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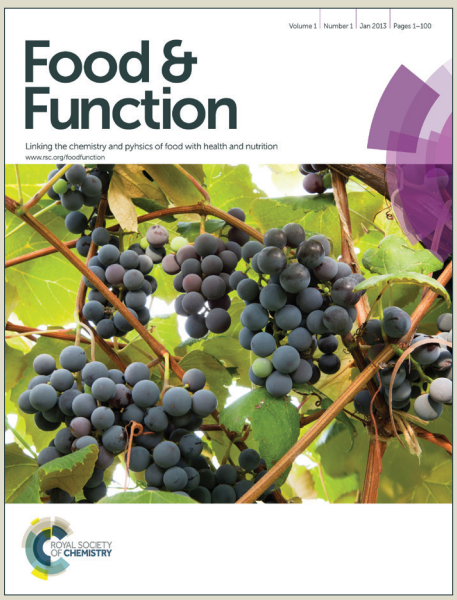

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Antihyperlipidemic Bioactivity of *Alpinia officinarum* (Hance) Farw Zingiberaceae Can Be Attributed to The Coexistance of Curcumin, Polyphenolics, Dietary Fibers And Phytosterols

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Running title: ***Alpinia officinarum*** **Exhibits Potent Antihyperlipidemic Bioactivity**

Abstract

Rhizoma *A. officinarum* (Hance) Farw, synonymously is called rhizoma galangae or smaller galangal (hereafter abbreviated as AO). Numerous studies reported AO to possess anti-inflammatory, anticancer, chemoprotective, antibacterial, antifungal and diuretic properties. To understand whether AO exhibit antihyperlipidemic bioactivity and what is the mechanism of action, we performed chemical and animal studies using hamsters (age 4 weeks, body weight 45±4 g). The grouping was: control, high fat (HF) diet, HF+AO2%, HF+AO4%, HF+AO6%, HF+AO8% and HF+AO10%. AO contained curcumin 5.67 mg/g (on wet basis), crude fiber 1.3±0.0%; soluble diet fiber 92±2 mg/g; insoluble diet fiber 502±5 mg/g; and phytosterols 63.9±1.6 mg/100g. Its methanolic extract consisted of high polyphenolics 4927.8±101.1 mgGAE/100g and flavonoids 593.2±22.2 mgQE/100g. The enlarged organs including liver, kidney, and spleen that were elevated by HF were completely alleviated by AO supplement diets. Levels of serum cholesterol, triglyceride, LDL-C, HDL-C and LDL-C/HDL-C ratio for the control origianlly were 138±6, 98±4, 40±5, 168±7 mg/dL and 0.24, which were elevated by HF to 319±12, 223±13, 108±11, 194±6 mg/dL and 0.05, and alleviated completely by HF+AO8% and HF+AO10%. *In vitro*, AO extracts showed potent DPPH free radical- and superoxide

anion- scavenging capabilities. *In vivo*, AO (at dose ≥8%) dose-dependently alleviated levels of superoxide dismutase, catalase, GSH, and MDA to 117±6.9 U/mL, 32.9±3.7 U/mL, 7.0±1.7 μmol/mL and 1.8±0.4 nmol/L, respectively, underlying the remarkable antioxidative and antihyperlipidemic effects of AO. Conclusively, we are the first who report the occurrence of curcumin in rhizoma *A. officinarum*. Curcumin synergistically elicits promising anti-dyslipidemic bioactivity with coexisting total polyphenolics, dietary fibers and phytosterols.

Introduction

Alpinia officinarum (Hance) Farw Zingiberaceae, synonymously named as *Languas officinarum* (Hance) Farw and commonly called lesser galangal (Fig. 1), is a plant from ginger family originated in China and cultivated in Southeast Asia. There are also a diversity of names locally used, like 'Southern Ginger', 'Lam Ginger', 'KaoLian Ginger', **'**China Root', 'India Root', 'East India Catarrh Root', 'Rhizoma Galangae', 'Gargaut', 'Colic Root', 'Kaempferia Galanga', or "Red Nutmeg'[1].

Rhizoma *A. officinarum* (named hereafter AO) is a world-renowned botanical, which has been used for decades because of its rich medicinal diversity. Numerous studies reported AO to possess anti-inflammatory, anticancer, chemoprotective, antibacterial, antifungal and diuretic properties[2]. Galangal improves the appetite, helps settle stomach upsets, stimulates the blood flow, health and vitality, and is frequently used as a sexual stimulant[3].

There are two different cultivars of galangal, one is "greater galangal or *A. galangal*" and the other is AO, also called "smaller galangal". The former, larger in size, lighter in color and subtler in aroma, is the cultivar mostly used in Thai cooking. As contrast, the latter exhibit aromatic smell and spicy taste and contains β-sitosterol, galangin, emodin[4], quercetin phoblaphenes, iron, vitamins A and C[3]. The hydroalcoholic percolates of AO contained mainly tannins, alkaloids, flavonoids and saponin[5] and

showed the concentration dependent radical scavenging activity[5]. Shin et al. (2002)[6] adopting the bioassay-guided fractionation demonstrated the presence of 8 antiemetic constituents in AO including two flavonoids, four diarylheptanoids, one sterol, and a new compound which also showed antiemetic activity, i.e. 5-hydroxy-7-(4-hydroxy-3-methoxyphenyl)-1-(4-hydroxyphenyl)-3-heptanone[6].

Curcumin (diferuloylmethane), a flavonoid and a major constituent of rhizoma turmeric (*Curcuma longa* Zinziberaceae), is a spice that shows rather promising neuroprotective activities for intervening bipolar disorder[7] and temporal lobe epilepsy [8]. *C. longa* is popularly formulated in Indian and Indonesian curries and widely used in European spicy condiments. Curcumin alone revealed a promising neuroactivity[9,10] via reducing oxidative products and proinflammatory cytokines as well as increasing the activity of antioxidant enzymes and preventing apoptotic cell death[11]. Curcumin influences oxidative and lipid-mediated stress in the vascular system[12].Up to date, the presence of curcumin in AO never has been cited. Whether AO contains curcumin and via which mechanism of action could AO exhibit antihyperlipidemic and antioxidative bioactivity? To verify this, we performed a series of chemical analyses and at the same time using the hamster model, we carried out the animal study.

Materials and methods

Chemicals

n-hexane, ethyl acetate, acetone, methanol, silica gel (particle size 70-230 mesh) and Na_2SO_4 anhydrous were purchased from E. Merck Co. (Dresden, Germany). Cayman GSH Assay Kit was supplied by Cayman Chemical Co. (Ann Arbor, MI, USA). Authentic gallic acid, quercetin, curcumin, and phytosterols were provided by Sigma-Aldrich Co. (St. Louis, MO, USA)

Plant source

Fresh rhizoma *A. officinarum* (AO), rhizoma *Alpinia galangal*, rhizoma *Curcuma longa,* and rhizoma *Zingiber officinale* were purchased from the Ginger Farm in Taichung, Taiwan.

Proximate compositional analysis

Method of AOAC (1984)[13] was followed to perform the analyses of moisture, ash, crude fiber, crude protein, crude fat, and carbohydrate.

Determination of dietary fibers

The determination of insoluble dietary fibers (IDF) and the soluble dietary fibers (SDF) in the fresh rhizoma *A. officinarum* were carried out as directed by the method of AOAC (1995)[14].

Preparation of desiccated crude powder (DCP)

Fresh AO was thoroughly rinsed twice with deionized water, blow-dried with

compressed air, cut into slices having a thickness of 2 mm, and lyophilized. The desiccated slices were blended into crude powder of mesh #10-#20 using a high speed crusher (TME2013, Taichung Mechanical and Electric Co., Taichiung, Taiwan) (named hereafter the desiccated crude powder, DCP).

Preparation of different solvent extracts

To 50 g of DCP the required amount of solvent (water, ethyl acetate, methanol or hexane) was added. The extraction was repeated thrice using solvents 400, 250, and 200 mL, respectively. For each extraction, the mixture was macerated at ambient temperature for 12 h in the dark and filtered through the fat-free ashless Whatman filter paper. Filtrates of same solvents were combined and evaporated under reduced pressure on a water bath held at 50^{o}C. The percent yield of each extract was calculated. The desiccated extracts were redissolved in each corresponding solvent to obtain stock solutions having strength of 10 mg/mL, filtered through 0.45 μM Micropore into brown vials, and stored at -20^{o}C (named hereafter as stock solution).

Determination of total polyphenolics

A modified method of Kujala et al. (2010)[15] was used for the determination of the total polyphenolic content (TPPC) using Folin-Ciocalteu's reagent. Briefly, to 500μL aliquot of each stock solution 1 mL of Folin-Ciocalteau phenol reagent was added and left to stand for 30 min at ambient temperature. The reaction mixture was

centrifuged at 3000g for 5-10 min. The supernatant was separated and the absorbance was measured at 760 nm. In parallel, gallic acid was employed as a calibration standard. In brief, the authentic gallic acid was dissolved in a methanol/water (60:40, v/v) mixed solvent previously acidified with HCl (0.3%) to obtain the desired concentrations as indicated. To each 0.2 mL aliquot 0.8 mL Na_2CO_3 solution (7.5%) was added and mixed well. The remaining procedures were conducted as mentioned in the above. The supernatant was separated and the absorbance was measured at 760 nm. The experiment was repeated in triplicates. The content of TPPC was expressed as mg gallic acid equivalent (GAE) per 100 g of dried extract weight.

Determination of total flavonoids

Quettier-Deleu et al. (2000)[16] was followed to carry out the determination of total flavonoids. Briefly, to 1 mL aliquot of each stock solution 1 mL methanolic $AlCl_3 \cdot H_2O$ (2%) was added. The mixture was vigourously agitated and left to stand for 10 min at ambient temperature. The optical density was read at 430 nm. The authentic quercetin was similarly treated to establish the reference curve. The total flavonoid content was calculated and expressed as mg quercetin equivalent (QE) per 100 g of dried extract weight.

Determination of the phytosterol content

To 5 g of DCP 50 mL hexane was added. The mixture was refluxed for 2 h and filtered. The residue was repeatedly refluxed twice. The filtrates were combined and evaporated to dryness under reduced pressure. To the residue 1 mL of the internal standard 5α-cholestane (1 mg/mL), and 15 mL of 6 M ethanolic HCl was added. The mixture was heated in a 90^{o}C water bath for 1 h. After cooled to ambient temperature, 50 mL of 2 M ethanolic KOH solution was added and left to react at ambient temperature for 20 h to facilitate the saponification reaction. To the reaction mixture 25 mL of chloroform was added. The mixture was agitated for 5 min vigorously to facilitate the extraction and then left to stand for phase separation. The lower layer was separated and stored in a 100 mL flask. The chloroform extraction was repeated for twice. The lower chloroform layers were combined and rinsed with purified water to neutral. The washing mixture was left to stand for phase separation. The lower layer was separated, to which 2 g anhydrous sodium sulfate was added and agitated vigorously to facilitate the dehydration. The mixture was filtered through a 0.45μm Micropore and the filtrate was subjected to evaporation at 40^{o}C to dryness. To the residue 100 μL of BSTFA/TMCS (99:1) was added. The mixture was left to react for 2 h at 70^{o}C. The final reaction product was analyzed for the phytosterol content using GC-FID and GC-MS.

The operation conditions for GC/FID

The product phytosterol derivatives were analyzed with GC/FID/GC/MS using the protocol as previously cited[17]. Briefly, a GC-FID chromatography GC HP 6890 attached to an HP5973SD detector and a capillary column DB-1 (ℓ=60 m; i.d. = 0.25 mm; thickness = 0.25 μm) was used. The carrier gas nitrogen was operated at a flow rate 1 mL/min. The temperatures of the injection port and detector were set at 250^{o}C and 280 ^{o}C, respectively. The ionization potential used was 70 eV. The initial temperature was set at and held 200^{o}C for 1 min, programmed at 0.5^{o}C/min up to 280^{o}C. Thereupon, the temperature was held at 280^{o}C for 1 min. then the temperature was programmed with an elevation of 10^{o}C/min up to 290^{o}C and held at which for 40 min. The flux ratio was set at 50:1[17].

The operation conditions of GC/MS

The equipment installation was the same as stated in the above[17]. However the initial temperature was set at and held 200^{o}C for 1 min and then was programmed at 10^{o}C/min up to 270^{o}C, and thereafter programmed at an elevation rate of 0.5^{o}C/min up to 280^{o}C and held at which for 1 min. The final elevation rate was set at 10^{o}C/min until 290^{o}C and held at 290^{o}C for 40 min. The flux ratio was set at 80:1[17].

HPLC determination of curcumin content

Curcumin was extracted according to Heath et al. (2003)[18]. In brief, to 2 g of DCP 15 mL methanol (80%) was added and the extraction was conducted at

4°C for 24 h and filtered. The extraction was repeated twice. The filtrates were combined and centrifuged at 3800g for 30 min. The supernatant was obtained by decantation and concentrated under vacuum to a final volume of 10 mL to remove methanol. Distilled water was added to make up a final volume of 50 mL (named herein methanol extract sample, MES). On the other hand, the mobile phase was prepared by mixing cyanomethane: methanol: deionized water: acetic acid at a ratio 41:23:36:1 (v/v). At the same time, the standard solution was prepared using the authentic curcumin, i.e. curcumin (10 mg) was dissolved in 100 mL of mobile phase to obtain the stock curcumin solution (SCS). SCS was serially diluted with mobile phase to obtain standard solutions of curcumin with concentrations 0.0, 10, 20, 40, 60, 80 and100 μg/mL. Aliquots 20 μL of the MES and the authentic solutions were respectively subjected to HPLC analysis using the Hitachi L-2130 HPLC (Hitachi Ltd., Tokyo, Japan). The flow rate of mobile phase was controlled at 0.8 mL/min to pass the separation column Tosoh TSK-GELODS-100S C18 column (ℓ×i.d. =250×4.6 mm, packed with 3μm particles). The effluent was monitored at 422 nm using the detector (L-2400 UV detector) (Hitachi, Japan).

Pulverized rhizoma *A. officinarum* for formulation of animal diet

The DCP obtained in the above was further pulverized to powders having mesh range #20-#40 (pulverized AO). The AO powder obtained was used for the formulation of the experimental animal meals according to AIN-76 (1978) [19] with a slight modification[17]. To prepare the high fat high cholesterol diet, corn oils were dispensed up to 12% with 0.5% of cholesterol (denoted as HF). AO 2%-10% were respectively incorporated with HF to obtain diets HF+AO2%, HF+AO4%, HF+AO6%, HF+AO8% and HF+AO10%, respectively. Each chow was thoroughly mixed previously to ensure the homogeneous composition.

Animal experiment

42 male Syrian hamsters (*Mesocricetus auratus*), age 4 weeks, were purchased from the National Laboratory Animal Centre. All studies performed with this hamster model were approved by the Hungkuang University Ethic Committee in accordance with Helsinki Declaration in 1975. The hamsters were acclimated for the first week and fed on regular Fu-So rodent chow only. The animal room was controlled at RH 65-75%, room temperature 22±2^{o}C with 12h/12h of light/dark cycle. The access of chow and water was *ad libidum*. In the second week the hamsters were divided into 7 groups based on their body weight, six in each and two in each cage. Group 1 was fed the regular chow (Fu-So Animal Feed Co., Taichung, Taiwan) (assigned as control group, C). Group 2 was fed HF chow. Groups 3 to 7 were respectively fed HF+AO2%,

HF+AO4%, HF+AO6%, HF+AO8% and HF+AO10%. The body weight and the amount of chows consumed were recorded every 2 days until the end of the experiment. The averaged change of body weight and the cumulative food intake were expressed as the amount changed per two weeks. At the end of week 9, the hamsters were fasted for 12 h, ether anethesized, and the blood samples were withdrawn immediately from the aorta abdomen.

Serum lipid determiantion

The assay for the serum cholesterol, serum triglyceride, LDL-C and HDL-C was conducted with the serum lipid assay kits (TECO DIAGNOSTICS) by folowing the instructions given by the manusfacturer as previously reported.[17]

Assay for serum superoxide dismutase (SOD) activity

SOD was measured with a SOD determination kit (Sigma, MO, USA) using Dojindo's highly water soluble tetrazolium salt, WST-1 (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disuLfophenyl)-2H-tetrazolium monosodoium)[20] (Fridovich, 1983). SOD activity was quantified by measuring the decrease in color development at 440 nm.

Assay for serum catalase activity

Activity of catalase in plasma and liver homogenates was determined by measuring the amount of decrease in absorption at 240 nm in a reaction mixture containing phosphate buffer (0.1 mmol/L, pH 7.0) and H_2O_2 (8.8 mmol/L) according to Aebi (1984)[21]. One catalase unit was defined as the amount of enzyme required to decompose 1 mmol/L of H_2O_2/min.

Assay for serum glutathione (GSH) level

The serum glutathione level was measured according to the instructions given by Cayman Chemical Co. (Ann Arbor, MI, USA).

Assay for serum malondialdehyde (MDA) level

The lipid peroxide level in plasma was determined using the thiobarbituric acid (TBA) assay (or MDA assay) kit as described by Santos et al. (1980)[22]. Briefly, to 100 µL of plasma 100 µL sodium dodecylsulphate (8.1% w/v), 750 µL acetic acid (20% v/v, pH 3.5), and 750 µL TBA (0.8%) were added. Water was added to make up a total volume 2 mL. The mixture was boiled in water bath for 30 min and cooled at ambient temperature. To the mixture 500 µL cold water, 2.5 mL butanol, and 2.5 mL pyridine were added, mixed well and centrifuged. The optical density of the supernatants was read at 532 nm using a spectrophotometer. Standard 1,1,3,3-tetra ethoxy propane (TEP) at different concentration was used to establish the calibration curve. Results

were expressed as nmol/mL TBA-reactive substances (TBARS; or MDA).

The DPPH free radical scavenging capability (FRSC)

The method of Shimada et al[23] was followed to determine FRSC as previously described by Lin et al. (2008)[17]. In brief, sample extract 0.2-1.0 mg/mL was mixed thoroughly with 4 mL methanol. To which 1 mL of methanolic DPPH (0.2 mM) was added and agitated to mix well. The reaction was allowed to proceed in the dark at ambient temperature for 30 min. The optical density was measured at 517 nm with a Hitachi U-2001 UV/Vis Spectrophotometer. Butylated hydroxytoluene (BHT) was used as the positive control.

The superoxide anions scavenging capability (SASC)

The method Robak and Gryglewski (1988)[24] was utilized to determine the superoxide radical scavenging capability. Briefly, the stock solution of each sovent extract was diluted with DMSO to prepare sample solutions having strength 0.2-1.0 mg/mL. To the DMSO sample solutions, 1 mL 60 μM phenazine methosulfate (PMS), 1 mL nitroblue tetrazolium (NBT, 150 μM), and 1 mL 468 μM of β-dihydronicotinamide adenine dinucleotide (β-NADH) were added. All these reagents were previously prepared by dissolving each chemical in 0.1M phosphate buffer (pH=7.4). The mixtures were left to stand in the dark avoiding direct sunlight for 5 min to facilitate the reaction. The

optical density was read at 560 nm and the SASC was calculated. DMSO alone was used as the vehicle control. The lower OD at 560 nm means the stronger the SASC.

Statistical analysis

Triplicate data obtained in the same group were analyzed by Student's *t* test with computer statistical software SPSS 10.0 (SPSS, Chicago, IL). The Statistical Analysis System software One-way Analysis of Variance (one-way ANOVA) was used to analyze the variances. Duncan's multiple range tests were used to test their significances of difference between paired means. Significance of difference was judged by a confidence level of $p < 0.05$.

Results

The proximate composition

AO exhibited contents of moisture, 71.1±0.1%; crude protein, 1.7±0.1%; crude fat 0.3±0.0%; crude ash 0.6±0.1%; crude fiber, 1.3±0.0%; and nitrogen free extract (NFE) 25.0±0.2%, respectively (Table 1). Curcuma longa has been found to have greater ash, fat, protein, carbohydrates and dietary fiber than that of Zingiber officinale. However, Zingiber officinale has greater moisture and ß-carotene[25].

The dietary fiber content was comparatively high

AO consisted of 93±2 mg/g soluble dietary fiber (SDF) and 502±5 mg/g insoluble dietary fiber (IDF) (Table 2).

The content of phytosterols was rather abundant

AO exhibited a total phytosterol content 63.9±1.6 mg/100g (Table 2). A diversity of phytosterol was occurring in AO, among which nine chemicals were identified (content in mg/100g), i.e. cholestane (16.3±0.1), cholesta-3,5-diene (0.4±0.1), cholesta-8,24-dien-3-ol (1.3±0.1), stigmastan-3,5,22-trien (2.4±0.3), stigmastan-3,5-diene (11.1±0.1), campesterol (1.4±0.1), cholest-5-en-3-ol (2.0±0.1), stigmasterol (2.6±0.2), and β-sitosterol (16.7±0.3) (Fig. 2, Table 2). The most abundant were β-sitosterol, cholestane, and stigmastan-3,5-diene (Fig. 2, Table 2).

The contents of total phenolics and flavonoids was unusually high

To compare, the yield methanolic extract of AO was 20.1±0.4%w/w, in which abundant total polyphenolics 4927.8±101.1 mgGAE/100g and total flavonoids 593.2±22.2 mgQE/100g were observed (Table 3). The yield of the aqueous extract was 35.8±2.8%w/w, and its total polyphenoilic and flavonoid contents reached 1354.3±23.1 and 59.1±0.5 mg/100g, respectively (Table 3). The next abundant was the ethyl acetate extract, which showed a percent (w/w) yield, total polyphenolic-, and total flavonoid contents of 4.6±0.2%w/w, 1834.7±211.9 mgGAE/100g, and 221.4±67.1 mgQE/100g, respectively (Table 3).

Occurrence of curcumin in AO was newly found for *A. officinarum*

Curcumin, a well known polyphenolics present in the rhizoma of turmeric (*Curcuma longa*) spice, was also identified in AO by HPLC with a peak retention time at 39.57 min (Fig. 3). The content of which reached 5.7±1.0 mg/g (wet basis). To our believe, this is the first report that indicated the abundant curcumin present in AO. To compare, we also explored the occurrence of curcumin in *Curcuma longa*, which has been frequently used as the positive rhizoma control. As seen, *Curcuma longa* contained a huge amount of curcumin reaching a level of 13.3±2.3 mg/g.. To our astonishment, the abundance of curcumin in AO was next to that of rhizoma *Curcuma longa*. While the curcumin content of rizoma *Zingiber officinale* was found to be only 1.6±0.4 mg/g, And amazingly, curcumin was totally absent in *Alpinia galangal* (Table

4).

***In-vitro* antioxidative capability of different solvent extracts**

Whether AO exhibits antioxidative bioactivity and which solvent extract could retain most part of the antioxidative constituents, the antioxidative bioactivity of different solvent extracts was explored. The methanolic- and ethyl acetate- extracts showed promising DPPH free radical scavenging capability (FRSC) (Fig. 4). Their *in vitro* antioxidative activities were comparable to the reference compound BHT (Fig. 4). The hexane extract was less effective than the methanolic- and ethyl acetate extarcts at doses < 0.4 mg/mL (p<0.05), but it comparably leveld off at doses ≥ 0.6 mg/mL (Fig. 4). As contrast, the aqueous extract did not reveal any significant effect with respect to FRSC (Fig. 4). Amazingly, the methanolic-, ethyl acetate- and the hexane-extracts showed rather strong superoxide anion scavenging capability (SASC) that even reached 25% better over the reference BHT (Fig. 5), while the aqueous extract was much less effective (Fig. 5). Results implicated that FRSC and SASC may be not only closely related with the contents but also depending on the polarity of the polyphenolics in respective solvent extracts (Table 3).

Variation of body- and organ weights of hamsters

When experimental hamsters were treated with the different meals (Table 5, modified from AIN-76, 1978)[17], the cumulative food intake in the control and the

HF group reached 475±5g and 518±6g at the end of week 9, starting from 98±2g and 108±4g at week 2 (Table 6). Feeding with AO appaently reduced the food intake of hamsters in a dose-dependent manner. At the end of week 9, the cumulative food intake was 504±4g, 488±3g, 476±3g, 470±3g, and 460±4g, respectively for HF+AO2%, HF+AO4%, HF+AO6%, HF+AO8%, and HF+AO10% (Table 6). Correspondingly, the cumuilative weigt gain was reduced form 44.0±5.4g for the HF group to 42.0±4.7g, 41.0±3.3g, 39.4±3.7g, 35.4±3,3g (p<0.01), and 34.6±4.3 (p<0.01), respectively (Table 7).

The liver weight of liver, kidney and spleen of the HF group reached high weights of 15.6±0.6g, 2.50±0.08g, and 0.78±0.03g, respectively. AO at 2%-10% dose-dependently reduced the liver weight to 15.0±0.4g, 13.3±0.5g (p<0.05), 11.1±0.2g (p<0.01), 10.4±0.7g (p<0.01), and 10.5±0.5g (p<0.01) (Table 8). And the weight of kidneys was reduced to 2.48±0.04g. 2.43±0.03g (p<0.05), 2.41±0.05g (p<0.05), 2.40±0.07g (p<0.05), and 2.40±0.06g (p<0.01), and similarly, the weight of spleen was alleviated to 0.76±0.02g, 0.73±0.03g, 0.72±0.03g (p<0.05), 0.70±0.03g (p<0.01), and 0.70±0.04g (p<0.01), respectively (Table 8).

AO exhibited potent hypolipidemic bioactivity

The serum cholesterol and triglyceride levels for the control were

138±6mg/dL and 98±4mg/dL and the HF group showed 319±12mg/dL and 223±13mg/dL, respectively ($p < 0.01$). The serum cholesterol values were apparently reduced after treated with AO in a dose-responsive manner, reaching 288±14mg/dL ($p <0.05$), 257±12mg/dL ($p <0.05$), 220±11mg/dL ($p <0.01$), 144±10mg/dL ($p <0.01$), and 116±9mg/dL ($p <0.01$) after treated with AO2%- AO10% respectively for 10 weeks ($p < 0.01$) (Table 9). Similar trend was found in the serum triglyceride levels, which were correspondingly reduced to 178±15 mg/dL ($p <0.05$), 133±10mg/dL ($p <0.01$), 110±11mg/dL ($p <0.01$), 99±7mg/dL ($p <0.01$), and 94±9mg/mL ($p<0.01$) (Table 9). As contrast, the levels of LDL-C and HDL-C in the HF group were respectively raised to 108±11mg/dL and 194±6mg/dL (Table 9). While the level of LDL-C was reduced and that of HDL-C was elevated by AO, the trend was also dose-responsively depending on the quantity of AO administered, i.e. the level of LDL-C was reduced by AO dose-dependently from 108±11 mg/dL for HF to 40±6 mg/dL ($p < 0.01$) while that of HDL-C was raised from 194±6mg/dL to 167±5mg/dL ($p < 0.01$) respectively after treated with AO2%-AO10% (Table 9). It is worth noting that the diet containing AO ≥ 8% completely abolished the adverse effects of HF. As seen, the diet containing AO ≥ 8%, effectively ameliorated the ratio of LDL-C/HDL-C to 0.25±0.0 compared to the value 0.05±0.0 of HF group (Table 9).

The *in vivo* antioxidative bioactivity was substantially alleviated by AO

We showed the levels of serum SOD and GSH of the HF group were reduced to 93.2±5.8 U/mL and 2.1±0.2 μmol/mL compared to the control values 118.8±6.7 U/mL and 6.9±0.6 μmol/mL, respectively (Table 10). Conversely, those of serum catalase and MDA were upregulated by HF to 123.3±13.7 U/mL and 8.7±1.3 nmol/L, respectively, contrasting with the control values 33.5±2.3 U/mL and 2.0±0.3 nmol/L (Table 8). The highly HF-altered values were variably ameliorated by AO. As seen, the levels of SOD and catatalse were dose-dependently alleviated by AO at doses ≥AO2%, GSH was only recovered when treated with AO at doses ≥AO4%, while MDA was only ameliorated by AO at dose doses ≥AO8% (Table 10).

Discussion

Huge amount of dietary fiber may have assisted the antihyperlipidemic bioactivity

High content of SDF and IDF have been shown to be a potent hypolipidemic agent (Table 2)[26]. Taking the refined konjac meal (RKM, a high content dietary fiber meal) as an example, RKM reduced the hepatic total cholesterol and triglyceride levels[26]. Dietary maneuvers lowered the serum cholesterol values in patients with diabetes mellitus[27]. Date waste (100 g/kg) reduced the total serum lipids and low density lipoprotein cholesterol (LDL-cholesterol) by 32-48%, while the serum triglycerides and total cholesterol levels were lowered by 23-35%[28]. And relavantly, AO treatments dose-dependently reduced cumulative food intake (Table 6) and cumilative weight gain (Table 7) and alleviated the enlargement of organs to normal values (Table 8).

The *in vitro* antioxidative capability, FRSC and SASC were associated with the polarity of polyphenolics

To understand the occurrence of main composition of AO that might reveal promising DPPH free radical- (FRSC) and the superoxide anion-scavenging capabilities (SASC), different solvent extracts of AO were compared. The methanolic- and ethyl acetate- extracts showed highly a promising FRSC, being rather comparable

to BHT. The hexane extract was slightly ineffective at lower dose. However, the effect became comparable at dose ≥ 0.6 mg/mL (Fig. 5). As contrast, aqueous extract was totally ineffective (p<0.001). Apparently, the FRSC was in parallel to the polyphenolic contents in each extract (Table 3).

Regarding the SASC, the methanolic-, ethyl acetate- and the hexane- extracts particularly showed promising SASC than BHT and the aqueous extract (Fig. 5). We suggest such an outcome could be ascribed to different polarity of the reacting molecules and the reaction system. Judging from the cited, the order of polarity of solvents used in this experiment is: DMSO >> water >methanol > hexane[29]. As many polyphenolics of AO were more methanol soluble (Table 3) and readily reacted with DPPH free radicals in the methanolic medium, the others could be more DMSO soluble and scavenged the superoxide anions more readily. As consequence, the results of FRSC and SASC were apparently uncompromised.

High fat diet induced oxidative stress

HF diet raised the levels of serum cholesterol, triglycerides (p<0.01), ratio LDL-C/HDL-C, and downregulated SOD and GSH (p<0.05) with stimulated production of oxidative stress (Table 9, Table 10). Golden Syrian hamsters when fed on a fat rich diet developed dyslipidemia and atherosclerotic plaques, the syptoms are similar in many respects to human atheroma[30].

A close correlation exists between the increased oxidative stress in accumulated fat and the pathogenic mechanism of obesity and obesity-associated metabolic syndrome[30]. Alternatively, the atherosclerosis oxidation theory shows that the inhibition of LDL oxidation may also inhibit atherosclerosis independent of lowering plasma cholesterol concentrations[30].

Polyphenolics present in rhizoma *A. officinarum* played the main role in ameliorating dyslipidemia

Dietary polyphenols (DPP) are associated with a lower cardiovascular disease risk[31]. DPP positively reduced fasting and postprandial triglyceride-rich lipoprotein (TRLs) and associated oxidative stress[31]. The concentrations of urinary 8-isoprostane decreased significantly with the polyphenol-rich diets[31].

Curcumin, the newly found polyphenolic in rhizoma *A. officinarum* contributed greatly to antihyperlipidemic effect.

Although cucumin is widely found in the rhizoma of turmeric (*Curcuma longa*) spices, to our believe, we are the first who report abundant occurrence of curcumin in AO (Fig. 3, Table 4). By comparing with the other selected species of ginger family, we found the abundance of curcumin in AO (5.67 mg/g wet basis) was only second to that of rhizoma *Curcuma longa* (13.34 mg/g d wet basis) (Table 4).

Curcumin influences oxidative and lipid-mediated stress in the vascular system[12]. Mounting experimental evidence suggests that curcumin may act chemically as scavenger of free radicals, and influences signal transduction and modulates the activity of specific transcription factors that regulate the expression of genes involved in free radicals scavenging, lipid homeostasis and carnitine palmitoyltransferase-I (CPT-1) etc.[12].

The administration of low-dose curcumin (15 mg/day, t.i.d.) showed a trend of reduction in levels of total cholesterol and LDL cholesterol in acute coronary syndrome (ACS) patients [32].

Babu and Srinivasan (1997)[33] reported that when the streptozotocin-induced diabetic rats were maintained on a diet containing 0.5% curcumin, promising effect on the reduction of blood cholesterol, triglyceride and phospholipid levels was observed. At the same time, the enhanced activity of acyl-CoA oxidase (a β-oxidation associated enzyme) and and inhibition of triglyceride accumulation in the liver were observed[34].

An adult hamster will eat about 10-12 g diet per day[35]. On the basis of the data obtained (Table 1, Table 2, and Table 4), the amount of daily uptake of curcumin was approximately 19.38 mg/day-23.26 mg/day at maximum by the HF+AO10% group, underlying the beneficial hypolipidemic effect of the formulated diets (Table 9).

High phytosterol content positively assisted with amelioration of dyslipidemia

Four crystalline substances from AO had been isolated and identified to be beta-sitosterol, galangin, emodin and quercetin[4]. Similarly, we showed the abundant occurrence of β-sitosterol, cholestane, and stigmastan-3,5-diene in AO (Fig. 2, Table 2).

Dietary phytosterol is protective against diet-induced hypertriglyceridemia, likely through multiple mechanisms that involve modulation of intestinal fatty acid metabolism and a reduction in hepatic lipogenesis[36] (Fig. 2, Table 2). Phytosterol dosages of 1.6-3 g daily have been shown to reduce LDL cholesterol by 4.1-15% versus placebo within the first month of therapy[37]. Several placebo-controlled trials found that the addition of phytosterols to statin therapy was associated with reductions of 7-20% in LDL cholesterol for up to 1.5 years[37].

The total phytosterol content in vegetables normally ranged within 1.1-53.7 mg/100 g edible portion[38], comparably, AO contained ratherr high phytosterols which may be associated with its promising hypolipidemic activity.

Taken together, chemicaly, AO uniquely contained abundant dietary fibers and phytosterols (Table 1, Table 2), polyphenolics (Table 3), and curcumin (Table 4), and biologically, AO effectively alleviated status of dyslipidemia.

Conclusively, we are the first who report the occurrence of curcumin in rhizoma *A. officinarum*. The presence of abundant curcumin can elicit promising dyslipidemic bioactivity with the coexisting total polyphenolics, dietary fibers and phytosterols.

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Conflict of interest

The authors declare no conflicts of interest. All authors have disclosed any financial, personal, and any relationships with other people or organization on submission of this work.

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Table captions

Table 1. Proximate composition of rhizoma *A. officinarum*.[1]

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Table 10. *In vivo* serum antioxidative power affected by rhizoma *A. officinarum* diets[1]

Table 1. Proximate composition of rhizoma *A. officinarum*.[1]

Composition	%
Moisture	71.1±0.1
Crude protein	1.7±0.1
Crude fat	0.3±0.0
Crude ash	0.6±0.1
Crude fiber	1.3±0.0
NFE[2]	25.0±0.2

[1]Data expressed in Mean±S.D. (n=3).

[2]NFE: Nitrogen-free extract.

%NFE (Nitrogen-free extract)

= %[100-(crude fat + crude protein + crude fiber + ash)]

Table 2. Contents of dietary fibers and phytosterols present in rhizoma *A. officinarum*.[1]

Sample	TDF (mg/g)[2]		
	SDF[3]	IDF[4]	TDF[c]
A. officinarum rhizoma	93±2	502±5	595±4

Peak Phytosterols	Content, (mg/100g)[4]	Retention time, min
Cholestane	16.3±0.1	22.81
Cholesta-3,5-diene	0.4±0.1	24.78
Cholesta-8,24-dien-3-ol	1.3±0.1	27.31
Stigmastan-3,5,22-trien	2.4±0.3	28.46
Stigmastan-3,5-diene	11.1±0.1	30.07
Campesterol	1.4±0.1	38.60
Cholest-5-en-3-ol	2.0±0.1	38.84
Stigmasterol	2.6±0.2	40.04
β-sitosterol	16.7±0.3	41.83
Other	9.7±1.2	dispersed
Total	63.9±1.6	-

[1]Data are expressed in Mean±SD (n=3). [2]TDF: total dietary fiber.

[3]SDF: soluble dietary fiber, [4]IDF: insoluble dietary fiber.

[4]On wet basis

Table 3. Total polyphenolic and flavonoid contents present in different extracts of rhizoma *A. officinarum*.[1]

sample	yield	polyphenolics	flavonoids
	(%w/w)	([2]mgGAE/100 g)	([3]mgQE/100 g)
aqueous extract	35.8±2.8	1354.3±23.1	59.1±0.5
hexane extract	3.8±0.1	1119.7±98.4	80.3±15.6
ethyl acetate extract	4.6±0.2	1834.7±211.9	221.4±67.1
methanol extract	20.1±0.4	4927.8±101.1	593.2±22.2

[1]All extractions except the essential oil were performed with the desiccated samples. [2]GAE：gallic acid equivalent. [3]QE：quercetin equivalents

Data are expressed in Mean±SD (n=3).

Table 4. Curcumin contents found in different genii of ginger family*

sample	Curcumin, mg/g
Rhizoma *Alpinia officinarum*	5.7±1.0[b]
Rhizoma *Alpinia galangal*	nd
Rhizoma *Curcuma longa*	13.3±2.3[a]
Rhizoma *Zingiber officinale*	1.6±0.4[c]

*nd: not detected. Data on wet basis were expressed in mean±SEM from triplicate experiment (n =3). Different superscripts in lower case indicate significant difference ($p<0.01$).

Table 5. Formulation of the experimental animal diets[1]

Ingredient	Group						
	control	HF[2]	HF+ AO2%	HF+ AO4%	HF+ AO6%	HF+ +AO8%	HF+ AO10%
Casein	20	20	20	20	20	20	20
sucrose	50	39.5	39.5	39.5	39.5	39.5	39.5
corn starch	15	15	15	15	15	15	15
corn oil	4	12	12	12	12	12	12
lard	1	3	3	3	3	3	3
cholesterol	0	0.5	0.5	0.5	0.5	0.5	0.5
AIN 76 mineral[3]	3.5	3.5	3.5	3.5	3.5	3.5	3.5
AIN 76 vitamin[4]	1	1	1	1	1	1	1
cellulose	5	5	5	5	5	5	5
methionine	0.3	0.3	0.3	0.3	0.3	0.3	0.3
choline bitartrate	0.2	0.2	0.2	0.2	0.2	0.2	0.2
AO%[5]	0	0	2	4	6	8	10

[1]Based on AIN-76 formula (American Institute of Nutrition, 1977). [2]HF: high fat. [3]Mineral premix：$CaHPO_4 \cdot 2H_2O$, NaCl, $K_3C_6H_5O_7$, K_2SO_4, MgO, MnO_3, Fe-citrate, $ZnCO_3$, $CuCO_3$, KI, $NaSeO_3$, and $K_2SO_4 \cdot Cr_2(SO_4)_3 \cdot 24H_2O$. [4]Vitamin premix: thiamine hydrochloride, pyridoxine hydrochloride, riboflavin, nicotinic acid, vitamin B_{12}, retinyl palmitate, and vitamins D_3, E, and K. [5]AO: desiccated *A. officinarum*.

Table 6. Cumulative food intake gain*

Body weight gain / group	Week 2	4	6	8	9
Control, (C)	98±2	220±5	300±6	440±5	475±5[d]
High fat, (HF)	108±4	280±5	388±7	506±5	518±6[a]
HF+AO2%	102±3	282±5	376±5	498±5	504±4[b]
HF+AO4%	100±2	248±4	356±4	475±4	488±3[c]
HF+AO6%	990±3	236±4	323±3	460±3	476±3[d]
HF+AO8%	980±3	221±3	302±4	443±4	470±5[e]
HF+AO10%	97±5	218±3	299±4	433±4	468±4[f]

*Data expressed in Mean±SEM (g) from triplicate experiments (n=3). The superscripts in lower case in column 'week 9' indicate significantly different.

Table 7. Cumulative weight gain*

Body weight gain / group	Week 2	4	6	8	9
Control, (C)	6.1±1.2	12.5±2.2	20.5±2.5	28.4±4.2	35.0±5.3[b]
High fat, (HF)	6.1±1.3	14.5±3.9	26.5±3.7	32.4±4.5	44.0±5.4[a]
HF+AO2%	6.2±1.2	13.6±3.2	25.7±2.7	31.6±3.5	42.0±4.7[a]
HF+AO4%	6.2±1.1	13.1±2.1	24.7±2.4	30.4±4.1	41.0±3.3[a]
HF+AO6%	6.2±1.1	12.8±2.4	23.0±3.2	29.4±3.4	39.4±3.7[a]
HF+AO8%	6.2±1.1	12.4±2.6	20.4±2.7	28.3±3.4	35.4±3.3[b]
HF+AO10%	6.2±1.1	12.3±1.9	20.1±2.1	28.0±3.3	34.6±4.3[b]

*Data expressed in Mean±SEM (g) from triplicate experiments (n=3). The superscripts in lower case in column 'week 9' indicate significantly different.

Table 8. Weight variation of some main organs affected by different rhizoma *A. officinarum* diets.

	Weight, g		
Group	Liver	Kidney	Spleen
Control, (C)	10.5±0.3$^{\#}$	2.40±0.05$^{\#}$	0.70±0.02$^{\#\#}$
High fat, (HF)	15.6±0.6**	2.50±0.08**	0.78±0.03**
HF+AO2%[2]	15.0±0.4**	2.48±0.04**	0.76±0.02**
HF+AO4%	13.3±0.5$^{**,\#}$	2.43±0.03$^{**,\#}$	0.73±0.03*
HF+AO6%	11.1±0.2$^{\#\#}$	2.41±0.05$^{\#}$	0.72±0.03$^{*\#}$
HF+AO8%	10.4±0.7$^{\#\#}$	2.40±0.07$^{\#}$	0.70±0.03$^{\#\#}$
HF+AO10%	10.5±0.5$^{\#\#}$	2.40±0.06$^{\#\#}$	0.70±0.04$^{\#\#}$

[1]Values are expressed as mean±SD (n = 6). Data with symbol "*" are significantly different (p<0.05) and "**" are highly significantly different (p< 0.01) compared to the control. "#" means significant different (p<0.05), and "##" means highly significant different compared to the high fat group (p<0.01).

[2]AO: desiccated *A. officinarum* rhizoma powder.

Table 9. Hypolipidemic effect of the rhizoma *A. officinarum* diet[1]

group	serum cholesterol, (mg/dL)	serum triglyceride, (mg/dL)	LDL-C, (mg/dL)	HDL-C, (mg/dL)	LDL-C /HDL-C
Control, (C)	138±6$^{\#\#}$	98±4$^{\#\#}$	40±5$^{\#\#}$	168±7$^{\#\#}$	0.24±0.0$^{\#\#}$
High fat, (HF)	319±12**	223±13**	108±11**	194±6**	0.05±0.0**
HF+AO2%[2]	288±14$^{**,\#}$	178±15$^{**,\#}$	90±10**	118±13**	0.76±0.0$^{**,\#\#}$
HF+AO4%	257±12$^{**,\#}$	133±10$^{**,\#\#}$	77±8$^{**,\#}$	125±12**	0.62±0.0$^{**,\#\#}$
HF+AO6%	220±11$^{**,\#\#}$	110±11$^{\#\#}$	53±9$^{\#\#}$	138±11*	0.38±0.0$^{**,\#\#}$
HF+AO8%	144±10$^{*,\#\#}$	99±7$^{\#\#}$	41±7$^{\#\#}$	164±8$^{\#\#}$	0.25±0.0$^{\#\#}$
HF+AO10%	116±9$^{\#\#}$	94±9$^{\#\#}$	40±6$^{\#\#}$	167±5$^{\#\#}$	0.24±0.0$^{\#\#}$

[1]Values are expressed as mean±SD (n = 6). Data with symbol "*" are significantly different (p<0.05) and "**" are highly significantly different (p< 0.01) compared to the control.

"#" means significant different (p<0.05), and "##" means highly significant different compared to the high fat group (p<0.01).

[2]AO: desiccated *A. officinarum* rhizoma powder.

Table 10. *In vivo* serum antioxidative power affected by rhizoma *A. officinarum* diets[1]

group	SOD[3] (U/mL)	Catalase[4] (U/mL)	GSH[4] (μmol/mL)	MDA[5] (nmol/L)
Control, (C)	118.8±6.7$^{\#\#}$	33.5±2.3$^{\#\#}$	6.9±0.6$^{\#\#}$	2.0±0.3$^{\#\#}$
High fat, (HF)	93.2±5.8**	123.3±13.7**	2.1±0.2**	8.7±1.3**
HF+AO2%[2]	95.6±4,3$^{**,\#}$	108.4±11.5$^{**,\#}$	3.4±0.4**	7.3±1.3**
HF+AO4%	99.8±6.2$^{**,\#}$	67.3±7.8$^{**,\#\#}$	4.7±0.8$^{**,\#}$	4.6±1.2**
HF+AO6%	111.5±4.6$^{**,\#\#}$	49.0±4.1$^{\#\#}$	6.0±0.9$^{\#\#}$	2.5±1.1*
HF+AO8%	117±6.9$^{*,\#\#}$	32.9±3.7$^{\#\#}$	7.0±1.7$^{\#\#}$	1.8±0.4$^{\#\#}$
HF+AO10%	120.4±6.9$^{,\#\#}$	30.4±3.4$^{\#\#}$	7.3±1.6$^{\#\#}$	1.7±0.5$^{\#\#}$

[1]Values are expressed as mean±SD (n = 6). Data with symbol "*" are significantly different (p<0.05) and "**" are highly significantly different (p< 0.01) compared to the control. "#" means significant different (p<0.05), and "##" means highly significant different compared to the high fat group (p<0.01).

[2]AO: desiccated *A. officinarum* rhizoma powder.

[3]SOD: superoxide dismutase. [4]GSH: glutathione (reduced form). [5]MDA: malondialdehyde.

Figure Legends

Fig. 1. Morphology of *Alpinia officinarum Zingiberaceae* (Hance) Farw

A. leaves and flowers. B. appearance, color and size of rhizoma. The size of rhizoma has a dimension 20 cm × 20 cm.

Fig. 2. The phytosterols in rhizoma *A. officinarum* verified by GC-FID.

At least 19 compounds were found coexisting as the "phytosterols". Among which only nine phytosterols were identifiable, that are (peak No; Retention Time, RT, min): cholestane (1; 22.81), cholesta-3,5-diene (2; 24.78), cholesta-8,24-dien-3-ol (3; 27.31), stigmastan-3,5,22-trien (4; 28.46), stigmastan-3,5-diene (5; 30.07), campesterol (6; 38.60), cholest-5-en-3-ol (7; 38.84), stigmasterol (8; 40.04), and β-sitosterol (9; 41.84).

Fig. 3. Curcumin identified by HPLC.

The peak with retention time 39.57 min was assigned to curcumin. Its content reached 5.67 mg/g rhizoma *A. officinarum* (wet basis).

Fig. 4. Comparison of the DPPH free radical scavenging capability among different solvent extracts of rhizoma *A. officinarum*.

Fig. 5. Comparison of the superoxide anion scavenging capability among different solvent extracts of rhizoma *A. officinarum*.

Fig. 1. Morphology of *Alpinia officinarum* Zingiberaceae
A. leaves and flowers. B. appearance, color and size of rhizoma.

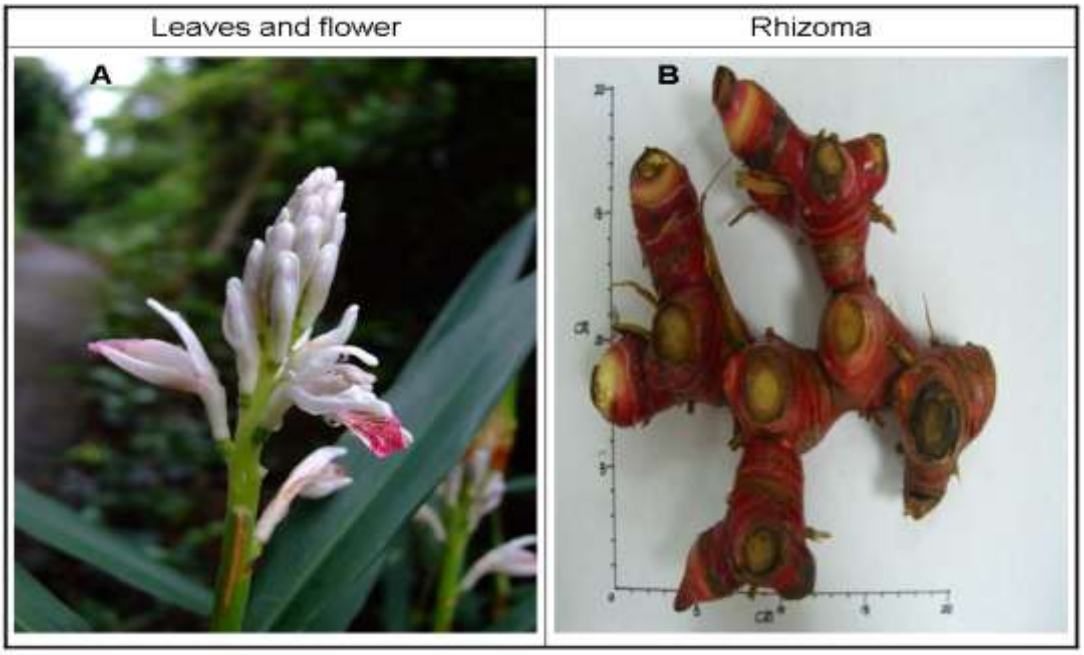

Fig. 2. Phytosterols found in rhizoma *A. officinarum*

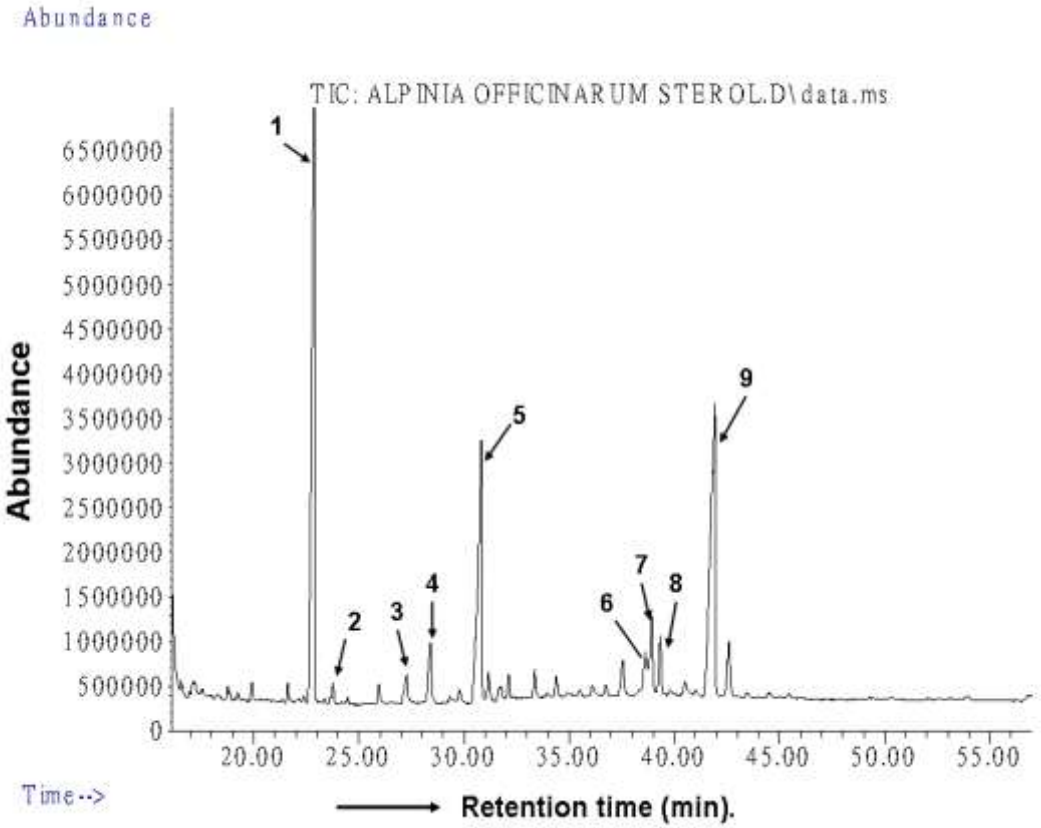

Fig. 3. curcumin

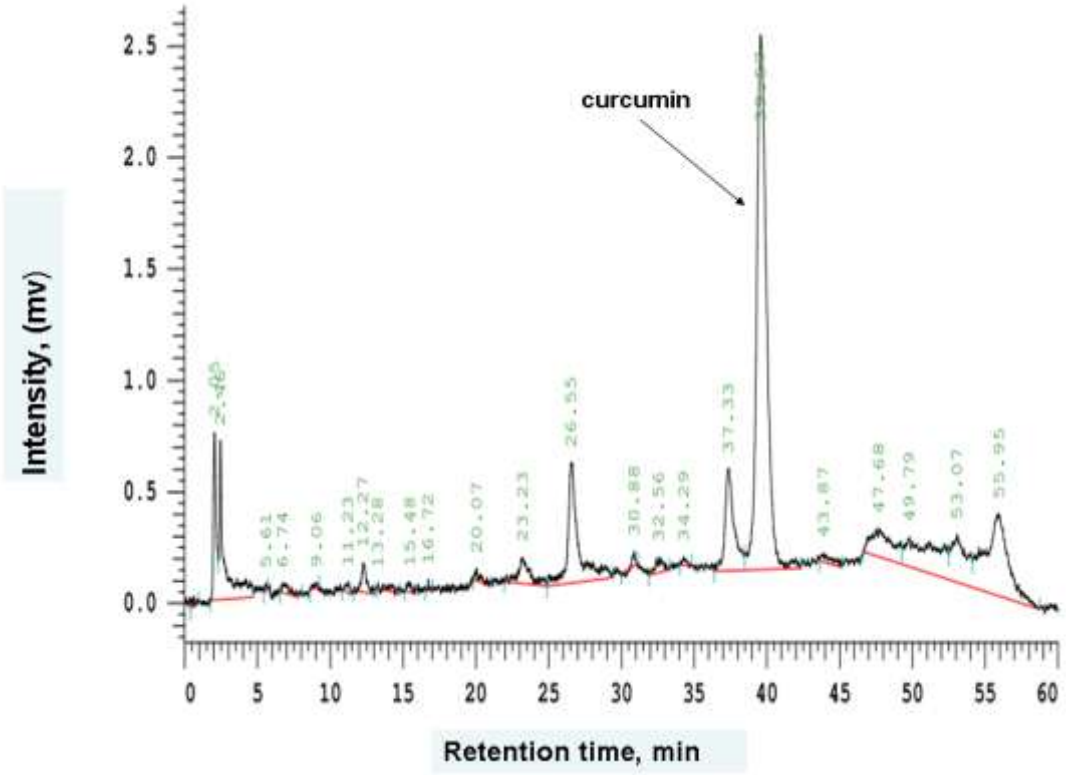

Fig. 4. The DPPH free radical scavenging capability

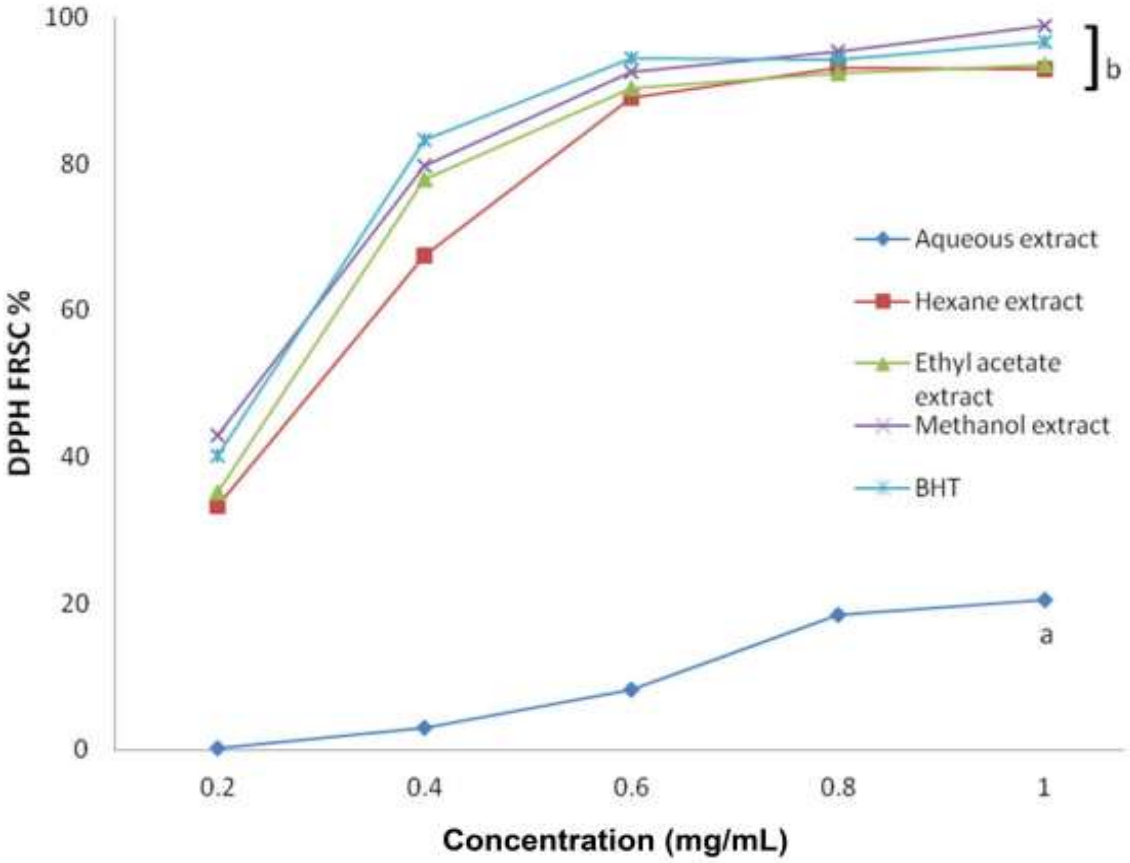

Fig. 5. The superoxide anion scavenging capability