Journal of Ethnopharmacology 134 (2011) 872–877

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

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

In vitro anti-inflammatory and anti-cancer activities of *Cuscuta reflexa* Roxb.

V. Suresh[a], V. Sruthi[b], B. Padmaja[c], V.V. Asha[a,*]

[a] *Bioprospecting and Molecular Pharmacology Lab, Rajiv Gandhi Centre for Biotechnology Thiruvananthapuram, Kerala 695014, India*
[b] *School of Biotechnology, Chemical & Biomedical Engineering VIT University, Vellore, Tamilnadu, India*
[c] *Ayurveda Research Institute for Mother and Child Health Care, Thiruvananthapuram, India*

ARTICLE INFO

Article history:
Received 25 March 2010
Received in revised form
14 December 2010
Accepted 25 January 2011
Available online 1 February 2011

Keywords:
Cuscuta reflexa Roxb.
Inflammation
Cancer
RAW264.7
Hep3B

ABSTRACT

Aim of the study: To determine anti-inflammatory and anti-cancer activities of *Cuscuta reflexa* in cell lines (*in vitro*).
Materials and methods: Anti-inflammatory activity of the water extract was analysed *in vitro* using lipopolysaccharide (LPS) induced inflammatory reactions in murine macrophage cell line RAW264.7. The expression of COX-2 and TNF-α genes involved in inflammation was analysed by SQ RT-PCR. EMSA was conducted to analyse the influence of the extract on NF-κB signalling. Anti-cancer activity was analysed on Hep3B cells by MTT assay, DAPI staining, annexin V staining and SQ-RT PCR analysis of BAX, Bcl-2, p53 and survivin.
Results: The extract down regulated LPS induced over expression of TNF-α and COX-2 in RAW264.7 cells; blocked NF-κB binding to its motifs and induced apoptosis in Hep3B cells as evidenced from MTT, DAPI staining and annexin V staining assays. The extract up regulated pro-apoptotic factors BAX and p53, and down regulated anti-apoptotic factors Bcl-2 and survivin.
Conclusions: The study showed that *Cuscuta reflexa* inhibits LPS induced inflammatory responses in RAW264.7 cells through interplay of TNF-α, COX-2 and NF-κB signalling. It induced apoptosis in Hep3B cells through the up regulation of p53, BAX and down regulation of Bcl-2 and survivin.

1. Introduction

Recent studies have shown that chronic inflammation can progressively lead to cancer and the role of inflammatory reactions leading to neoplasia is also well documented (Aggarwal et al., 2006b). The progression of cancer from a single cell involves the interplay of numerous inflammatory modulators (Coussens and Werb, 2002). These complex set of reactions decide the fate of a cell-malignancy or normal growth. Chronic inflammation has been reported to be linked with steps involved in tumorigenesis and metastasis (Coussens and Werb, 2002; Mantovani, 2005). Compounds that can block or alter inflammatory reactions can have tremendous potential in the management, prevention and treatment of cancer (Aggarwal et al., 2006a,b). If a drug could interfere with the inflammatory reactions and induce apoptosis at the same time in cancer cells, it could be useful in both cancer prevention and therapy (Aggarwal et al., 2006b).

Cuscuta reflexa Roxb., commonly known as dodder, belongs to the family Convolvulaceae. Interestingly, the folk medicine of Bangladesh uses this herb to cure tumours (Costa-Lotufo et al., 2005). The Tripura tribe of Bangladesh and Satar tribes of Nepal has attributed a multitude of functions to this plant – for the treatment of oedema and body ache, as an aphrodisiac, maintenance of hepatic system, for alleviation of skin infections and also for curing jaundice (Siwakoti and Siwakoti, 1999; Hossan et al., 2009). Based on this tribal information, this plant was analysed for its anti-inflammatory and anti-cancer activities *in vitro*. Since this plant is used to cure inflammation, cancer and liver disorders, we have used *in vitro* models for hepatic inflammation and hepatic cancer for the study. The macrophages in liver (also called Kupffer cells) have important roles in mediating the inflammatory response in liver (Knolle and Gerken, 2000). We have used the macrophage cell line as a model in inflammatory studies and hepatocellular carcinoma cell lines for anti-cancer studies.

Abbreviations: DMEM, Dulbecco's modified Eagle medium; TNF-α, tumour necrosis factor α; COX-2, cyclooxygenase-2; dNTPs, di nucleotide triphosphates; EMSA, electrophoretic mobility shift assay; NF-κB, nuclear factor κB; DTTD, di thiothretol; DAPI, 4′,6-diamidino-2-phenylindole; G3PDH, glyceraldehyde 3 phosphate dehydrogenase; BAX, Bcl2 associated X protein; LPS, lipopolysaccharide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

* Corresponding author. Tel.: +91 471 2529457; fax: +91 471 2348096.
E-mail addresses: vvasha@rgcb.res.in, ashavv@rediffmail.com (V.V. Asha).

0378-8741/$ – see front matter © 2011 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.jep.2011.01.043

2. Materials and methods

2.1. Cell lines and reagents

RAW264.7, an immortalised murine macrophage cell line, and human hepatocellular carcinoma cell line Hep3B were obtained from American Type Culture Collection (ATCC), Manassas, USA. DMEM, Antibiotic–antimycotic mixture and HEPES were obtained from Gibco – BRL, USA. Trypsin, ethidium bromide, FBS, DEPC, glycerol, bromophenol blue, xylene cyanol, primers, lipopolysaccharide, dithiothretol, nonidet P40, phenyl methane sulphonyl fluoride (PMSF), ficoll 400, bradford reagent, labelled primer and SDS were obtained from Sigma, USA. DMSO, hexane, chloroform, isopropanol, acetic acid are from Merck India pvt. Ltd. Oligo dT, RT buffer, dNTPs, RNase inhibitor, AMV-RT enzyme, PCR master mix, agarose, poly (dI-dC), and T4 kinase were from Promega, Madison, USA. TRIzol, $Na_2EDTA{\cdot}2H_2O$ and EDTA were from Invitrogen, Carlsbad, USA. $NaHCO_3$, KH_2PO_4, and NaH_2PO_4 from Himedia, Mumbai, India. NaCl, $MgCl_2$, EGTA and KCl from Qualigen's, Mumbai, India.

2.2. Collection of the plant and extraction

The plant *Cuscuta reflexa* Roxb., was collected from the outskirts of Trivandrum, Kerala, India. A voucher specimen was kept in the institute herbarium (Ethno 31). It was dried in shade, powdered and sieved. 10 g of the powder was extracted with 300 ml of double distilled water in room temperature. The decoction is then lyophilized (Model Freezone, Labconco Corporation, MO, USA) at a very low temperature (−45 °C) and pressure (30 psi). The powdery residue obtained was stored at −20 °C and used for the experiments.

2.3. Anti-inflammatory assays

2.3.1. Cell culture

Murine macrophage cell line RAW264.7 was used for the anti inflammatory assays. The cells were cultured and maintained on DMEM with 10% fetal calf serum and 1% antibiotic–antimycotic in a humidified atmosphere of 5% CO_2 at 37 °C in an incubator (Sheeba and Asha, 2009).

2.3.2. Cytotoxicity assay

The cytotoxicity of the extract to RAW264.7 cells was analysed by MTT assay (Liu and Schubert, 1998; Sheeba and Asha, 2009). Briefly, the cells were seeded in to 96 well culture plates and treated with different concentrations of CR (100, 200, and 300 μg/ml) and Silymarin (50 μg/ml) for 24 and 48 h. After the treatment, the cells were rinsed with PBS and incubated in 5 mg/ml MTT reagent dissolved in PBS for 4 h. Cells were then permeabilized by the addition of SDS (10% in DMSO). The absorbance at 570 nm was read on a microplate reader (Biorad Model 680; Bio-Rad Laboratories, Hercules, CA, USA) after 10 s of vigorous machine shaking. The percentage of cell viability was calculated as:

$$\text{Percentage growth inhibition} = 100 - (\text{mean OD of individual test group} \times 100)$$

Mean OD of the control:

$$\text{Percentage cell viability} = 100 - \text{percentage inhibition}$$

2.3.3. PCR amplification and quantification (SQ-RT-PCR)

The study was conducted in 6 well plates and were divided into control, DMSO control, LPS control, LPS + CR (25, 50 and 100 μg/ml) treatment groups in triplicates. The normal group was kept untreated and DMSO was added in DMSO group. The extract treatment groups were treated with respective doses of the CR. The final concentration of DMSO in the medium was adjusted to 0.1% in all cases. After 30 min of the treatment with extract, LPS (lipopolysaccharide, 10 μg/ml) was added and incubated for 4 h. The total RNA was then isolated from different treatment groups by Trizol method (Suresh and Asha, 2008; Sheeba and Asha, 2009). The RNA was quantified using a spectrophotometer (Perkin Elmer, USA) at 260 nm. cDNA was prepared from the total RNA (2 μg) by reverse-transcription using an oligo (dT) 18 mer as a primer and M-MLV reverse transcriptase. The expression levels of COX-2, TNF-α and β-actin (internal control) were analysed by PCR amplification of these genes by using specific primers at specific annealing temperatures (Supplementary Table 1) in a thermal cycler (iCycler, Biorad, USA). The PCR reactions (25 μl) were carried out in 2 mM $MgCl_2$, 10 mM dNTPs, PCR buffer and 2 units of Taq DNA polymerase in the thermal cycler. After amplification, the amplicons were electrophoresed in 1.2% agarose gel stained with ethidium bromide. The bands were quantified by densitometry on Gel documentation System using quantity one software (Biorad, USA). Values were normalised by calculating the densitometry ratio with that of internal control β-actin as base line.

2.3.4. Electrophoretic mobility shift assay (EMSA) for NF-κB binding

The assay was performed as described previously (Sheeba and Asha, 2009). Briefly RAW264.7 cells were seeded into 6 well plates and incubated with various concentrations of CR (25, 50 and 100 μg/ml) for 1 h along with untreated normal control and DMSO control. The cells were then treated with 10 μg/ml of LPS for 1 h. The nuclear extract from the cells were prepared using a high-salt detergent buffer (totex buffer). Cells were harvested by centrifugation, washed once in ice cold PBS and re-suspended in four cell volumes of totex buffer. The cell lysate was incubated on ice for 30 min and then centrifuged at 13,000 rpm at 4 °C for 5 min. NF-κB oligonucleotide was labelled using [^{32}P] ATP (3000 Ci/mmol). Equal amounts of protein was (10–20 μg) added to a reaction mixture containing 20 μg of bovine serum albumin, 2 μg of poly (dI-dC), 2 μl of buffer D, 4 μl of buffer F and 100,000 cpm (cerenkov) of [^{32}P] – labelled oligonucleotide, made up to a final volume of 20 μl with distilled water. Samples were incubated at room temperature for 25 min. The electrophoresis was performed in a non-denaturant polyacrylamide gel (4%) in TBE buffer at pH 8.0, which was then developed using a Kodak XAR-5 film (Kodak Corporation, Rochester, NY, USA). Only active NF-κB can bind to the DNA, therefore, disappearance of the band indicates inhibition of DNA binding by the drug.

2.4. Anticancer assays

2.4.1. Cell culture

The hepatocellular carcinoma cell line, Hep3B was used for the anticancer assays. This cell line was maintained in DMEM as described above for the RAW264.7 cells.

2.4.2. MTT assay for the analysis of growth inhibition by the extract

Hep3B cells were seeded on to 96 well micro plates and were treated with different concentrations of water extract of CR (25, 50, and 100 μg/ml) for 24 and 48 h. The cytotoxicity was analysed and the percentage of growth inhibition was calculated as described above.

2.4.3. Visual detection of apoptosis by DAPI staining

The assay was performed on Hep3B cells treated with different concentration of the extracts (CR 20, 50, and 100 μg/ml),