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Research Paper

Effects of *Kaempferia parviflora* rhizomes dichloromethane extract on vascular functions in middle-aged male rat

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ABSTRACT

Ethnopharmacological relevance: In Thai traditional medicine, rhizomes of *Kaempferia parviflora* (KP) have been used for treating hypertension and for the promotion of longevity with good health and well being. Ageing is one of the most important risk factors for development of cardiovascular disease. To investigate whether a 6 weeks oral administration of a dichloromethane extract of fresh rhizomes of *Kaempferia parviflora* (KPD) had any effects on vascular functions, on the accumulation of lipid, as well as on any signs of gross organ toxicity in middle-aged rats.

Materials and methods: Fresh rhizomes of *Kaempferia parviflora* were first macerated twice with 95% ethanol to remove the dark color before extracting three times with 100% dichloromethane. The dichloromethane extract was evaporated under reduced pressure to obtain the dried *Kaempferia parviflora* dichloromethane extract (KPD). The rats were orally administered with the KPD at a dosage of 100 mg/kg body weight, or with the same volume of the vehicle (tween 80, 0.2 g: carboxymethylcellulose sodium, 0.2 g: distilled water 10 ml) once or twice a day for 6 weeks. Vascular functions were studied on isolated thoracic aorta and the mesenteric artery. The vascular eNOS enzyme was measured by Western blot analysis. Blood chemistry was measured by enzymatic methods. Liver cell lipid accumulation was measured using oil red O staining.

Results: A 6 weeks treatment of KPD once a day had no significant effects on any of the studied parameters. When the KPD was given twice a day, the contractile responses to phenylephrine of the thoracic aorta and mesenteric artery were lower than the vehicle control group, and this effect was abolished by N^G-nitro-L-arginine or by removal of the vascular endothelium. Vasorelaxation to acetylcholine, but not to glyceryl trinitrate, by the thoracic aortic and mesenteric ring precontracted with phenylephrine was higher from the KPD treated rats than those from the vehicle control groups. Western blot analysis showed a higher quantity of thoracic- and mesenteric-eNOS protein obtained from the KPD treated rats. In addition, the body weight, serum glucose and triglycerides levels, visceral and subcutaneous fat, as well as liver lipid accumulation were all significantly decreased in the KPD treated rats compared to those of the vehicle control. No differences were found between the KPD treated-, and the vehicle-control for animal food intake, internal organ weight, serum ALP, SGOT, SGPT, BUN and creatinine levels, serum cholesterol, HDL-C and LDL-C levels, nor total blood cell counts.

Conclusions: The chronic oral administration of KPD extract, to middle aged rats, caused a decrease in vascular responsiveness to phenylephrine with an increase in the acetylcholine induced vasorelaxation, due to an increase in nitric oxide production from their blood vessels. The extract also caused a decrease in visceral and subcutaneous fat, fasting serum glucose and triglyceride levels and liver lipid accumulation, with no changes to liver and kidney functions or to total blood cell counts. It is possible that these KPD extracts could be developed as a health product for mid-aged humans to reduce obesity, diabetes type II and cardiovascular disease.

1. Introduction

Age is one of the most important risk factors for the development of metabolic syndrome and/or cardiovascular diseases.

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Advancing age is associated with increased body fat especially the accumulation of lipid in the viscera and liver (Park et al., 2006), and this can be responsible for the age-related risks of diabetes type II, hypertension, thrombosis and atherosclerosis (Barbagallo et al., 2001; Okosun et al., 2001; Blokhin and Lentz, 2013). It has been shown that reduction of visceral fat even with a modest loss in weight (18%) could prevent the insulin resistance and glucose intolerance of aging in F344/Brown Norway and Zucker Diabetic Fatty rats (Gabriely et al., 2002). In addition, there was an increase in plasma insulin level in Sprague-Dawley rats that led to a reduced weight and also decreased the risk factors associated with diabetes type II and cardiovascular disease (Barzilai and Gupta, 1999; Resnick et al., 2000; Wing et al., 2011). For humans the careful use of suitable micronutrients that prevent lipid accumulation is likely to prevent or reduce disease and prolong our healthy vascular functions.

Kaempferia parviflora (KP) Wall. Ex Baker belongs to the family Zingiberaceae and is found in the northern part of Thailand. In Thai traditional medicine, rhizomes of this plant have been used for treating various symptoms including erectile dysfunction, hypertension, inflammation, abdominal pain, as well as for the promotion of longevity with good health and well being (Wutythamawech, 1997; Yenjai et al., 2004). In Laos folk medicine, it has been used for lowering blood glucose levels, improving blood flow and increasing vitality (Akase et al., 2011). In Japan, KP extract is commercially available as a food supplement for the treatment of metabolic syndrome (Nakao et al., 2011). A number of scientific investigators have reported on the activities of an ethanolic extract from KP that included: acting as an aphrodisiac in male rats (Sudwan et al., 2006; Chaturapanich et al., 2011), as an agent to prevent gastric ulcers (Rujjanawate et al., 2005), as an anti-inflammatory agent (Tewtrakul and Subhadhirasakul, 2008; Sae-wong et al., 2009), and to prevent myocardial ischemic-reperfusion injury in an isolated rat heart (Malakul et al., 2011). In an *in vivo* experiment, a *Kaempferia parviflora* ethanolic extract caused an increase in testicular blood flow and an alteration in the blood clotting mechanisms by activation of fibrinolysis after chronic oral administration to young male rats (Chaturapanich et al., 2008; Murata et al., 2013). When a less polar organic solvent was used for extraction of a *Kaempferia parviflora*, plant rhizome powder with ethyl acetate, Akase et al. (2011) and Shimada et al. (2011) reported that it had an anti-obesity activity in spontaneously obese type II diabetic mice (Tsumura, Suzuki, Obese Diabetes TSO mice), but not in the Non-Obesity (TSNO) mice, by suppressing accumulation of visceral and subcutaneous fat and plasma triglycerides. On the other hand, Horikawa et al. (2012) found that an ethyl acetate extract of the *Kaempferia parviflora* rhizomes and its purified compounds: 3,5,7,4′-tetramethoxyflavone and 3,5,7,3′,4′-pentamethoxyflavone caused enhancement of adipogenesis. However, there has been no evidence for any effect of extracts from *Kaempferia parviflora* rhizomes on vascular functions and lipid accumulation especially in middle-age animals. Middle-aged rats do tend to accumulate body and liver lipid, and have an advanced risk of developing metabolic syndrome and cardiovascular disease, and become more susceptible to certain chemical substances (Park et al., 2006; Einstein et al., 2010; MacPhail et al., 2012). Thus, it was of interest to investigate whether extracts from the *Kaempferia parviflora* rhizome had any effect on vascular functions, as well as on lipid accumulation and gross organ abnormality in middle-aged rats. Therefore the first aim of the present study was to investigate the effects of chronic treatment of middle-aged rats with a dichloromethane extract of *Kaempferia parviflora* rhizomes (KPD) on vascular functions. The second aim of the study was to determine whether this chronic treatment with the KPD extract had any effects on the accumulation of lipid, the clinical chemical profiles and observations of abnormal gross organ developments. The following parameters were therefore investigated: (1) a study of the vascular functions of isolated thoracic aortic and mesenteric rings on their constrictor and dilatory responses to phenylephrine and acetylcholine, respectively. In addition, vascular eNOS protein expression was studied by Western blot analysis, (2) animal body weight and food intake, (3) visceral and subcutaneous fat, liver lipid accumulation, (4) gross abnormalities of internal organs, (5) liver and kidney functions, and (6) fasting serum glucose and serum lipid profiles. We would therefore expect that if the KPD extract led to an improvement of vascular functions and/or a lipid lowering activity in middle-aged rats with no signs of toxicity, the KPD extract would be a good choice for further development for use as a health product for preventing or delaying the development of obesity, metabolic syndrome and/or cardiovascular disease.

2. Materials and methods

2.1. Plant material

Fresh Rhizomes of *Kaempferia parviflora* were collected in Ampur Phurua, Loei Province, Thailand in April 2009. Authentication was achieved by comparison with the herbarium specimens in the Department of Biology Herbarium, Faculty of Science, Prince of Songkla University, Thailand, where a voucher specimen (Collecting no. 2548-03) of the plant material used has been deposited.

2.2. Extraction, drug preparation for oral administration and detection of the drug in the blood

Fresh rhizomes of *Kaempferia parviflora* (20 kg) were blended and extracted successively by macerating twice with 95% ethanol (2×20 L) for two days to extract most of their dark color, followed by extracting three times with 100% dichloromethane (3×20 L). The dichloromethane soluble part was filtered and evaporated under reduced pressure. The dried residue from the dichloromethane extract was further treated by suction under high pressure with an oil vacuum pump in order to remove residual dichloromethane and a yellowish gummy dichloromethane *Kaempferia parviflora* extract (KPD) was obtained with a 2.6% yield.

The KPD was analyzed by high performance liquid chromatography (HPLC) in order to obtain a chemical profile. Analytical HPLC was carried out on a HP1100 system equipped with a photodiode array detector (Agilent Technologies). The extract was analyzed on a Symmetry® C_{18} column (5 μm, 3.9×150 mm i.d.; Waters), with a gradient of CH_3OH:H_2O + 0.05% of trifluoroacetic acid (10:90 → 100:0). The flow rate was 1 ml/min; the UV traces of the eluants were measured at 210 and 254 nm and the UV spectra (DAD) were recorded between 200 and 500 nm. The HPLC chromatograms of the KPD and its three major pure compounds: 5,7-dimethoxyflavone (DMF), 5,7,4′-trimethoxyflavone (TMF) and 3,5,7,3′,4′-pentamethoxyflavone (PMF) together with their retention times and corresponding UV spectra are shown in Fig. 1. Quantitative analysis by HPLC chromatography was carried out by measuring the area under the peak of each of the three major compounds, as determined by comparison with the standard curve of known concentrations of the pure compounds and showed 84.88 mg/g for DMF, 68.98 mg/g for TMF, and 70.03 mg/g for PMF, which together amounted to 223.89 mg/g of dried KPD extract.

For oral administration, the KPD was suspended in a mixture of tween 80, 0.2 (g): carboxy-methylcellulose sodium salt, 0.2 (g): distilled water, 10 ml, at a concentration of 100 mg/ml. The control rat was orally administered with this vehicle using the same volume as that for the KPD treated rat.