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Anti-inflammatory and preventive activity of white mulberry root bark extract in an experimental model of pancreatitis

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ABSTRACT

Pancreatitis is characterized by highly morbid inflammation in the pancreas. Currently, there is no specific drug available for pancreatitis except supportive medicines. The present study assessed the pancreato-protective effect of *Morus alba* root bark extract by using alcohol and cerulein-induced model of pancreatitis. The study also investigated the phytochemical profile through GC-MS and HPLC. Methanolic extract of *Morus alba* root bark extract (MEMARB) was subjected to GC-MS and HPLC studies. Male albino Wistar rats were administered ethanol (0%–36%) and cerulein (20 μg/kg b.wt. i.p.) with or without MEMARB. Serum lipase, amylase, caspase-1, lipid peroxidation products, glutathione and enzymatic antioxidants were determined. Histological changes in the pancreas were assessed. Cudraflavone B in MEMARB was quantified by HPLC. Significant amount of Cudraflavone B was detected by quantitative HPLC. Marked increase in the levels of serum amylase, lipase, caspase-1, IL-18 and IL-1β were observed in ethanol and cerulein administered rats than in MEMARB co-administered rats. In MEMARB co-administered rats, the antioxidant status was restored to near normal levels. Histological examinations showed that MEMARB significantly reduced the inflammatory and fibrotic changes. The results reveal the potent pancreato-protective effects of *Morus alba* root bark. The anti-inflammatory effect of *Morus alba* root bark extract might be due to the presence of various phytonutrients including Cudraflavone B.

1. Introduction

Pancreatitis is an inflammatory response initiated in the pancreatic parenchyma due to acinar cell injury. The underlying pathomechanism is the premature ectopic activation of the master proteolytic zymogen trypsinogen, to active trypsin, precipitating a cascade of zymogen activation and 'auto digestion' of the pancreas.[1]

Persistent, irreversible inflammation and fibrosis of the pancreas are characteristics of chronic pancreatitis (CP). Symptoms of CP include abdominal pain, frequent acute pancreatitis (AP) episodes, exocrine and endocrine insufficiency.[2] Alcohol abuse causes 70% of CP while smoking, gene mutations, hyperparathyroidism and autoimmunity are other etiologies.[3] Chronic pancreatitis has a worldwide prevalence of 50/100,000 persons. Tropical calcific pancreatitis (TCP), a rare form of CP, has a prevalence of 20–125/100,000 persons in southern India. 5% of CP patients and 40–50% of hereditary pancreatitis patients develop highly lethal pancreatic cancer.[4]

Current management guidelines recommend only supportive measures like hospitalization, intensive fluid resuscitation, bowel rest, parenteral nutrition, enzyme supplements and pain management through non-steroidal anti-inflammatory drugs (NSAIDs).

In both acute and chronic pancreatitis injured and dying pancreatic acinar cells are the primary drivers of inflammation and initiators of necroptosis and pyroptosis, key determinants of disease severity. Acinar cells elaborate immune responses by secreting cytokines (TNF-α, IL-1β, IL-6, and IL-10), chemokines (MCP-1) and endogenous damage associated molecular patterns (DAMPs).

NLRP3 inflammasome is a multiprotein intracellular innate immune sensor consisting of NLRP3, apoptosis-associated speck-like protein (ASC) and procaspase-1. It assembles in response to

Abbreviations: AP, acute pancreatitis; CP, chronic pancreatitis; TCP, tropical calcific pancreatitis; MCP 1, monocyte chemotactic protein 1; DAMPS, damage associated molecular patterns; PSC, pancreatic stellate cell; NF-kappa B, nuclear factor kappa-light-chain-enhancer of activated B cells; AP1, activator protein 1; MEMARB, methanolic extract of *Morus alba* root bark.

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diverse stimuli and forms the scaffold for the activation of pro-inflammatory cytokines IL-1beta and IL-18 and induces the release of HMGB1 expression important in pancreatic inflammation, parenchymal cell injury and disease resolution. Pro-inflammatory cytokines IL-1 beta and IL-18 are triggers of pyroptosis, a highly lytic form of cell death, which appears to be predominant in pancreatitis.[5–7]

In the face of a threat from rising pancreatitis cases and the absence of a specific licensed drug, inflammatory pathways have been under scrutiny to find suitable targets for potential anti-inflammatory drug molecules. Various anti-inflammatory effectors that have been tested include thalidomide (targets TNF-alpha), panhaematin (decreases leukocyte infiltration), IL-R antagonist montelukast, MCP 1 inhibitors, COX - 2 inhibitor flavocoxid, vitamin K3 (inhibits autophagy) and the broad anti-inflammatory effects of agents like quercetin, resveratrol and curcumin.[1]

Morus alba L. or white mulberry is native to northern China and has been naturalized and cultivated throughout Asia and Europe. It has long-standing ethno medicinal significance. Various parts of the plant have been used in traditional Asian medicine. Phytochemical analyses have identified alkaloids, flavonoids, flavones, flavanones, stilbenes, benzophenones, coumarin derivatives and terpenoids in the root bark of *M. alba*[8]. *Morus* is one of the few genera to contain prenylated flavonoids. Prenyl flavonoids are credited with enhanced biological effects attributed to the prenyl side-chains.[9] But, their scientific validation should be taken care of.

The bioactive principles from *Morus alba* root bark are reported to have antibacterial, antiviral, antioxidant, hypoglycemic, neuroprotective, nephroprotective, antiulcer, analgesic and anti-inflammatory properties.[10]

The aim of the present investigation is to assess the therapeutic efficacy of phytonutrients of white mulberry roots with special reference to their influence on the level of cytokine production and their impact on acinar cell damage monitored in terms of serum and pancreatic marker enzymes and cellular antioxidants.

2. Materials and methods

2.1. Chemicals

Reference standards for HPLC - gallic acid, galangin, rutin, quercetin and thymoquinone - were obtained from LGC Promochem India Pvt. Ltd., (Bangalore, India). HPLC grade solvents were purchased from Merck India. ELISA kit for IL-Iβ was purchased from Abcam and Invitrogen ELISA kit for IL-18 was purchased from Thermo Fisher Scientific. Lipase and alpha-amylase assay kits were procured from Coral Clinical Systems, Goa, India. All other chemicals used were of analytical grade.

2.2. Plant material collection, identification and extraction

Fresh *Morus alba* roots were collected from the Plant Sciences department, University of Madras, Guindy Campus, Chennai, department of Sericulture, Vitchanthangal, Kancheepuram District, Tamil Nadu and a private mulberry farm at Purisai, Kancheepuram District, Tamil Nadu. The plant material was authenticated by Professor P. Jayaraman, Director, Plant Anatomy Research Centre, West Tambaram, Chennai. The herbarium specimen (PARC/2015/3144) was preserved for future reference in the Department of Biochemistry, Bharathi Women's College. The roots were pooled, cleaned under tap water and air-dried for 2–3 weeks. Completely dry roots were lightly scraped to reveal the yellowish layer underneath. The root bark or cortex was peeled off with the help of a knife, cut into small pieces and powdered in a blender. The crude powder was sieved to obtain a fine homogenous powder. *M.alba* root bark powder was soaked in methanol and left to agitate on a shaker for 24hrs. The extract was filtered. The residue was extracted again with fresh methanol to ensure complete extraction. The filtrates were air dried to a powder, sieved and stored in an air-tight container at 4 °C. For animal experimentation, a homogenous suspension of the bark powder was prepared with 0.1% DMSO.

2.3. Animals

Adult male albino Wistar rats (175–200g, seven-eight weeks old) used for the study were housed under hygienic conditions [22-24 °C] in polypropylene cages under 12 h light/12 h dark cycle. The animals were allowed free access to water and standard pelleted rat chow during the acclimatization period. Animal maintenance and experimentation protocols conformed to the guidelines of the Institutional Animal Ethics Committee constituted by the Committee for the Purpose of Control and Supervision of Experiments on Animal (CPCSEA), Government of India, [XVII/VELS/PCOL/02/2000/CPCSEA/IAEC/06.10.15].

2.4. Experimental protocols

2.4.1. Preliminary phytochemical screening by GC-MS

The methanolic extract of the root bark of white mulberry (*M.alba*) was subjected to GC-MS analysis. The analysis was carried out on Agilent 6890N gas chromatograph with HP-5ms column coupled to a mass spectrometer JEOL GC-MATE II in the electron ionization (EI) mode with ionization voltage set to 70eV. The mass spectral scan range of the mass analyser was set to 50–600 amu. Helium was used as a carrier gas at a constant flow of 1 mL/min. The front inlet temperature was 220 °C. The GC-oven was set for the following temperature profile: ramp rate from 50 °C-250 °C at 10 °C/min. NIST (National Institute of Standards and Technology) GC-MS mass spectral database was used to interpret the mass spectrum using the retention time.

2.4.2. HPLC for identification of flavonoids and quantification of Cudraflavone B

Accurately weighed quantities of the standards and the sample were transferred to separate volumetric flasks and dissolved in methanol and diluted to a specific concentration. A specific quantity of *Morus alba* root bark powder was refluxed with extraction solvent (methanol: water: hydrochloric acid) for 135 min to hydrolyse the flavonoid glycosides and contents were made up with methanol. An equal volume of the standard and sample (20 μL) was then used for the HPLC analysis on Shimadzu (Japan), HPLC Class VP series with a UV–vis detector. The samples were run on a C18 column (100 Å pore size, 3.5 μm, 4.6 mm × 250 mm) 40 min run time. The mobile phase used was a mixture of methanol, water and phosphoric acid mixed in the ratio (100:100:1). The elution was isocratic with the flow rate set at 1.5 mL/min. The flavonoids were monitored by the ultraviolet detector set at 270 nm. EZChrom Data System was used for data acquisition, processing and report generation. The resulting chromatograms were recorded and the areas under the major peaks measured. Flavonoids were identified by matching the retention time and their spectral characteristics against those of the standards.

2.4.3. Ethanol and cerulein-induced chronic pancreatitis

After one-week of acclimatization, the animals were randomly divided into four groups of six animals each. Group 1: received normal diet (standard rat chow) for 5 weeks; Group 2: received the normal diet and MEMARB (300 mg/kg body weight/day) orally for the last 3 weeks of the experimental period; Group 3: received an ethanol containing isocaloric diet, and 20 μg/kg body weight of

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