OPEM
www.opem.org
Oriental Pharmacy and Experimental Medicine 2004 **4(3)**, 179-185

Biological activity of an Indian medical plant, *Indigofera cordifolia*

Bhattiprolu Kesava Rao[1], Masami Kawase[2,*], Toru Tanaka[2], Satoru Tani[2], Noboru Motohashi[3], Kazue Satoh[4], Hiroshi Sakagami[5], Shigemi Terakubo[6], Hideki Nakashima[6], Krisztina Wolfard[7] and Joseph Molnar[7]

[1]Department of Chemistry, Nagarjuna University, Andhra Pradesh, India; [2]Faculty of Pharmaceutical Sciences, Josai University, Sakado, Saitama 350-0295, Japan; [3]Meiji Pharmaceutical University, Kiyose, Tokyo 204-8588, Japan; [4]School of Medicine, Showa University, Shinagawa, Tokyo 142-8555, Japan; [5]Department of Dental Pharmacology, Meikai University School of Dentistry, Sakado, Saitama 350-0283, Japan; [6]Department of Microbiology, St. Marianna University School of Medicine, 2-16-1 Sugao, Miyamae-ku, Kawasaki, Kanagawa 216-8511, Japan; [7]Faculty of Medicine, Institute of Medical Microbiology, Albert Szent-Gyorgyi Medical Centrum, University of Szeged, Szeged, Dom ter 10, H-6720, Hungary

SUMMARY

The ethanol extract of *Indigofera cordifolia* was studied for *in vivo* gastroprotective activity, cytotoxic activity against oral tumor and normal cells, multidrug resistance (MDR) reversal activity, anti-human immunodeficiency virus (HIV) activity and radical scavenging activity. The extract of *I. cordifolia* showed potent gastric mucosal protective activity against stomach injury induced by HCl/EtOH solution. However, the gastroprotective activity could not be related to the radical mechanism, because the extract weakly scavenged both·OH radical and $O_2\cdot^-$. The extract also showed promising levels of MDR-reversing activity. This study demonstrates the tumor-specific cytotoxic action of the plant extract. However, the extract had no anti-HIV activity. From above results, the study suggests the medicinal importance of *I. cordiforia* extract.

Key words: *Indigofera cordifolia*; Gastroprotective activity; Cytotoxic activity; MDR; Anti-HIV; Radical intensity

INTRODUCTION

Herbal remedies used in the traditional folklore medicine provide an interesting source for the development of potentially new drugs for chemotherapy (Samy *et al.*, 1999). The use of medicinal plants plays an important role to cover the basic health needs in the developing countries. Therefore, it is of interest to carry out a screening of these plants in order to validate their use in folklore medicine and to reveal the active principle by isolation and characterization of their constituents.

Indigofera species have been used as forage crops, pulp crop or seed crop. Some species of *indigofera* such as *I. endecaphylla, I. patens, I. enneaphylla* and *I. subulata* are noted as toxic to cattle and other animals (Miller and Smith, 1973). Several toxic constituents of plants of *Indigofera* genus are aliphatic nitro-compounds such as 3-nitropropanoic acid (Finnegen and Mueller, 1965; Garcez *et al.*, 1989).

Seeds of *Indigofera* species also have toxic

*Correspondence: Dr Masami Kawase, Faculty of Pharmaceutical Sciences, Josai University, Sakado, Saitama 350-0295, Japan. Fax: +81-49-271-7984; E-mail: kawasema@josai.ac.jp

amino acids such as canavarine, an antimetabolite of arginine (Miller and Smith, 1973). The hepatotoxic and teratogenic amino acid, indospicine, was isolated from *I. endecaphylla* (Finnegan and Mueller, 1965; Miller and Smith, 1973).

Some species of *Indigofera* have been used in folklore medicine (Hartwell, 1970). Phytochemical analyses of *Indigofera* genus, such as *I. hebepetala* (Hasan *et al.*, 1994; 1996) and *I. microcarpa* (Souza *et al.*, 1988), have resulted in the isolation of flavonoids, flavonol glycosides and 2-arylbenzofurans. The extract of *I. oblongifolia* leaves has significant antimicrobial activity against Gram-positive, Gram-negative and fungal species (Dahot, 1999). The extract of *I. tinctoria* showed significantly the hepatoprotective activity against CCl_4-induced liver injury in rats and mice (Singh *et al.*, 2001) and an antioxidant effect on D-galactosamine/endotoxin-induced acute hepatitis in rodents (Sreepriya *et al.*, 2001a; 2001b). From the stems of *I. longoracemosa*, a novel abietane diterpenoid of indigoferabietone was isolated and showed antituberculous and antibacterial activity (Thangadurai *et al.*, 2002).

Indigofera cordifolia has been still used as a remedy for Malarial fevers in India. The white seeds resemble poppy seeds, used in a mixture with bajra or jawar for making bread. The seeds are harmful if consumed alone. However, no detailed study of the biological activity of *I. cordifordia* extract has been reported so far.

We investigated here the antiulcer activity, cytotoxic activity, anti-human immunodeficiency virus (HIV) activity, multidrug resistance (MDR) reversal activity and radical scavenging activity of the ethanol extract of *I. cordifolia*.

MATERIALS AND METHODS

Plant material

The plant material (*Indigofera Cardifolia*, Heyne, Family: Papilionaceae, Fabaceae) was collected by one of the authors (B. K. Rao) in November 1991 from a Holy derive place, Meharabad upper hills very nearer to the recent God-Man and the Avatar of the Age AVATAR MEHER BABA Derine tomb, Maharashtra State 414 001, India (Rao *et al.*, 2003). The plant was authenticated by taxonamist Professor Rao P. N. of Nagarjuna University, India. A voucher specimen was deposited in the herbarium of Nagarjuna University.

Preparation of extracts

The air dried plant material (300 g) was extracted with 80% ethanol by maceration and percolation. The solvent was removed and the green extract (1.8 g) was obtained.

Animals

Male Wistar rats purchased from Tokyo Laboratory Animals, Inc. (Tokyo Japan) were housed under controlled conditions (temperature, 23 ℃; light from 07:00 to 19:00) and were provided with standard rat chow and water. Rats weighing about 220 - 280 g were used *in vivo* studies.

Protective effect on gastric mucosal injury

Protective effect of ethanol extract of *I. cordifolia* on HCl/ethanol-induced gastric mucosal injury was assessed in rats. Rats were divided into groups of 7 animals and fasted 24 hr. The ethanol extract of *I. cordifolia* dissolved in 10 % Tween 80 was administrated orally at the dose of 250 mg/kg. After 30 min, 5 ml/kg of HCl/ethanol solution (150 mM HCl / absolute ethanol = 1 : 9 v/v) was orally administrated to the rat. After 1 h, the animals were sacrificed, and the stomachs were removed and fixed by inflation with 10 ml of 2 % formalin solution in phosphate-buffered saline (PBS). Then, they were incised along the greater curvature. The length

of each lesion formed on the glandular portion was measured. The sum of the lengths of lesions in each animal was calculated and expressed as a lesion index (mm).

Assay for anti-HIV activity

Human T cell leukemia virus 1 (HTLV1)-bearing CD4 positive human T cell lines, MT-4 cells, were infected with HIV-1_{IIIB} at a multiplicity of infection (m.o.i.) of 0.01. HIV- or mock-infected MT-4 cells (1.5×10^5/mL, 200 μL) were placed into 96-well microtiter plates in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS) and incubated in the presence of varying concentrations of the compounds tested. After incubation for 5 days at 37 ℃ in a CO_2 incubator, cell viability was quantified by a colorimetric assay (at 540 nm and 690 nm), monitoring the ability of viable cells to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a blue formazan product (Nakashima *et al.*, 1992). All data represent the mean values of triplicate measurements. The values are translated into percentage cytotoxicity and percentage antiviral protection, from which 50 % cytotoxic concentration (CC_{50}) and 50 % effective concentration (EC_{50}) are calculated. The selectivity index (SI) was defined as follows: $SI=CC_{50}/EC_{50}$.

Cell culture

Human squamous cell carcinoma (HSC-2) cells and human submandibular gland tumor (HSG) cells were maintained as monolayer cultures at 37 ℃ in Dulbecco's modified Eagle medium (DMEM) (Gibco BRL, Grand Island, NY, U.S.A.) supple- mented with 10 % heat-inactivated FBS in a humidified 5 % CO_2 atmosphere, and subcultured by trypsinization. Human gingival fibroblasts (HGF) were isolated from the periodontal tissue of healthy gingival biopsy of a 10-year-old female, as described previously (Sakagami *et al.*, 2000), according to the guideline of Meikai University Ethic Committee, after obtaining the informed consent from the patients. Cells between the fifth and seventh passages were used.

Cytotoxic activity

Cells were incubated for 24 hr with the indicated concentrations of test samples in culture medium, and the viable cell number was determined by MTT method (Sakagami *et al.*, 2000). In brief, the cells were washed with PBS, and incubated for 4 hr with fresh culture medium containing 0.2 mg/mL MTT (Sigma Chem. Ind., St. Louis, MO). After removing the medium, cells were lysed with 100 μL DMSO and the absorbance at 540 nm of the cell lysate was measured with Labsystems Mutiskan^R (Biochromatic) with Star/DOT Matrix printer JL-10. The A_{540} values of control HSC-2, HSG and HGF cells were 1.519, 0.884, and 0.283, respectively. The 50 % cytotoxic concentration (CC_{50}) was determined from the dose-response curve.

Radical intensity

Radical intensity was determined at 25 ℃ using electron spin resonance (ESR) spectroscopy (JEOL JES REIX, X-band, 100 kHz modulation frequency). Instrument settings: center field, 336.0 ± 5.0 mT; microwave power, 8 mW; modulation amplitude, 0.1 mT; gain, 630; time constant, 0.03 s; scanning time, 2 min. Radical intensity was determined in the indicated buffer and the radical intensity was defined as the ratio of peak heights of these radicals to that of MnO (Satoh *et al.*, 1997).

Radical scavenging activity against superoxide anion ($O_2^{\cdot-}$) and hydroxy radical (·OH)

$O_2^{\cdot-}$ was generated by hypoxanthine (HX) and xanthine oxidase (XOD) reaction (total volume: 200 μL) [2 mM HX in 0.1 M phosphate buffer

(PB) (pH 7.4) 50 μL, 0.5 mM DETAPAC 20 μL, DMPO (15 %) 30 μL, sample (in H_2O) 50 μL, XOD (0.5 U/mL in PB) 50 μL]. The gain and constant time were changed to 400 and 0.1 s, respectively. The radical intensity was determined by ESR spectro- scopy after 1 min mixing of them. The $O_2^{\cdot-}$ scavenging activity was expressed as super-oxide dismutase (SOD) unit/mg sample, by calibration with standard curve of SOD.

The ·OH was produced by Fenton reaction (200 μL)(1 mM $FeSO_4$ containing 0.2 mM DETAPAC) 50 μL, PB (0.1 M, pH 7.4)] 50 μL, 92 mM DMPO 20 μL, sample (in H_2O) 50 μL, 1 mM H_2O_2, 30 μL]. The gain was changed to 160.

Cell and fluorescence uptake

The *MDR1*/A expressing cell lines were selected by culturing the infected cells with 60 ng/mL colchicine to maintain the expression of the MDR phenotype (Kessel, 1989). The L5178 MDR cell line and the L5178 Y parent cell line were grown in McCoy's 5A medium supple- mented with 10 % heat-inactivated horse serum, L-gluta-mine and antibiotics. The cells were adjusted to a concentration of 2×10^6/mL and resuspended in serum-free McCoy's 5A medium, and 0.5 mL aliquot of the cell suspension were distributed into each Eppendorf centrifuge tube. Then, 10 μL of 2 mg/mL test compounds were added and incubated for 10 min at room temperature. Then, 10 μL rhodamine 123 (R123) as indicator of drug accumulation was added to the extracts (5.2 μM final concentration) and the cells were incubated for a further 20 min at 37 ℃, washed twice and resuspended in 0.5 mL PBS (pH 7.4) for analysis. The fluorescence of cell population was measured by flow cytometry using Beckton Dickinson FACScan instrument. (±)-Verapamil was used as the positive control in R123 accumulation experiments (Weaver *et al.*, 1993). The R123 accumulation was calculated from fluorescence of one height values. Then, the percentage of mean fluorescence intensity was calculated in treated *MDR1* and parental cell lines, compared to untreated cells. The fluorescence activity ratio was calculated by the following equation (Kessel, 1989; Weaver *et al.*, 1993):

MDR1 reversal activity = (*MDR1* treated/*MDR1* control)/(parental treated/parental control)

RESULTS

Antiulcer activity

The antiulcer activity of ethanol extract (**E16**) of *I. cordifolia* was tested. As shown in Figure 1, the extract did show potent gastric mucosal protective activity against stomach injury induced by HCl/EtOH solution. It reduced the lesion index by 81 %.

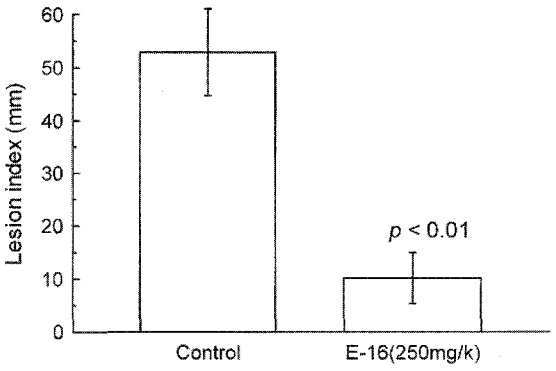

Fig. 1. Protective effect of **E16** extract of *I. cordiforia* against gastric injury induced by HCl/EtOH (1:9) solution. The gastric lesion index is shown as the mean value ±SD. The sample was given orally at a dose of 250 mg/kg.

Cytotoxic activity

Cytotoxic activity of ethanol extract (**E16**) of *I. cordifolia* against two human oral tumor cell lines (HSC-2 and HSG) and human gingival fibro-blasts (HGF) was investigated. The ethanol extract was relatively cytotoxic to two human oral tumor cells, as compared with human

Table 1. Cytotoxic and anti-Hiv activity of **E16** extract of *I. cordifolia*

Compound	50% Cytotoxic concentration (CC_{50}, μg/mL)			SI[a] = CC_{50} (normal)/ CC_{50} (tumor)	Anti-HIV activity		
	Human tumor cell		Normal cells		CC_{50} (μg/mL)	EC_{50} (μg/mL)	SI (CC_{50} /EC_{50})
	HSC-2	HSG	HGF				
E16	279	188	>500	>2.14	= 86.61	> 200	< 1
Gallic acid	19	38	81	2.84	-	-	-
A_{540}	1.444	1.600	0.337	-	-	-	-
DS	-	-	-	-	> 1000	= 3.6784	> 272
CRDS	-	-	-	-	> 1000	= 0.6677	> 1498
AZT (μM)	-	-	-	-	= 284.47	= 0.0129	= 22043
ddc (μM)	-	-	-	-	= 2274.83	= 33.3017	= 689

Near confluent cells were incubated for 24 hours without or with various concentration of each sample, and the relative viable cell number (absorbance at 540 nm of the MTT-stained cell lysate) was determined by the MTT method. The CC_{50} was determined from the dose-response curve. Each value represents the mean from duplicate determination.
[a]determined by the equation: SI=[CC_{50}(HGF)/CC_{50}(HSC-2)+CC_{50}(HSG)]×2

Table 2. Effect of **E16** extract of *I. cordifolia* on the multidrug resistance of L-5178 cells

Compound	Concentration (μg/mL)	FSC[a]	SSC[a]	FL-1[a]	Fluorescence activity ratio
Par (control)[b]	-	582.22	142.22	839.68	30.25
MDR + R123 (control)[c]	-	629.12	212.82	27.76	1.00
(±)-Verapamil	5	626.09	228.71	233.50	8.41
DMSO	20	642.49	234.00	21.47	0.77
E16	20	638.15	221.73	662.31	23.86

[a] FSC: Forward scatter count; SSC: Side scatter count; FL-1: Fluorescence intensity.
[b] Par: a parental cell without MDR gene.
[c] MDR: a parental cell transfected with MDR gene.

normal cell (Table 1). The cytotoxicity of E16 extract was much weaker than that of gallic acid.

Anti-HIV activity

The inhibition of HIV-induced cytopathic effects by ethanol extract of *I. cordifolia* was studied (Table 1). However, there was not a significant inhibition by **E16** extract of the cytopathic effects on HIV infection in MT4 cells using effective concentrations of > 200 μg/mL (Selectivity Index (SI) < 1), compared with four positive controls-dextran sulfate (DS) (SI > 272), curdlan sulfate (CRDS)(SI > 1498), AZT (SI > 22043) and dide-oxycytidine (ddC) (SI > 689).

MDR reversal on tumor cells

The MDR reversing effect of **E16** extract was

compared to that of (±)-verapamil (positive control), using a mouse leukemia cell line (L-5178 cells) (Table 2). The effects were measured by fluorescence ratio (FR) between treated and untreated group cells. **E16** extract of *I. cordifolia* showed exceptionally promising levels of MDR-reversing activity. The extract (FR=23.86) was 3-fold more potent than (±)-verapamil (FR=8.41) (Table 2). Then, the **E16** might be an anti-MDR inducing agent of great interest (Szabo *et al.*, 2000; Kawase and Motohashi, 2003).

Radical

The ESR spectra showed that **E16** extract of *I. cordifolia* dose-dependently reduced the intensity of DMPO-OH, which was a spin adduct of ·OH generated *via* the Fenton reaction. However, the ·OH radical scavenging activity of the extract was weak (IC_{50}=0.31 mg/mL). On the other hand, the extract scavenged weakly the $O_2^{\cdot-}$ produced by hypoxanthine-xanthine oxidase reaction (0.44 SOD unit/mg).

DISCUSSION

It is clearly noted that **E16** extact of *I. cordiforia* possesses the significant both gastroprotective activity and MDR modulating activity. It is suggested that active oxygen species play an important role in gastric lesions (Yoshikawa *et al.*, 1989). Antioxidant might inhibit the mucosal injury by trapping and/or quenching of the oxygen-derived free radical or radical peroxide by radical scavenging effect on gastric mucous. The ESR studies showed that **E16** extract of *I. cordifolia* scavenged both ·OH radical and $O_2^{\cdot-}$, however, the scavenging activities are weak. Therefore, the significant gastroprotective activity of **E16** could be not solely related to the radical-mediated mechanism.

The **E16** showed the promising levels of MDR-reversing activity and might restore the drug accumuration in cancer cells by inhibiting the Pgp-mediated efflux pump (Szabo *et al.*, 2000; Kawase and Motohashi, 2003). The study also demonstrates the tumor-specific cytotoxic action of the plant extract.

The results apparently indicate the existence of therapeutically useful substances and the chemotherapeutic value of *I. cordiforia*. Further work is necessary to isolate active principles and elucidate the actual mechanism involved in the gastroprotective and MDR reversal activities of this plant.

REFERENCES

Dahot MU. (1999) Antibacterial and antifungal activity of small protein of *Indigofera oblongifolia* leaves. *J. Ethnopharm.* **64,** 277-282.

Finnegan RA, Mueller WH. (1965) Chemical examination of a toxic extract of *Indigofera endecaphylla. J. Pharm. Sci.* **54,** 1136-1144.

Hartwell JL. (1970) Plants used against cancer. *Survey Lloydia* **33,** 288-392.

Hasan A, Ahmad I, Khan MA, Chudhary MI. (1996) Two flavonol triglycosides from flowers of *Indigofera Hebepetala. Phytochem.* **43,** 1115-1118.

Hasan A, Farman M, Ahmed I. (1994) Flavonoid glycosides from *Indigofera herbepetala. Phytochem.* **35,** 275-276.

Kawase M, Motohashi N. (2003) New multidrug resistance reversal agents. *Current Drug Targets* **4,** 31-43.

Kessel D. (1989) Exploring multidrug resistance using rhodamine 123. *Cancer Commun.* **1,** 145-149.

Miller RW, Smith CR Jr. (1973) Seeds of *Indigofera* species: their content of amino acids that may be deleterious. *J. Agric. Food Chem.* **21,** 909-912.

Nakashima H, Murakami T, Yamamoto N, Sakagami H, Tanuma S, Hatano T, Yoshida T,

Okuda T. (1992) Inhibition of human immunodeficiency viral replication by tannins and related compounds. *Antiviral Res.* **18,** 91-103.

Rao BK, Motohashi N, Kawase M, Spengler G, Molnar J. (2003) Multidrug resistance reversal in mouse lymphoma cells by Indian tea leaves, Indian coffee seeds and chicory. *Orient. Pharm. Exp. Med.* **3,** 100-105.

Sakagami H, Jian Y, Kusama K, Atsumi T, Ueha T, Toguchi M, Iwakura I, Satoh K, Ito H, Hatano T, Yoshida T. (2000) Cytotoxic activity of hydrolyzable tannins agaist human oral tumor cell lines-A possible mechanism. *Phytomed.* **7,** 39-47.

Samy RP, Ignacimuthu S, Raja DP. (1999) Preliminary screening of ethnomedical plants from India. *J. Ethnopharm.* **66,** 235-240.

Satoh K, Sakagami H, Motohashi N. (1997) Radical modulation activity of benz[*a*]phenothiazine. *Anticancer Res.* **17,** 2539-2544.

Souza MA, Bieber LW, Chiappeta AA, Maciel GM, Mello JF, Monache FD, Messana I. (1988) Arylbenzofurans from *Indigofera microcarpa. Phytochem.* **27,** 1817-1819.

Singh B, Saxena AK, Chandan BK, Bhardwai V, Gupta VN, Suri OP, Handa SS. (2001) Hepatoprotective activity of indigtone: a bioactive fraction from *Indigofera tinctoria* Linn. *Phytother. Res.* **15,** 294-297.

Sreepriya M, Devaki T, Balakrishna K, Appараnantham T. (2001a) Effect of *Indigofera tinctoria* Linn on liver antioxidant defense system during D-galactosamine / endotoxin-induced acute hepatitis in rodents. *Indian J. Exp. Biol.* **39,** 181-184.

Sreepriya M, Devaki T, Nayeem M. (2001b) Protective effects of *Indigofera tinctoria* L. against D-galactosamine and carbon tetrachloride challenge on *in situ* perfused rat liver. *Indian J. Physiol. Pharmacol.* **45,** 428-434.

Szabo D, Keyzer H, Kaise HE, Molnar J. (2000) Reversal of multidrug resistance of tumor cells. *Anticancer Res.* **20,** 4261-4274.

Thangadurai D, Viswanathan MB, Ramesh N. (2002) Indigoferabietone, a novel abietane diterpenoid from *Indigofera longeracemosa* with potential antituberculous and antibacterial activity. *Pharmazie* **57,** 714-715.

Weaver JL, Szabo G, Pine PS, Gottesman MM, Goldenberg S, Aszalos A. (1993) The effect of ion channel blockers, immunosuppressive agents, and other drugs on the activity of the multidrug transporter. *Int. J. Cancer* **54,** 456-461.

Yoshikawa T, Ueda S, Naito Y, Takahashi S, Oyamada H, Morita Y, Yoneta T, Kondo M. (1989) Role of oxygen-derived free radicals in gastric mucosal injury induced by ischemia or ischemia-reperfusion in rats. *Free Rad. Res. Commun.* **4,** 285-291.