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ORIGINAL ARTICLE

In Vivo Antioxidant Activity of Different Fractions of *Indigofera Barberi* Against Paracetamol-induced Toxicity in Rats

Sıçanlarda Parasetamol ile İndüklenen Toksisiteye Karşı *Indigofera Barberi*'nin Farklı Fraksiyonlarının *İn Vivo* Antioksidan Aktivitesi

Shaik AMINABEE[1]*, Atmakuri Lakshmana RAO[1], Maram Chinna ESWARAIAH[2]

[1]V. V. Institute of Pharmaceutical Sciences, Department of Pharmacology, Gudlavalleru, India
[2]Anurag College of Pharmacy, Department of Pharmacognosy, Kodad, India

ABSTRACT

Objectives: To evaluate the *in vivo* antioxidant activity of chloroform extract fractions of *Indigofera barberi* (whole plant) against paracetamol-induced toxicity in rats.

Materials and Methods: For 7 days, rats were treated with different chloroform extract fractions and toxicity was induced with a single dose of paracetamol by intraperitoneal injection. The group of animals pretreated with 100 mg/kg p.o of fraction D of *Indigofera barberi* improved significantly in terms of hepatic superoxide dismutase (SOD), catalase and peroxidase activities, and glutathione levels compared to the control group.

Results: The hepatic SOD, catalase, peroxidase activities, and glutathione levels in the animal groups treated with paracetamol were 33.6±0.09 µ/mg protein, 5.5±0.23 µ/mg protein, 0.131±0.15 µ/mL, and 46.1±5.81 µM, respectively. Hepatic SOD, catalase, peroxidase, and glutathione in the fraction D treated group were 61.8±0.07 µ/mg protein, 10.6±0.16 µ/mg protein, 0.913±0.23 µ/mL, and 87.6±1.4 micro molar, respectively. Therefore, the present study revealed that fraction D of *Indigofera barberi* has significant in vivo antioxidant activity and can be used to protect tissue from oxidative stress.

Conclusion: From the results, fraction D of *Indigofera barberi* at a dose of 100 mg/kg, p.o., improved the SOD, catalase and peroxidase activities, and glutathione levels significantly. Based on this study, we can conclude that fraction D of *Indigofera barberi* possesses *in vivo* antioxidant activity and can be employed in protecting tissue from oxidative stress.

Key words: *Indigofera barberi*, paracetamol, silymarin, radical scavenging

ÖZ

Amaç: Sıçanlarda parasetamol ile indüklenen toksisiteye karşı *Indigofera barberi*'nin (tüm bitki) kloroform ekstre fraksiyonlarının *in vivo* antioksidan aktivitesinin belirlenmesi.

Gereç ve Yöntemler: Yedi gün boyunca sıçanlara farklı kloroform ekstrakları uygulanmıştır ve toksisite intraperitoneal tek doz parasetamol uygulaması ile indüklenmiştir. 100 mg/kg p.o. fraksiyon D ile ön uygulaması alan hayvanlar hepatik süperoksit dismutaz (SOD), katalaz ve peroksidaz aktiviteleri ve glutatyon düzeyleri açısından kontrol grubuna göre belirgin bir şekilde iyileşmişlerdir.

Bulgular: Parasetamol uygulanan grupta hepatik SOD, katalaz, ve peroksidaz aktiviteleri ve glutatyon düzeyleri sırasıyla 33,6±0,09 µ/mg protein, 5,5±0,23 µ/mg protein, 0,131±0,15 µ/mg protein ve 46,1±5,81 µM olarak bulunmuştur. Fraksiyon D uygulanan grupta hepatik SOD, katalaz ve peroksidaz aktiviteleri ve glutatyon düzeyleri sırasıyla 61,8±0,07 U/mg protein, 10,6±0,16 µ/mg protein, 0,913±0,23 µ/mg protein ve 87,6±1,4 µM bulunmuştur. Bu nedenle, bu çalışma *Indigofera barberi*'de elde edilen fraksiyon D'nin belirgin bir *in vivo* antioksidan aktivitesi olduğunu ortaya koymuştur ve dokuyu oksidatif stresten korumak için kullanılabilir.

Sonuç: Bu sonuçlar, 100 mg/kg, p.o. dozda *Indigofera barberi*'den elde edilen fraksiyon D, SOD, atalaz ve peroksidaz aktiviteleri ve glutatyon düzeylerini belirgin bir şekilde düzeltmiştir. Bu çalışmaya dayanarak, *Indigofera barberi*'den elde edilen fraksiyon D'nin *in vivo* antioksidan aktivitesinin olduğu sonucuna varabiliriz ve dokuyu oksidatif stresten korumak için kullanılabileceği söylenebilir.

Anahtar kelimeler: *Indigofera barberi*, parasetamol, silimarin, radikal süpürücü

*Correspondence: **E-mail:** aminaammi786@gmail.com, **Phone:** +949158458 **ORCID-ID:** orcid.org/0000-0001-9256-0897
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INTRODUCTION

Today 80% (approximately) of the total world population rely purely on plants for their health and welfare. Recently, there was an increase in interest in the therapeutic prospective of curative plants as antioxidants in minimizing free radical instigated tissue damage. In reaction to this increased acceptance and significant demand for curative and herbal plants, several supervising organizations and groups have mentioned that indigenous curative plants have been introduced into agriculture.[1] Many plants generate various bioactive molecules and this makes them a principle and plentiful source of distinct novel kinds of medicines. A huge heritage of scrutiny and knowledge regarding prophylactic and remedial medicines was accessible in ancient scholastic efforts included in the Charaka, Atharva veda, Sushruta etc.[2] Above 50% of all current clinical medicines are of natural product source[3] and many natural products play a vital part in the medicine evolution process in pharmaceutical production.[4] Herbal medicines and products have acquired significance in modern years because of their capability and financial value.

Therefore, there was an increasing fascination in the quantification and utilization of plant antioxidants for systematic investigation as well as commercial (cosmetic, pharmaceutical, and dietary) purposes. The different antioxidant responses include many steps involving the initiation, propagation, dividing, hindrance, and cessation of free radicals. Oxidative free radicals are produced when cells utilize oxygen for different physiological procedures. Generally by-products are reactive oxygen species (ROS) like hypochlorous acid, superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical that evolve by the cellular redox action. At modest concentrations but at high levels, ROS have favorable effects on cellular operations and immune reactions and are pivotal for life; ROS produce oxidative stress that causes cell structure and function impairment, involving DNA, lipids, sugars, and proteins.[5] Oxidative pressure plays a crucial part in the evolution of degenerative and chronic ailments like skin diseases, cancer, autoimmune disorders, altitude sickness, cataracts, rheumatoid arthritis, osteoarthritis, coronary heart disease, aging, and various neurodegenerative diseases.[6] Antioxidants, either *in situ* (naturally produced) or externally supplied via diet, hinder oxidative pressure in the human body. These antioxidants efficient in scavenging free radicals react by prohibiting injury caused by ROS, and thereafter enhance the cellular response and immune defense, and lower the risk of degenerative diseases and cancer.[5]

Recently, many herbal medicines have been gaining much attention as alternative drugs[7] applicable as prophylactics for lifestyle-associated disorders but comparatively very little knowledge is accessible regarding their mechanism of action. There was extended interest in the investigation of plant outcomes, which has prompted enormous research on their possible health benefits. Traditional usages (Ayurveda) of plants are most familiar in aqueous extracts form. Recently, some papers have explored the focus for examining these plants in aqueous or ethanolic extracts and some have described activity in petroleum ether, chloroform, and benzene extracts.[8-10]

Indigofera barberi (Fabaceae) of the Tirumala Hills is a commonly assessed endemic herb. Vernacularly it is known as Adavineelimanadu mokka. It grows up to 1 m tall (under shrub). Branchlets are faintly angled. Leaves are 3 and they are foliolate. Leaflets are pubescent, ovate-oblong, mucronate, obtuse. Flowers (pink) are organized in axillary congested racemes. Pods are appressed, deflexed, sub-terete, sharply pointed, white-tomentose. Seeds are 2 to 4 in number. September to December is the flowering and fruiting season.[11]

Orally, leaf powder (5 g) is administered with butter milk for controlling diabetes. Leaves (50 g), pepper (1 g), and garlic (1 g) are made into paste and formulated into pills of peanut size, and for 5 days 5 pills are administered once a day to cure jaundice as authorized by Nakkala and many tribal physicians. Whole plant powder (5 g) is administered through rice washed water once a day for 10-15 days to remove intestinal worms and as a remedy for various types of peptic ulcers and skin diseases.[12] It is also used as a coloring agent and dye. Its leaf juice is utilized as an antiseptic to cure burns, cuts, wounds, and boils. Keeping these considerations in mind, the aim of the current study was to fabricate a scientific base for the use of the fractions of chloroform extract of *Indigofera barberi* as an antioxidant agent.

MATERIALS AND METHODS

Collection of materials

The *Indigofera barberi* (whole plant, Herbarium number: VVIPS/PCL/011) was collected from the evergreen forest of the Tirumala Hills in Andhra Pradesh state, India. Samples were authenticated and certified by Dr. K. Madhava Chetty, Plant Taxonomist, Department of Botany, Sri Venkateswara University, Tirupati, India. For one week, the *Indigofera barberi* (whole plant) was sorted, ranked, cleaned, and air-dried at room temperature. By utilizing a laboratory hammer mill the plant was finely ground into powder. Finely powdered samples were gathered and tightly stored in water- and air-proof containers shielded from heat and direct sunlight until needed for extraction.

Preparation of extracts

After the *Indigofera barberi* (whole plant) was completely ground into fine powder, it was successively extracted for 18 h with various solvents of increasing polarity, i.e. with petroleum ether, ethyl acetate, chloroform, ethanol, and distilled water in a Soxhlet apparatus. The obtained extracts were concentrated to dryness in a rotary evaporator until free of the solvents.

Isolation of fractions

Thin-layer chromatography (TLC) was carried out using silica gel aluminum plates, 60F-254, 0.5 mm (TLC plates, Merck). The obtained spots were visualized in ultraviolet light and 10% H_2SO_4 in methanol. More spots were seen with chloroform extract. Thus, for further purification, the chloroform extract was subjected to column chromatography using silica gel of pore size 60-100. The silica gel column was equilibrated and counterbalanced for 1 h with petroleum ether at flow rate 5 mL/min. The chloroform extract (1 g dissolved in methanol) was

loaded onto the column and 11 fractions were collected using different solvents of varying concentrations like petroleum ether (100%), petroleum ether: ethyl acetate (4:1), petroleum ether: ethyl acetate (2:3), petroleum ether: ethyl acetate (3:2), ethyl acetate (100%), chloroform: methanol (2:3), and chloroform: methanol (3:2).

Phytochemical analysis

After isolation of fractions from chloroform extract, phytochemical analysis[13] of fractions was carried out for the presence of alkaloids, tannins, saponins, glycosides, terpenoids, carbohydrates, flavonoids, proteins, amino acids, fixed oils, steroids, and sterols by different methods.

Animals

Albino Wistar rats of both sexes weighing 180-200 g were procured from the National Institute of Nutrition, Hyderabad, Telangana, India. The animals were kept in polypropylene cages (5 in each cage) at a relative humidity of 55-65% and medium temperature of 25±2°C. A 12 h light and dark cycle was retained in the air conditioned animal house. After arrival, all the rats were nourished with a common diet and distilled water for at least 1 week and then they were equally divided into categories with free access to food and distilled water.

Acute toxicity studies

Acute toxicity studies were performed according to the Organization for Economic Co-operation and Development guidelines.[14] The animals were divided in groups and each group contained 5 animals. These grouped animals were fasted for 4 h with free access to distilled water only. The fractions were administered orally in doses of 100, 300, 1500, and 3000 mg/kg to different groups of rats and they were observed over 14 days for mortality and physical/behavioral changes. All these experimental studies on the animals were conducted after permission was obtained from the IAEC (Ref: P2/IAEC/2/2017/VVIPS/SAB/Rats).

Experimental

Group I animals served as normal controls; they receive only vehicle (gum acacia 3% solution)

Group II animals served as toxic controls, treated with paracetamol in a single dose of 2 g/kg orally to produce acute toxicity

Group III served as the standard group and was treated with silymarin (100 mg/kg)

Group IV was treated with fraction A 50 mg/kg

Group V was treated with fraction A 100 mg/kg

Group VI was treated with fraction B 50 mg/kg

Group VII was treated with fraction B 100 mg/kg

Group VIII was treated with fraction C 50 mg/kg

Group IX was treated with fraction C 100 mg/kg

Group X was treated with fraction D 50 mg/kg

Group XI was treated with fraction D 100 mg/kg

The animals in groups III to XI were treated with a single dose of paracetamol 2 g/kg, orally, 6 h after the last treatment. On day 8 the rats were sacrificed by carotid bleeding and the liver was rapidly excised, rinsed in ice-cold saline, and a 10% w/v homogenate was prepared using 0.15 M KCl. Centrifugation was conducted at 800 rpm for 10 min at 4°C. The supernatant obtained was used for the estimation of catalase and peroxidase and other enzymes. Furthermore, the homogenate was centrifuged at 1000 rpm for 20 min at 4°C and the supernatant was used for biochemical estimation.

Biochemical estimation

Estimation of superoxide dismutase (SOD)

The assay of SOD was based on the reduction of nitro blue tetrazolium (NBT) to water insoluble blue formazan, per the method of Beauchamp and Fridovich[15] Liver homogenate (0.5 mL) was taken and 1 mL of 50 mM sodium carbonate, 0.4 mL of 24 mM NBT, and 0.2 mL of 0.1 mM ethylene diamine tetra acetic acid (EDTA) were added. The reaction was initiated by adding 0.4 mL of 1 mM hydroxylamine hydrochloride. Zero time absorbance was taken at 560 nm followed by recording the absorbance after 5 min at 25°C. The control was simultaneously run without liver homogenate. Units of SOD activity were expressed as the amount of enzyme required to inhibit the reduction of NBT by 50%. The specific activity was expressed in terms of units per milligram of proteins.

Estimation of catalase

Catalase activity was measured as described by Aebi.[16] Supernatant liquid (0.1 mL) was added to a cuvette containing 1.9 mL of 50 mM phosphate buffer (pH 7.0). The reaction was started by the addition of 1.0 mL of freshly prepared 30 mM H_2O_2. The rate of decomposition of H_2O_2 was measured spectrophotometrically from changes in absorbance at 240 nm. Activity of catalase was expressed as units/mg protein.

Estimation of peroxidase

The peroxidase assay was carried out per the method reported by Nicholos[17] Liver homogenate (0.5 mL) was taken, and to this were added 1 mL of 10 mM KI solution and 1 mL of 40 mM sodium acetate solution. The absorbance of potassium periodide was read at 353 nm, which indicates the amount of peroxidase. Twenty microliters of H_2O_2 (15 mM) was added and the change in the absorbance in 5 min was recorded. Units of peroxidase activity were expressed as the amount of enzyme required to change the OD by 1 unit per minute. The specific activity was expressed in terms of units per milligram of proteins.

Estimation of glutathione (GSH)

The procedure to estimate the reduced GSH level followed the method described by Ellman[18] The homogenate (in 0.1 M phosphate buffer, pH 7.4) was added with an equal volume of 20% trichloroacetic acid containing 1 mM EDTA to precipitate the tissue proteins. The mixture was allowed to stand for 5 min prior to centrifugation for 10 min at 200 rpm. The supernatant liquid (200 µL) was then transferred to a new set of test tubes and 1.8 mL of Ellman's reagent added (5.5'-dithio-(*bis*2-nitrobenzoic acid) (0.1 mM) prepared in 0.3 M phosphate buffer

with 1% of sodium citrate solution). Then all the test tubes were made up to the volume of 2 mL. After completion of the total reaction, solutions were measured at 412 nm against a blank. Absorbance values were compared with a standard curve generated from known GSH. The GSH level in the liver was calculated as micromole/g liver.

RESULTS

Acute toxicity studies

Acute toxicity studies were carried out by the up-and-down regulation method. It was found that the extract at a limit dose from 1500 to 3000 mg/kg is safe and does not show any mortality.

Isolation of fractions

TLC was carried out using silica gel aluminum plates, 60F-254, 0.5 mm (TLC plates, Merck). Eleven fractions were collected. The yielded products were pooled into four fractions based on TLC. The yield and appearance of the four fractions are given in Table 1.

Preliminary phytochemical screening

Phytochemical screening revealed the presence of alkaloids and carbohydrates in fraction A, saponins in fraction B, glycosides in fraction C, and flavonoids in fraction D (Table 2).

Table 1. Appearance and yield of the 4 fractions of *Indigofera barberi*

Fraction	Appearance	Yield
Fraction A	Yellow	150 mg/g
Fraction B	Dark brown greenish	200 mg/g
Fraction C	Light green	150 mg/g
Fraction D	Saffron	300 mg/g

Table 2. Preliminary phytochemical screening of fractions of chloroform extract of *Indigofera barberi*

S. no.	Phytochemicals	Fraction A	Fraction B	Fraction C	Fraction D
1	Alkaloids	+	-	-	-
2	Tannins	-	-	-	-
3	Saponins	-	+	-	-
4	Glycosides	-	-	+	-
5	Terpenoids	-	-	-	-
6	Carbohydrates	+	-	-	-
7	Flavonoids	-	-	-	+
8	Proteins	-	-	-	-
9	Amino acids	-	-	-	-
10	Fixed oils	-	-	-	-
11	Steroids and sterols	-	-	-	-

In vivo antioxidant activity

Phytochemical screening of the plant showed the presence of flavonoids in fraction D. The present study was undertaken to assess the *in vivo* antioxidant effect of different fractions of chloroform extract of *Indigofera barberi* whole plant on paracetamol-induced toxicity in rats. The results showed that the levels of SOD, catalase, peroxidase, and GSH levels in the control group were 65.2±0.11, 14.31±0.97, 0.967±0.13, and 98.2±1.14, and in the paracetamol treated group were 33.6±0.09, 5.5±0.23, 0.131±0.15, and 46.1±5.81, respectively. The levels of SOD, catalase, peroxidase, and GSH in the paracetamol-treated group were significantly lower than those in the normal group. With co-administration of fraction D of *Indigofera barberi* at a dose of 100 mg/kg, the levels of SOD, catalase, peroxidase, and GSH were 61.8±0.07, 10.6±0.16, 0.913±0.23, and 87.6±1.4, respectively. This markedly prevented paracetamol-induced alterations and maintained enzyme levels near their normal values (Table 3). The standard treated group also had significantly increased levels of SOD, catalase, peroxidase, and GSH (63.9±4.8, 12.1±0.81, 0.938±0.32, and 91.6±1.6, respectively).

The results are expressed as mean ± standard error of the mean for each group. The data were analyzed by one-way analysis of variance (ANOVA); $p<0.01$, $p<0.05$ indicated statistical significance.

Statistical analysis

All analyses were run in triplicate. The statistical analysis was performed by Student's t-test and ANOVA.

DISCUSSION

In paracetamol-induced toxicity, fraction D of chloroform extract of *Indigofera barberi* treatment increased the depleted levels of cellular GSH significantly in rats. Fraction D of chloroform extract of *Indigofera barberi* also restored the levels of antioxidant enzymes such as SOD and catalase almost back to their normal levels. SOD plays a vital role in the depletion and elimination of ROS and protects cells against the deleterious effects of the O_2^- derived from the peroxidative process in liver and kidney tissues[19] and the observed increase in SOD activity suggests that fraction D chloroform extract of *Indigofera barberi* has an efficient protective mechanism in response to ROS. Catalase is considered the most important H_2O_2 removing enzyme and is a key component of the antioxidative defense system.[20] Here catalase activity was increased and then restored to normal levels on administration of fraction D of chloroform extract of *Indigofera barberi*. Peroxidase is an enzyme that catalyzes the reduction of hydroperoxides, including H_2O_2, and functions to protect the cell from peroxidative damage.[21] We propose that the additive and synergistic antioxidant activity of phytochemicals such as flavonoids present in *Indigofera barberi* is responsible for its potent antioxidant activity.

CONCLUSION

Phytochemical screening of the fractions showed the presence of flavonoids in fraction D. In our investigation on *Indigofera barberi*, enzymatic oxidants such as GSH, SOD, catalase, and peroxidase were improved in the drug-treated group

Table 3. Radical scavenging activity of fractions of chloroform extract of *Indigofera barberi*

Group	Treatment	Dose	SOD (U/mg protein)	Catalase (U/mg protein)	Peroxidase (U/mL)	Glutathione (µM)
I	Control	2 mL/kg	65.2±0.11	14.31±0.97	0.967±0.13	98.2±1.14
II	Paracetamol treated	2 g/kg	33.6±0.09	5.5±0.23	0.131±0.15	46.1±5.81
III	Silymarin	100 mg/kg	63.9±4.8	12.1±0.81	0.938±0.32	91.6±1.6
IV	Fraction A	50 mg/kg	42.4±0.31	5.67±0.15	0.551±0.24	77.1±0.9
V	Fraction A	100 mg/kg	51.6±0.13	6.51±0.19	0.71±0.41	82.1±0.11
VI	Fraction B	50 mg/kg	41.2±0.07	6.5±0.27	0.416±0.27	73.6±0.61
VII	Fraction B	100 mg/kg	44.8±0.09	6.8±0.29	0.519±0.23	79.1±0.74
VIII	Fraction C	50 mg/kg	33.6±0.08	6.32±0.27	0.321±0.17	58.4±1.7
IX	Fraction C	100 mg/kg	38.1±0.06	6.50±0.2	0.481±0.19	61.3±1.8
X	Fraction D	50 mg/kg	55.9±0.11	9.6±0.13	0.851±0.14	83.5±0.6
XI	Fraction D	100 mg/kg	61.8±0.07	10.6±0.16	0.913±0.23	87.6±1.4

SOD: Superoxide dismutase

as compared to the control. Based on this we conclude that fraction D of chloroform extract of *Indigofera barberi* possesses *in vivo* antioxidant activity and may be employed in protecting tissues from oxidative stress.

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