Journal of Ethnopharmacology 193 (2016) 195–206

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

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

Chemopreventive effect of *Phaleria macrocarpa* on colorectal cancer aberrant crypt foci *in vivo*

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ARTICLE INFO

Article history:
Received 20 August 2015
Received in revised form
26 July 2016
Accepted 1 August 2016
Available online 2 August 2016

Keywords:
Phaleria macrocarpa
Azoxythemane
Aberrant crypt foci
Antioxidant activity
PCNA
Bax

ABSTRACT

Ethnopharmacological relevance: Natural products are important ingredients for pharmaceutical applications specifically new entities for treating cancer and other diseases. *Phaleria macrocarpa* is native of Indonesia and considered as a prolific source of bioactive substances useful for chemoprevention.
Aim of the study: To investigate the chemopreventive properties of *Phaleria macrocarpa* on azoxymethane (AOM)-induced aberrant crypt foci (ACF) in rats.
Methods: The biological activities of the ethanol extract of *P. macrocarpa* fruits were evaluated both *in vitro* and *in vivo*. First the extract was investigated for its *in vitro* antioxidant activity by the total phenolic content and ferric reducing antioxidant power assay. Then the chemopreventive effect of *P. macrocarpa* was performed on AOM-induced aberrant crypt foci as colorectal carcinoma model in rats.
Result: the crude ethanolic extract of *P. macrocarpa* has high antioxidant activity and modulated the oxidative stress as proved by the up-regulation of glutathione-s-transferase and superoxide dismutase. Immunohistochemical staining of the treated sections showed overexpression of PCNA and Bax, reduced crypt sizes and numbers, indicating the characteristic feature of apoptotic cancer cells. PCNA is a landmark of cell damage and turn-over and can be associated with clinical cancer mutation. The most potent doses were 250 mg/kg and 500 mg/kg as compared to 35 mg/kg 5-fluorouracil.
Conclusion: In this sense, the potential modulation of the colorectal pathophysiological pathway by *P. macrocarpa* natural compounds mostly flavonoids offer a great possibility for the discovery of new leads towards the colorectal cancer.

1. Introduction

Excluding breast cancer in female, colorectal cancer is the most common cancer diagnosed. More than million new cases of CRC are identified global every year and also the second most common cause of cancer deaths in the US and other countries (Jemal, 2011). While in Malaysia; a newest study reviewing 1212 CRC patients undertaking treatment between January 2001 and December 2010 (Magaji, 2014) with majority of patients are above 40 years old (Shah, 2014; Yusoff, 2012). The numbers are slightly lower in women than in men with increasing death rate in both male and female survivors. A number of factors can affect the overall risk for developing colorectal cancer; *i.e.*, processed food and high red meats diet, physical inactivity, overweight, smoking, heavy alcohol use, among others. The frequent and prolonged exposure to certain risk factors plays a fundamental role in acquiring consequent

Abbreviations: ACF, aberrant crypt foci; AOM, azoxymethane; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ANOVA, analysis of variance; BUN, blood urea nitrogen; FRAP, ferric reducing antioxidant power; GST, glutathione S-transferase; H&E stain, hematoxylin & eosin stain; HD, high dose; HPLC, high performance liquid chromatography; KRAS, Kirsten Rat Sarcoma Viral Oncogene Homolog; LC-MS, liquid chromatography-mass spectrometry; LD, low dose; MDA, malondialdehyde; MTT, 3-(4,5-Dimethylthiazol2-yl)-2,5-diphenyltetrazoliubromide assay; P. macrocarpa, Phaleria macrocarpa; ROS, reactive oxygen species; RAS, reticular activating system; SD, sprague-dawley; SEM, standard error of the mean; SOD, superoxide dismutase; TP, total protein

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http://dx.doi.org/10.1016/j.jep.2016.08.002
0378-8741/

gene mutations. The gene mutation attributes to imbalanced cellular homeostasis and subsequent gene alteration. Adenomatous polyposis coli (APC) gene and the RAS gene are the earliest two genes to get affected in the colon cancer (Fearon and Vogelstein, 1990). The overexpression of APC gene leads to increase of the growth rate of colorectal cells followed by uncontrollable expansion and spread whereas further up- and down-regulation of other genes, including KRAS and TP53 continues (Xavier, 2010).

Colorectal cancer is a slowly developing tumor originating from the mucosal layer of the colon or rectum and growing as adenomatous polyps. The mucosal layer, shaped as microvilli is constantly shedding out old cells and replenish new by division. The development of the adenomatous polyps, despite benign at the start, can be a sign of a greater cancer risk when they are growing into the walls and invading into lymph nodes or blood vessels. Moreover, the tumor can penetrate all layers of the colon wall and press the nearby organs or extend to the abdominal cavity. Surgical excision with local removal of the draining lymph nodes is the first line of treatment once the cancer is diagnosed. Local recurrence and distance metastasis are very common in most of the cases. Once the cancer penetrates adjacent systems or sends metastasis onto the lymph or blood vessels, surgical intervention is not the optimal solution. Chemotherapy stands as the second option to incorporate in the treatment strategy. Commonly 2 or more chemotherapeutic drugs are combined to eradicate colorectal cancer. The treatment required several chemotherapeutic cycles with each cycle takes often about 4 weeks and in recent reports up to 6 months are required for adjuvant chemotherapy. Very often, 5-Fluorouracil (5-FU), followed by capecitabine, irinotecan, oxaliplatin are the main choices but they are highly cytotoxic chemicals that have adverse effects on the normal cells.

Phaleria macrocarpa (Scheff.) Boerl (Family: Thymelaceae) is commonly known as crown of god and its local name in Malaysia is mahkota dewa. It originates from Papua Island, Indonesia and it grows in tropical areas. This plant is one of the most popular medicinal plants in Indonesia. *P. macrocarpa* grows throughout the year in tropical areas reaching a height of around 1–6 m. It is a complete tree (stem, leaves, flower and fruit) and the fruit shape is eclipse with a diameter of around 3 cm. The color of the fruit is green before ripening and red when fully ripe. Traditionally, *P. macrocarpa* (Scheff) Boerl is used to control cancer, impotency, diabetes mellitus, allergies, liver and heart disease, kidney disorders, blood diseases, acne, stroke, migraine, and various skin diseases (Zhang, 2006). Studies about the effects of *P. macrocarpa* on colon cancer are scarce, particularly dietary chemoprevention studies with respect to colon carcinogenesis.

Various components of mahkota dewa have been traditionally used for ethnomedical properties. The stems, fruits, seeds and bark were believed to contain variety of bioactive compounds such as patuletin 3-(6″-acetylglucoside), trans-BTP dioxolane, 2, 3, 4′-trihydroxy-4-methoxybenzophenone, vitexin 2″-p-hydroxybenzoate, 2,4-dimethyl-tetradecanoic acid, schizonepetoside E, nonoxynol 9 and other alkaloids, terpenoids, flavonoids, polyphenols, saponins, resins, lignin, and benzophenones (Tambunan and Simanjuntak, 2006; Tjandrawinata, 2010). In the course of our continuing studies on natural products, the plant materials derived from the *P. macrocarpa* have been found to exhibit high chemopreventive and antioxidant activity comparable to those of conventional drugs, and this activity has been attributed to the high phenolic content. That was in agreement to studies performed in 2003, where the anti-proliferative, anti-inflammatory and anti-carcinogenic properties of the plant extracts were proven *in vitro*. The species was investigated as part of our on-going project on chemical and biological studies of herbal plants used in traditional medicine (El-Seedi, 2007; El-Seedi, 2013).

In this study, the activity of the ethanol extract was studied using several *in vivo* test systems to complement the underlying mechanisms following the colorectal cancer induction standard characterized by the multi aberrant crypt foci. Moreover, the impact of the ethanolic extract on the antioxidant markers activity was investigated.

2. Materials and methods

2.1. Plant specimen and extract preparation

Dried fruits of *P. macrocarpa* plant were obtained from Ethno Resources Sdn Bhd, Malaysia, and identified by comparison with the voucher specimen deposited at the Herbarium of Rimba Ilmu, Institute of Science Biology, University of Malaya, Kuala Lumpur. The fruits were grounded and soaked in 900 mL of 95% ethanol at room temperature (30 ± 2 °C). The suspension was shook from time to time to allow complete dissolve of the powder in the ethanol. Subsequently, the ethanol extract was filtered (and evaporated under low pressure using (Buchi, Switzerland) rotatory evaporator to obtain the final crude-dried extract. The percentage yield of ethanol extracts of *P. macrocarpa* was found to be 12.0% (w/w). The dry extract was then dissolved in 5% Tween 20 and administered orally to the rats in concentration of 250 and 500 mg/kg, respectively.

2.2. Chemicals

Azoxymethane (AOM) is a potent carcinogen used to induce ACF in the rat colon. It was purchased from (Sigma-Aldrich, Switzerland) in 100 mg vial and stored at −20 °C until further use. It was diluted with normal saline to 10 mL and given subcutaneously at 15 mg/kg body weight to the rats once a week for two weeks (Andersson, 2008). 5-Fluorouracil (Sigma Chemical Co., St. Louis, MO, USA) was used as reference drug and dissolved in normal saline prior to the intraperitoneal injection to the rats at a dose of 35 mg/kg body weight (Tanaka, 2001).

2.3. Antioxidant measurement in vitro

2.3.1. Total phenolic content (TPC)

The concentration of phenolics in crude extract of *P. macrocarpa* was determined by the Folin–Ciocalteu method and the content of phenolics in crude extract was expressed as mg of gallic acid equivalent per g of extract (Gan, 2010). In brief the measurement of TPC was performed by mixing equal volumes of the ethanol extract (1 mg/mL DMSO) and 10% Folin-Ciocalteu reagent in a 96-well plate, incubated for 5 min then added 10% sodium carbonate solution. After 90 min incubation, the total phenolic content was read at 750 nm and the measurement was compared to a standard curve of prepared gallic acid (GA) solution. The TPC was expressed as milligrams of gallic acid equivalents (GAE) per gram of dry plant extract (mg GAE/g) (AOAC, 1995).

2.3.2. Ferric reducing antioxidant power (FRAP) assay

The ferric reducing antioxidant power (FRAP) assay had been used to determine antioxidant activity according to the method developed by (Benzie and Strain, 1996). The reaction mixture contained 10 mmol/l TPTZ (2, 4, 6-tripyridyl-s-triazine) with 300 mmol/L acetate buffer, in 20 mmol/L of $FeCl_3 \cdot 6H_2O$ and 40 mmol/l of HCl. The FRAP reagent was prepared by mixing 25 mL of acetate buffer, 2.5 mL of TPTZ solution and 2.5 mL of $FeCl_3 \cdot 6H_2O$. The prepared mixture was incubated in water bath at 37 °C for five minutes followed by a blank read at 593 nm using spectrophotomer. 10 μL of standard ethanol extract and 90 μL of distilled water were added to 900 μL of the working FRAP reagent.