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Protective effect of ethyl acetate fraction of *Drynaria quercifolia* against CCl_4 induced rat liver fibrosis via Nrf2/ARE and NFκB signalling pathway

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ABSTRACT

Ethnopharmacological relevance: Drynaria quercifolia rhizome is traditionally used as hepatoprotective drug especially in chronic jaundice.

Aim of the study: The present study was undertaken to scientifically evaluate the efficacy of *D. quercifolia* rhizome against liver fibrosis.

Materials and methods: D. quercifolia rhizome crude extract (DQ) and its fractions of hexane (HDQ), ethyl acetate (EDQ), butanol (BDQ) were evaluated *in vitro* using primary hepatocytes and RAW 264.7 cells. *In vivo* anti-liver fibrotic activity of EDQ was assessed using CCl_4 induced liver fibrosis in Wistar rats and serum biochemical parameters (AST, ALT, ALP, SB, cholesterol), MDA, PT, INR, GSH, SOD, CAT, liver glycogen, serum albumin levels were monitored. qRT-PCR analysis of TNF-α, COX-2, iNOS were performed. ELISA method was used to estimate TNF-α, COX-1 & 2. Histopathological studies like H & E, Masson's trichrome, immunohistochemistry staining for α-SMA, TIMP-1, Nrf2 were conducted. LC-Q-TOF-MS analysis of EDQ was conducted.

Results: In vitro activity guided fractionation of *D. quercifolia* revealed EDQ as active fraction when compared to other extracts. EDQ treatment significantly inhibited the expression of α-SMA, TIMP-1, COX-2, TNF-α, iNOS and increased the levels of Nrf2 in rat liver fibrosis. LC-Q-TOF-MS analysis of EDQ confirmed the presence of naringin and naringenin.

Conclusion: The anti-liver fibrotic activity of EDQ is via inhibition of NFκB signalling pathway, antioxidant response through Nrf2 activation and further inhibition of HSC activation.

1. Introduction

Liver fibrosis is one of the leading causes of morbidity which results from an excessive accumulation of extracellular matrix proteins that are produced due to liver injury. Liver fibrosis is most commonly caused by alcoholism, viral hepatitis, fatty liver disease, etc. Untreated liver fibrosis further progress to cirrhosis, portal hypertension and liver failure. The terminal outcome of liver fibrosis is the development of liver cirrhosis and hepatocellular carcinoma. In this stage, liver transplantation is the only possible treatment.

Liver fibrosis is induced by free radical injury, oxidative stress, chronic inflammatory response, activation of hepatic stellate cell, deposition of collagen and formation of fibrillar scar matrix. The transdifferentiation of quiescent hepatic stellate cell (HSC) to activated HSC and its transformation into myofibroblasts is the key event in liver fibrosis (Bataller and Brenner, 2001). Activated HSCs express α-smooth muscle actin (α-SMA), cause increased synthesis of extracellular matrix proteins, promotes the expression of tissue inhibitor of metalloproteinase (TIMP), loss of retinoid-storing capacity, enhanced cell migration, increased proliferation, production of chemotactic proteins and acquisition of fibrogenic capacity (Atzori et al., 2009; Moreira, 2007; Robert et al., 2016; Ghazwani et al., 2013).

The currently available anti-fibrotic therapies focus on suppression of hepatic inflammation and removal of the causative agents rather

Abbreviations: AST, Aspartate transaminase; ALT, Alanine transaminase; ALP, Alkaline phosphatase; SB, Serum Bilirubin; H & E, Hematoxylin and eosin; COX-1 & 2, Cyclooxygenase 1 & 2; LOX, Lipoxygenase; TNF- α, Tumour necrosis factor-α; α-SMA, α-smooth muscle actin; MMP, matrix metalloproteinases; TIMP-1, Tissue inhibitor of metalloproteinase-1; iNOS, Inducible nitric oxide synthase; GSH, Reduced Glutathione; SOD, Superoxide Dismutase; CAT, Catalase; MDA, Malondialdehyde; HSC, Hepatic stellate cells; PT, prothrombin time; INR, international normalized ratio; Nrf2, Nuclear factor erythroid 2-related factor 2; NF-κB, Nuclear factor κB; NFκBP65, p65 subunit of NFκB; ARE, antioxidant response element; NBT, Nitroblue tetrazolium; qRT-PCR, quantitative real-time polymerase chain reaction; IHC, Immuno histochemistry; LPS, Lipopolysaccharide; Keap1, kelch-like ECH-associated protein-1; ECM, extra cellular matrix; CCl_4, Carbon tetrachloride; LC-QTOF-MS, Liquid Chromatography Quadrupole Time-of-Flight Mass; ANOVA, Analysis of Variance; cDNA, complementary deoxyribonucleic acid

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than subduing fibrosis (Iredale, 2003). Hence, anti-fibrotic treatment which prevents the progression of liver cirrhosis would be an effective therapeutic approach. The reversal of HSC activation terminates the fibrogenesis (Troeger et al., 2012). The reversal of HSC activation can take place at different levels such as inhibition of HSC activation, inactivation of profibrogenic cytokines, inhibition of matrix synthesis and increased matrix degradation by the drugs (Beljaars et al., 1999; Feng and Wang, 2009). Thus HSC are attractive targets for antifibrotic therapy.

Drynaria quercifolia (L.) J. Smith (Polypodiaceae) is an epiphytic/epilithic medicinal pteridophyte, widely distributed in Asia and Australia. It is locally called 'Marappannakizhangu' or 'Attukalkizhangu' in Western Ghats, Kerala, India. The Tribes of Western Ghats use the rhizomes as a vegetable as well as hepatoprotective drug especially in chronic jaundice. Pounded mass of about 5–10 g of the rhizome with previous day's rice gruel/juice of coconut kernel is administered thrice daily for four days by Kani people to treat chronic jaundice along with a salt free diet (Asha et al., 1992). The rhizome soup is consumed as food supplement and as a remedy for rheumatism (Saravanan et al., 2013). In traditional Indian system of medicine, the rhizome is used as astringent, tonic and to treat fever, gonorrhoea, malaria, inflammation, migraine (Warrier et al., 1995). Tribes of Kolli hills, India, use the rhizome as an anti-inflammatory agent (Irudayaraj and Senthamarai, 2004).

We have already reported the anti-inflammatory, analgesic, anti-allergic properties of *D. quercifolia* and the presence of naringin and naringenin in *D. quercifolia* (Anuja et al., 2010, 2014a, 2014b). Compounds like β-sitosterol, β-amyrin, friedelin, epifriedelinol, 3-β D-glucopyranoside were also reported from *D. quercifolia* (Ramesh et al., 2001). The hepatoprotective effect of fronds of *Drynaria quercifolia* was earlier reported by Kamboj and Kalia (2013). *In vitro* hepatoprotective activity of crude methanolic extract of *Drynaria quercifolia* L. rhizome was reported by Devika and Prasanna (2016).

In the present study, we have conducted the activity guided study of *D. quercifolia* rhizome extracts using *in vitro* primary rat hepatocytes and murine macrophage RAW 264.7. The nuclear translocation of nuclear factor-κB (NFκB) p65 subunit in lipopolysaccharide (LPS) treated RAW 264.7 was investigated using confocal microscopy. For further elucidation of the therapeutic potential of ethyl acetate fraction of *D. quercifolia* rhizome against carbon tetrachloride (CCl_4) induced rat liver fibrosis, studies were carried out with the special focus on HSC activation markers like α-SMA, TIMP-1; anti-inflammatory markers such as tumour necrosis factor-α (TNF-α), cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), and antioxidant marker Nuclear factor erythroid 2-related factor 2 (Nrf2).

2. Materials and methods

2.1. Chemicals

Silymarin, naringenin (PubChem CID 439246), naringin (PubChem CID 4441) and custom primers were obtained from Sigma Chemicals Co., USA. Carbon tetrachloride was obtained from Hi-Media, India. Serum biochemical assay kits were procured from Crest Bio systems, India. ELISA Kit for TNF-α was purchased from Ray Biotech, USA and COX kit from Cayman Chemical, USA. Trizol was procured from Ambion Life technologies, USA. Qubit 3.0 was obtained from Life technologies, USA. cDNA preparation kit and High Capacity cDNA synthesis Kit were obtained from Thermo Fisher Scientific, USA. SYBR Green Master Mix was procured from Bioline, London, UK, α-SMA antibody was purchased from Elabscience, USA. TIMP-1 antibody was purchased from Biorbyt, UK and NF-κB p65 antibody, Nrf2 antibody from Thermo Fisher Scientific, USA. Goat anti-rabbit IgG-horseradish peroxidase (HRP) was obtained from Santa Cruz Biotechnology Inc., USA.

2.2. Collection of plant material

The rhizomes of *D. quercifolia* were collected from Thiruvananthapuram, India and authenticated by Dr. N. Mohanan, plant taxonomist of JNTBGRI. A voucher specimen was deposited at JNTBGRI, herbarium (TBGT 57025).

2.3. Preparation of plant extract and fractions

The rhizomes were washed thoroughly, shade-dried and powdered. 100 g powder was extracted with 1 L of 70% aqueous ethanol at room temperature for 24 h, the procedure was repeated twice and filtered. The filtrate was concentrated using rotary evaporator, vacuum dried in desiccator and the yield (w/w %) of extract was found to be 18%. This crude extract was referred to as DQ, which was further fractionated by liquid-liquid partition. 10 g of DQ was suspended in 100 mL of distilled water, re-extracted five times with equal volumes (100 mL) of hexane. The hydrophilic phase was then extracted five times with equal volumes (100 mL) of ethyl acetate. Remaining hydrophilic phase was further extracted five times with 100 mL of butanol. Each fractions were concentrated using rotary evaporator, and vacuum dried in desiccator. The yields (w/w %) of the fractions were found to be 6%, 2%, 1% for hexane, ethyl acetate and butanol respectively. Hexane fraction was referred to as HDQ, ethyl acetate fraction as EDQ and butanol fraction as BDQ. The crude extract and fractions were suspended in 1% Tween-80 to required concentrations and used for the pharmacological experiments.

2.4. Liquid chromatography quadrupole time-of-flight mass spectrometry (LC-Q-TOF-MS) analysis of EDQ

EDQ was subjected to chromatographic separation on Xevo G2 (Waters) Quadrapole-Time-of-Flight (Q-TOF) fitted with UPLC BEH C18 column (50 mm × 2.1 mm × 1.7 μm; Acquity H class, Waters). Gradient elution was started from 0.1 min using 0.1% formic acid in water (Solvent A; 95%) and methanol (Solvent B; 5%), continued up to 6 min (solvent A; 5% and solvent B; 95%). Initial solvent system was applied after 6 min to re-equilibrate the column, operated at flow rate 0.3 mL/min. Mass spectra was obtained in positive and negative electronic spray ionization mode. Source temperature was set at 135 °C and desolvation temperature at 350 °C.

2.5. In vitro primary hepatocyte culture study with plant extract and fractions

Hepatocytes were isolated from rat liver by collagenase perfusion and suspended in calcium free Hank's balanced salt solution (HBSS) containing anhydrous disodium hydrogen phosphate, potassium chloride, potassium dihydrogen orthophosphate, sodium chloride, D-glucose, and hepatocytes were cultured using Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, penicillin (100 IU/mL), streptomycin (100 μg/mL) and insulin (0.8 IU/mL). Hepatocyte attachment to the culture dish was complete at 2 h after plating and medium was changed to remove unattached or dead cells. At 18 h after plating, hepatocytes were treated with plant extract and fractions (DQ/HDQ/EDQ/BDQ) in different concentrations (25, 50, 100 μg/mL). For dose standardization we have used concentrations of different extracts starting from 5 μg/mL upto 100 μg/mL. No significant protection was observed in 5–20 μg/mL concentrations when compared to CCl_4 control. Hence, we have selected 25, 50, 100 μg/mL doses. After 24 h, hepatocytes were incubated with 5 mM CCl_4 for 1.5 h in the presence or absence of plant extract, fractions dissolved in 0.1% dimethyl sulphoxide (DMSO). Standard drug silymarin (100 μg/mL) was used as positive control. Hepatocytes cultured without CCl_4 treatment served as normal control. Hepatocyte damage was assessed by measuring levels of AST, ALT leakage into the medium by fully automatic