission and promotes oxidative damage especially to the airways and cause obstructive pulmonary disease (Ross and Glen, 2014) [26].

Enzyme, SOD creates a first line of defense to eliminate or neutralize free radicals like superoxide whose level mounted during the stress by converting it to H₂O₂ and O₂ (Raina *et al.*, 2015) [27] generated by the action of NPYR. The level of SOD enzyme elevated in the NPYR treated group because of the overproduction of the enzyme to cope the rising numbers of oxidant molecules. Overutilization of catalase enzyme ended up as decreased level of CAT in NPYR treated animals and this suggests boosting of endogenous antioxidant system to hold back oxidative stress as it is directly allied through inflammation and development of cancer.

Protein is the main organic building molecule of the body. The increased level of protein in group II may also be implicated to be due to the NPYR-induced proteins which endorse carcinogenesis in the system and stirred protein synthesis of detoxification enzymes. Soaring level of cholesterol in group II animals is linked to ATP-binding cassettes (ABCs) and Lecithin: cholesterol acyltransferase (LCAT) (Chou *et al.*, 2015) [29] as ABCs is responsible for efflux of cholesterol. Administration of NPYR in male mice increased the TCC by down regulation of ABCs, elevated cholesterol level and promotes pulmonary cancer. Oxidized environment decreased the catalytic activity of LACT resulted in the accumulation of cholesterol.

Post treatment of plant enhanced the activity of glutathione metabolizing enzymes which decreased in NPYR administered

mice, indicating antitumor and chemopreventive effect of hydromethanolic extract of *Indigofera tinctoria* due to the anti-oxidant properties. G6PD is the first enzyme in pentose phosphate pathway and thus an important source of antioxidant NADPH. Elevated activity of GST and GPx in plant and PTX treated groups indicate an increase in GSH recycling which an endogenous tripeptide is known for its function in sustaining redox status of pulmonary tissue by phytochemicals present in the plant extract as a sign of chemoprevention from NPYR induced toxicity.

The elevated level of glycoproteins in NPYR treated mice subjects is the indication of development of cancer as sialic acid containing oligosaccharides present in lung tissue play an important role in the adhesion between cancer cells and endothelial cells and also demonstrated that metastatic potential of tumor cells is proportional to cell surface sialylation. Hexose and hexosamine aid in eradication of reactive metabolites formed due the carcinogenesis induced by NPYR.

Antioxidant role of *Indigofera tinctoria* hydromethanolic extract is proved by its total antioxidant capacity, FRAP and DPPH radical scavenging activity.

5. Conclusion

In conclusion, the results of our study revealed that *Indigofera tinctoria* hydromethanolic extract can alter various biochemical, endogenous antioxidant enzymes, lung specific markers and glycoprotein levels. At present, though the precise mechanism of the protective effects of this plant is not clearly understood, the results suggests that lung toxicity treatment by plant extract could be through varying biochemical parameters, enhancing glutathione metabolizing enzymes and reducing lung specific makers and glycoproteins levels.

Table 1: Effects of *I. tinctoria* hydromethanolic extract on biochemical/ oxidative stress parameters in lungs of control and experimental animals.

Groups Parameters	Control	NPYR	ITHML	ITHMH	PTX
LPO	55.995±6.51	64.941±1.46	38.234±1.9*	46.532±1.74*	32.107±3.94*
SOD	0.106±0.006	0.500±0.003 ^a	0.100±0.03*	0.090±0.03*	0.077±0.03*
CAT	108.615±1.59	56.427±0.36 ^a	95.855±12.88*	84.267±1.79*	82.155±8.99*
TPC	0.145±0.001	0.381±0.045 ^a	0.171±0.004*	0.180±0.002*	0.185±0.00*
TCC	38.66±2.16	86.13±2.92 ^a	50.31±3.12	46.78±2.89*	47.91±1.71*

Each value is expressed as mean ± S.D. for six mice in each group. LPO: lipid peroxidation (nmole MDA/mg tissue); SOD: superoxide dismutase (U/mg protein); CAT: catalase (μmole of H₂O₂ consumed/mg protein); TPC: Total Protein Content (mg of BSA equivalent); TCC: Total Cholesterol Content (mg/mg of tissue); *p<0.01

Table 2: Effects of *I. tinctoria* hydromethanolic extract on glutathione metabolizing enzymes in lungs of control and experimental animals.

Groups Parameters	Control	NPYR	ITHML	ITHMH	PTX
GPx	4.74±0.533	0.809±0.165 ^a	1.634±0.516	2.088±1.000	1.979±0.137
GSH	2.507±0.108	0.348±0.006 ^a	1.008±0.047*	1.078±0.061*	1.206±0.085*
GST	2207±4.14	158.57±1.46 ^a	1575±1.92*	1562±1.13*	1197±4.51*
G6PD	47.466±9.32	30.800±1.203	39.162±6.198	37.087±8.57	37.88±6.676

Each value is expressed as mean ± S.D. for six mice in each group. GPx: Glutathione peroxidase (μmole of NDAH oxidized/mg protein); GSH: Reduced glutathione content (μmole/mg of protein); GST: Glutathione-s-transferase (nmole CDNB formed/mg protein); G6PD: Glucose-6-Phosphate Dehydrogenase (nmole NADH reduced/mg protein); *p<0.01

Table 3: Effects of *I. tinctoria* hydromethanolic extract on toxicity specific biomarkers in lungs of control and experimental animals.

Groups Parameters	Control	NPYR	ITHML	ITHMH	PTX
LDH	0.155±0.03	0.394±0.002 ^a	0.126±0.017*	0.120±0.029*	0.142±0.016*
ADH	0.301±0.01	0.631±0.047 ^a	0.473±0.03	0.352±0.01*	0.346±0.007*
GGT	26.31±0.07	65.23±0.01	32.78±1.72*	28.83±0.91*	43±0.61*

Each value is expressed as mean ± S.D. for six mice in each group. LDH: Lactate Dehydrogenase (U/L); ADH: Alcohol Dehydrogenase (U/ml); GGT: γ -glutamyl transpeptidase (nmole of p-nitroaniline formed/mg protein); *p<0.01

Table 4: Effects of *I. tinctoria* hydromethanolic extract on glycoprotein level in lung tissue of control and experimental animals.

Groups Parameters	Control	NPYR	ITHML	ITHMH	PTX
Hexose	33.666±0.203	45.133±2.259 ^a	44.453±1.49	38.88±1.135*	39.873±0.565*
Hexosamine	12.603±1.391	27.656±8.427	16.673±3.121	15.065±2.73	16.616±0.805
Sialic Acid	2.290±0.001	38.64±4.59 ^a	6.53±4.56*	1.42±0.75*	7.55±3.49*

Each value is expressed as mean ± S.D. for six mice in each group. Hexose: mg/g tissue; Hexosamine: µg/g tissue; Sialic Acid: mg/g tissue; *p<0.01

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