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Research Paper

Swietenia macrophylla King induces mitochondrial-mediated apoptosis through p53 upregulation in HCT116 colorectal carcinoma cells

Bey Hing Goh [1], Chim Kei Chan, Muhamad Noor Alfarizal Kamarudin, Habsah Abdul Kadir *

Biomolecular Research Group, Biochemistry Program, Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia

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ABSTRACT

Ethnopharmacological relevance: Swietenia macrophylla King is a traditional herb used to treat various diseases including hypertension, diabetes and cancer. Previous study demonstrated its anti-tumor effect but the potential mechanisms have not been clearly defined. The current study was to further investigate the underlying mechanism of ethyl acetate fraction of *Swietenia macrophylla* (SMEAF)-induced anti-proliferative effect and apoptosis in HCT116 colorectal carcinoma cell.

Materials and methods: Cell viability was evaluated in HCT116 cells by trypan blue exclusion assay. Apoptotic cell death was detected by Hoechst 33342/propidium iodide (PI) staining and intracellular reactive oxygen species (ROS) was analyzed by flow cytometry. The apoptotic gene and protein expression were determined by Real-time quantitative PCR (q-PCR) and immunofluorescence staining using flow cytometry, respectively.

Results: SMEAF significantly inhibited HCT116 cell viability and induced apoptosis in a dose-dependent manner. SMEAF-induced apoptosis was triggered by the activation of p53 and intracellular reactive oxygen species (ROS) production. Moreover, the significant increase in p53 was accompanied by a decrease murine double minute 2 (MDM2) expression. SMEAF significantly increased the expression of the Bax protein resulting in a markedly elevated Bax/Bcl-2 ratio which may have triggered the mitochondrial apoptotic pathway, resulting in caspase-3/7 and caspase-9 activation.

Conclusion: These results suggested that SMEAF exerts its antitumor activity in HCT116 cells by activating proapoptotic signaling pathway through intracellular ROS formation triggering the mitochondrial-mediated pathway via p53 activation.

1. Introduction

Colorectal cancer (CRC) is currently one of the most prevalent malignancies in the world making it a major public health concern (Johnson, 2012). In the last 10 years, CRC has overtaken lung cancer as the second most common cancer among Malaysians, raising serious concerns about the poor dietary habits, especially the consumption of foods high in saturated fats. Although early diagnosis often leads to a complete cure, in most cases the polyps go undetected. In such cases, therapies such as surgical intervention, chemotherapy and radiation are often insufficient to treat the disease, thus needing other preventive or unconventional therapeutic strategies. Consequently, there is an emerging interest in exploring the use of phytochemicals in cancer therapy. Phytochemicals have been widely reported to mediate diverse machineries against cancer, comprising cell cycle arrest, immune response, induction of apoptosis and DNA damage (Chiang et al., 2010). Therefore, controlling the growth and proliferation of cancerous cells and inducing apoptosis are major goals in cancer chemoprevention.

The most classic feature of cancer cells is to evade apoptosis, a form of programmed cell death which regulates the development and homeostasis of mammalian cells under a variety of physiological and pathological conditions including oxidative stress. Thus, promotion of apoptotic cell death is a potential therapeutic approach for prevention and treatment of cancer (Hanahan and Weinberg, 2000). The mechanisms implicated are diverse and appeared to involve a combination of cell signaling pathways at multiple levels including inhibition of NF-kB activation, upregulation of the proapoptotic protein levels, increase in cytochrome c release, cell cycle arrest, DNA damage, activation of p53 and caspase-3 (Shishodia et al., 2005; Sahu et al., 2009). Apoptosis can be initiated by two major signaling pathways, namely, the death receptor (extrinsic) pathway and the mitochondrial (intrinsic) pathway (Fulda and Debatin, 2006) that involves the activation of a series of molecular events leading to cell death. Stimulation of intrinsic mitochondrial mediated pathway by reactive oxygen

* Corresponding author. Tel.: +60 3 79674363; fax: +60 3 79614178.
E-mail address: habsah@um.edu.my (H. Abdul Kadir).
[1] Present address: Brain Research Institute, Jeffrey Cheah School of Medicine and Health Sciences, Monash University Malaysia, Jalan Lagoon Selatan, Bandar Sunway, Selangor 46150, Malaysia.

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species (ROS) will eventually activate the apoptotic cascade, consequently facilitates further release of apoptogenic factors (Pourova et al., 2010).

p53 is a tumor suppressor protein encoded by the TP53 gene in human. It has been described as "the guardian of the genome" since its activation induces cell cycle arrest, senescence, differentiation and response to cellular stress such as DNA damage, oncogene activation, telomere erosion and hypoxia (Vousden, 2000). Thus, p53 is especially related to the initiation of apoptotic cell death and its activation in tumor cells has been recognized as a promising strategy for cancer treatment and several drugs targeting p53 are recently tested in preclinical and clinical trials (Cheok et al., 2010). One of the crucial events for initiating p53 activation is through ROS generation, where the ROS generated by stressed cells would trigger expression of p53 which is sensitive to redox change and eventually leads to apoptosis (Cardaci et al., 2008). Studies also have shown physical and functional interactions of p53 with various members of Bcl-2 family providing the basis for an alternative route of p53-mediated cell death (Speidel, 2010).

Swietenia macrophylla is an endangered forest timber plant, locally known as "sky fruit", belonging to the Meliaceae family (50 genera and 1400 species) and growing natively throughout the tropical regions of the Americas, in particular Central and South American countries such as Mexico and Bolivia and in west India, Malaysia, and southern China (Moghadamtousi et al., 2013). *Swietenia macrophylla* has been used in Asia and many other countries to treat diverse ailments based on its antimicrobial, anti-inflammatory, antioxidant effects, antimutagenic, anticancer, antitumor and antidiabetic activities. Almost all parts of the plant are used in traditional medicine for the treatment of various human ailments. The seed of *Swietenia macrophylla* in particular has significant medicinal properties and has been reported to possess anti-inflammatory, antimutagenic and antitumor activity (Goh and Kadir, 2011; Moghadamtousi et al., 2013). Various species of *Swietenia* particularly *Swietenia macrophylla*, *Swietenia humilis* and *Swietenia mahagony* have been used traditionally to treat cancer in countries such as Mexico and Malaysia (Graham et al., 2000; Camacho et al., 2003; Divya et al., 2012; Roy et al., 2013). In Malaysia, *Swietenia macrophylla* is considered as a common and well known medicinal plant for treatment of various cancers including colon cancer (Jin, 2012). Previous pharmacological investigations of these species have provided the scientific basis for the ethnomedicinal use of these plants for cancer treatment. The methanolic extract of the leaves and bark of *Swietenia macrophylla,* and the bark of *Swietenia humilis* showed cytotoxic effect on nasopharyngeal carcinoma (KB). In addition, *Khaya senegalensis* (syn: *Swietenia senegalensis*) bark extract was reported to possess anti-proliferative, anti-inflammatory and pro-apoptotic effects on HT-29, HCT-15 and HCA-7 human colorectal cells and hypothesized to be useful in the prevention and treatment of colorectal cancer (Androulakis et al., 2006). Limonoids is the major class of phytochemicals that are commonly purified and identified from *Swietenia* plants and has proven to be responsible for majority of their bioactivities including cytotoxicity (Moghadamtousi et al., 2013). The limonoids derived from *Swietenia humilis*, namely, humilinolide A, B, C and D when tested against lung carcinoma (A-549), breast carcinoma (MCF-7) and colon adenocarcinoma (HT-29) cell lines revealed selective cytotoxic activity against HT-29 cells (Jimenez et al., 1997).

The above studies have demonstrated that *Swietenia* species and their bioactive constituents have a significant role in disease prevention including cancer and especially colon cancer. Our previous study revealed that *Swietenia macrophylla* ethyl acetate fraction was selectively cytotoxic against HCT116 cells as evident by the apoptotic characteristic features such as cell shrinkage, chromatin condensation, membrane blebbing and phosphatidylserine externalization (Goh and Kadir, 2011), suggesting that the ethyl acetate fraction may induce apoptosis in HCT116 cells. However, the underlying mechanisms for the antiproliferative and apoptosis-inducing effects of *Swietenia* species and *Swietenia macrophylla* in particular have not been investigated. These mechanistic data are crucial not only in validating the ethnomedicinal uses of these plants as anticancer agents but more importantly to provide novel molecular target sites that are imperative to the current targeted chemopreventive strategies. In this context, this study was conducted for the first time to analyze potential apoptosis-inducing mechanisms on human adenocarcinoma cells HCT116.

2. Materials and methods

2.1. Preparation of ethyl acetate fraction from Swietenia macrophylla seeds

From our previous report (Goh and Kadir, 2011), seeds of *Swietenia macrophylla* were purchased from a local supplier and upon authentication, a voucher specimen (No. KLU046901) was deposited at the herbarium in the Institute of Biological Sciences, University of Malaya, Kuala Lumpur, Malaysia. In brief, the crude ethanol extract of *Swietenia macrophylla* seeds was prepared by decanting and concentrating the ethanol that was used in soaking the powdered seeds for 72 h. The crude ethanol extract was further extracted repeatedly using hexane. The hexane insoluble residue was then partitioned by using ethyl acetate and water (1:1). Finally, the *Swietenia macrophylla* ethyl acetate fraction (SMEAF) was obtained by evaporating the ethyl acetate soluble fraction to dryness.

2.2. Cell culture

The human colorectal carcinoma (HCT116, ATCC number CCL-247) was purchased from American Type Culture Collection (ATCC, USA). HCT116 cells were maintained in RPMI 1640 Medium (Sigma). The medium was supplemented with 10% (v/v) heat-inactivated fetal bovine serum (PAA Lab, Austria), 100 μg/mL streptomycin and 100 unit/mL penicillin (PAA Lab, Austria) and 50 μg/mL amphotericin B (PAA Lab, Austria). The medium was filter-sterilized by using a 0.2 μm filter membrane (Minisart, Sartorius Stedim). The cells were cultured and conditioned in 5% CO_2 incubator at 37 °C in a humidified atmosphere.

2.3. Cell viability and morphological change analysis by trypan blue dye exclusion assay

Cell viability was determined by a trypan blue dye exclusion assay. Cells (1×10^6 cells) in 60 mm^2 culture dishes were incubated overnight, and then the medium was replaced with new medium with various concentrations of SMEAF (0.05–1 mg/mL) and incubated for 24 h. After the incubation period, each cell suspension was mixed with an equal volume of 0.4% Trypan blue solution for 5 min. The cells were then observed under a microscope, and viable cells were counted using hemocytometer. For morphological observation, cells were seeded and incubated for 24 h before further incubated with or without 0.05 mg/mL of SMEAF for another 6, 12, 24, 48 and 72 h. Following incubation period, the morphological changes were observed using phase contrast inverted microscope (Leica DMI 3000B, Germany) at 400 × magnifications.