Journal of Ethnopharmacology 141 (2012) 424–431

Contents lists available at SciVerse ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

In vitro and *in vivo* immunomodulatory activities of iridoids fraction from *Barleria prionitis* Linn

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ARTICLE INFO

Article history:
Received 10 September 2011
Received in revised form 8 December 2011
Accepted 3 March 2012
Available online 13 March 2012

Keywords:
Barleria prionitis
Iridoids fraction
Immunostimulant activity
HPTLC standardization

ABSTRACT

Ethnopharmacological relevance: Barleria prionitis Linn. (Family: Acanthaceae), one of the important Ayurvedic medicinal plant in India, has long been used to treat variety of ailments including swellings, gout, arthritic and rheumatic disorders, nervine and skin diseases, and also acts as immunorestorative.
Aim of the study: The present study was aimed to explore *in vitro* and *in vivo* immunomodulatory activities of the iridoids fraction *i.e.* n-butanol fraction of methanol extract from *Barleria prionitis* aerial parts (IFBp).
Materials and methods: IFBp was studied for *in vitro* [nitroblue tetrazolium (NBT) test and neutrophils candidacidal assay] and *in vivo* immunomodulatory activity on cellular and humoral immune responses to the antigenic challenge by sheep red blood cells (SRBCs) and by neutrophil adhesion test, phagocytic activity and cyclophosphamide-induced myelosuppression. The study comprised the preliminary phytochemical screening, HPTLC standardization and maximum tolerable dose determination of IFBp.
Results: IFBp (50, 100 and 200 μg/ml) significantly ($P \leq 0.01$) increased the intracellular killing activity of stimulated neutrophils assayed by *in vitro* NBT reduction test and neutrophils candidacidal assay. Pretreatment of IFBp (100 and 200 mg/kg; p.o.) evoked a significant increase in percent neutrophils and neutrophils adhesion to nylon fibres. Oral administration of IFBp augmented the humoral immune response to SRBCs, evidenced by increase in antibody titres and dose dependently potentiated the delayed-type hypersensitivity reaction induced by SRBCs in mice. IFBp potentiated significantly ($P \leq 0.01$) the macrophage phagocytic activity and ameliorated the red blood cells, total white blood cells and platelets count and haemoglobin concentration, and also restored the myelosuppressive effects induced by cyclophosphamide. The content (% w/w; mean ± SD, $n = 3$) of main iridoids *i.e.* shanzhiside methyl ester and barlerin was found to be 21.55 ± 2.40 and 10.03 ± 1.69 in IFBp of BP, respectively.
Conclusion: The present investigation reveals that IFBp is a potent immunostimulant, stimulating both the specific and non-specific immune mechanisms.

1. Introduction

Modulation of immune responses to alleviate disease has been of interest for many years and the concept of 'Rasayana' in Ayurveda is based on related principles (Charak Samhita, 1949). Many medicinal plants classified as "Rasayana" in Ayurveda are believed to be useful in strengthening the immune system of an individual (Patwardhan et al., 1991). The concept of immunomodulation relates to a non-specific activation of the immune system. It implies primarily a non-antigen dependent stimulation of the function and efficiency of macrophages, granulocytes, complement, natural killer cells, lymphocytes and also the production of various effector molecules by activated cells (para-immunity). Being non-specific, it is expected to give protection against different pathogens including bacteria, fungi, viruses *etc.* and constitutes an alternative or adjunct to conventional chemotherapy (Wagner, 1984).

Barleria prionitis Linn. (BP) (Family: Acanthaceae) is found throughout the tropical regions of India, Sri Lanka and South Africa. BP is an annual shrub, locally known as "Vajradanti" in India and "Katukaradu" in Sri Lanka, and is widely used in folk medicines. The herb's extract is prescribed for massage in toothache, swellings, arthritis and gout. In folk medicine, BP is widely used to treat nervine disorders, boils and glandular swellings, leprosy and other skin diseases, rheumatic affections, internal abscesses, chronic sinusitis *etc.* (Nadkarni, 1954; Chopra et al., 1966; Asolkar et al., 1992; The Aurvedic Pharmacopoeia of India, 1999; Khare, 2004).

Abbreviations: BP, *Barleria prionitis*; IFBp, iridoids fraction *i.e.* n-butanol fraction of methanol extract from *Barleria prionitis* aerial parts; HPTLC, high performance thin layer chromatography; NBT, nitroblue tetrazolium; SRBCs, sheep red blood cells; HBS, Hank's balanced salt; NaCl, sodium chloride; RBCs, red blood cells; PBS, phosphate buffer saline; MTD, maximum tolerable dose; TLC, total leukocyte cells; DLC, differential leukocyte cells; HA, haemagglutinating antibody; DTH, delayed-type hypersensitivity; RES, reticulo-endothelial system; WBCs, white blood cells; CMI, cell-mediated immunity.

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doi:10.1016/j.jep.2012.03.005

In line with these traditional medicinal uses, BP has been reported to possess anti-respiratory syncytial virus (Chen et al., 1998), antiarthritic, anti-inflammatory and hepatoprotective activities (Sing et al., 2003, 2005), antihepatotoxic, antistress and immunorestorative (Suri et al., 2003), glutathione *S*-transferase, acetylcholinesterase inhibitory and antibacterial (Kosmulalage et al., 2007) activities. Previous work on the iridoid constituents (monoterpene lactone glucosides) of BP have led to the isolation and identification of shanzhiside methyl ester, 8-O-acetyl shanzhiside methyl ester (barlerin), 6,8-O,O-diacetyl shanzhiside methyl ester (acetylbarlerin) (Taneja and Tiwari, 1975; Damtoft et al., 1982; Byrne et al., 1987; Fathalla et al., 2009) and 6-O-trans-*p*-coumaryl-8-O-acetyl shanzhiside methyl ester and its cis isomer (Chen et al., 1998).

The present study was therefore undertaken to explore *in vitro* [nitroblue tetrazolium (NBT) test and neutrophils candidacidal assay] and *in vivo* immunomodulatory activities of the iridoids fraction *i.e.* n-butanol fraction of methanol extract from *Barleria prionitis* aerial parts (IFBp) on cellular and humoral immune responses to the antigenic challenge by sheep RBCs and by neutrophil adhesion test, phagocytic activity and cyclophosphamide-induced myelosuppression.

2. Materials and methods

2.1. Materials

A Camag HPTLC (high performance thin layer chromatography) system (Muttenz, Switzerland) including a Linomat V sample applicator was used. HPTLC precoated Silica gel 60 F_{254} plates (20 cm × 20 cm) (Merck, Darmstadt, Germany) were used. Double beam UV visible spectrophotometer (Shimadzu Corporation, Kyoto, Japan), vaccum evaporator (Bio-Technics India^{TM}) and digital plethysmometer were used. Shanzhiside methyl ester and barlerin, isolated and identified in our laboratory, were used as biomarkers. Hanks balanced salt (HBS) solution, Sabouraud's 2% dextrose broth and *Candida albicans* (ATCC 10231) fungal culture were purchased from HiMedia, Mumbai, India. All other chemicals like dimethylsulphoxide, sodium chloride, trypan blue, eosin, sodium deoxycholate, methylene blue, nitroblue tetrazolium (NBT), glucose, sodium citrate, citric acid, sodium carboxymethyl cellulose, gelatin, sodium carbonate *etc.* were purchased from Loba Chemie (Mumbai, India) and HiMedia (Mumbai, India). All the organic solvents and chemicals were of analytical grade and used as obtained.

2.2. Plant material

At the flowering stage, aerial parts of *Barleria prionitis* Linn. (BP) (Family: Acanthaceae) were collected from Wardha district, Maharashtra State, India during the month of November–December and authenticated at Department of Botany, Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur. A voucher specimen (number 9188) is deposited in the Institute of Pharmaceutical Education and Research, Wardha for the future reference. Fresh aerial parts were cleaned, shade dried and coarse to finely powdered by grinder and then sieved through mesh sieve # 20 and stored in air tight container until further use.

2.3. Extraction of plant material and preparation of iridoids fraction (IFBp)

The shade dried and coarse-finely powdered aerial parts (1 kg) of BP were extracted successively with petroleum ether (60–80 °C) and methanol by Soxhlet extraction method. The completion of extraction was ensured by taking a few ml of extractant from the thimble of Soxhlet apparatus in a porcelin dish and ensuring that no residue remains after evaporating the solvent. Methanol extract was dried at 50 °C under vaccum, the extractive value (% w/w) was found to be 16.82 of dry weight of plant material. Previous works on BP have led to the isolation and identification of mainly iridoid constituents (Taneja and Tiwari, 1975; Damtoft et al., 1982; Byrne et al., 1987; Chen et al., 1998). Wagner and Bladt (1996) suggested that iridoids can be enriched into n-butanol fraction from the parent extract. Therefore methanol extract (100 g) was suspended in 200 ml of 20% v/v methanol in distilled water and fractionated successively, in a separating funnel, with different volumes of n-butanol (50, 50, 50, 40, 40, 40, 35, 35, 30, 20, 20 ml) until complete fractionation. The n-butanol fractions were combined and vaccum evaporated to dryness to obtain iridoids fraction (IFBp). The extractive value of IFBp was found to be 42.83% w/w of the methanol extract from BP.

2.4. Preliminary phytochemical screening

IFBp was subjected to preliminary phytochemical screening (Harborne, 1984; Trease and Evans, 2008) for the detection of various phytoconstituents.

2.5. Compositional analysis of IFBp by HPTLC method

A Camag HPTLC system (Muttenz, Switzerland) including a Linomat V sample applicator, a Camag Twin-Trough TLC chamber, Camag TLC scanner III and Wincats integration software was used. Aluminium-backed HPTLC plates (10 cm × 20 cm) with 200 nm thickness of silica gel 60 F_{254} (Merck, Darmstadt, Germany), prewashed with methanol, were used. Shanzhiside methyl ester and barlerin being main iridoids present in BP were used as biomarkers for the standardization of methanol extract and IFBp from BP by HPTLC method. Methanol solvent was used to prepare stock solutions of the samples and the standard markers. From stock solutions of methanol extract (3 mg/ml) and IFBp (2 mg/ml) of BP, different concentrations (5, 10 and 15 μl) were spotted in the form of bands of width 6 mm by means of a Linomat V sample applicator to the plate. Similarly from stock solutions of shanzhiside methyl ester and barlerin (100 μg/ml), different volumes *i.e.* 2, 4, 6, 8 and 10 μl were spotted on the TLC plates to obtain concentration of 200, 400, 600, 800 and 1000 ng per spot. A constant application rate of 1 μl/15 s was employed and the chromatogram was developed upto 80 mm under chamber saturation (20 min) conditions with chloroform-methanol (80:20, v/v) as the mobile phase in a Camag twin-trough TLC chamber. Subsequent to the development, TLC plates were dried in a current of air with the help of air dryer. Densitometric scanning was performed on Camag TLC scanner III in the absorbance mode at 240 nm. The source of radiation utilized was a Deuterium lamp. The data of peak area plotted against the corresponding concentrations were treated by linear regression analysis.

2.6. In vitro immunomodulatory activity

2.6.1. Preparation of neutrophils

Neutrophils were isolated from venous blood of healthy volunteers. The heparinized blood (5 ml containing 100 units of heparin) was added to 1 ml of 4.5% dextran B in physiological saline. The mixture was gently shaken and allowed to stand for 60 min at room temperature to sediment erythrocytes. Neutrophils were isolated by Ficoll–Hypaque density gradient centrifugation according to Ferrante and Thong (1980). After removal of the residual erythrocytes by hypotonic lysis, the neutrophils were washed with HBS solution. The cells were suspended at a final concentration of 5×10^6 neutrophils/ml in HBS solution for NBT reduction test and