Journal of Functional Foods 18 (2015) 423–431

Available online at www.sciencedirect.com

ScienceDirect

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

Antioxidant and anti-tumour evaluation of compounds identified from fruit of *Amomum tsaoko* Crevost et Lemaire

Tian-Tian Zhang [a], *Chuan-Li Lu* [a,b], *Jian-Guo Jiang* [a,*]

[a] *College of Food and Bioengineering, South China University of Technology, Guangzhou 510640, China*
[b] *Nutritional Immunology & Functional Foods, Key Laboratory of Tropical Plant Resources and Sustainable Use, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Menglun, Mengla, Yunnan 666303, China*

ARTICLE INFO

Article history:
Received 26 May 2015
Received in revised form 21 July 2015
Accepted 12 August 2015
Available online 27 August 2015

Keywords:
Amomum tsaoko Crevost et Lemaire
Antioxidant
Anti-tumour
Diarylheptanoids
Identification

ABSTRACT

This research was aimed to isolate the active compounds with antioxidant and anti-tumour properties from *Amomum tsaoko* Crevost et Lemaire, a traditional health food in China. Ethanol (95%) extract and ethyl acetate fraction was found to have significant DPPH radical scavenging activities and cytotoxicities against cervical cancer cell Hela, hepatoma cells HepG-2 and SMMC-7721, and lung cancer cell A549. Four compounds were isolated from ethyl acetate fraction, and compounds III and IV are identified as new compounds. Compounds III and IV showed high DPPH radical scavenging activities. Compound II, III and IV exhibited good anti-tumour activity against HepG-2, SMMC-7721, Hela and A549 cell lines. Compound I showed a strong anti-tumour activity only against Hela cell lines. Compounds III and IV were concluded to be the active compositions responsible for potent antioxidant and anti-tumour properties of *A. tsaoko*, whose molecular mechanism and pathways of inhibiting tumour cell need to be further studied.

1. Introduction

Amomum tsaoko Crevost et Lemaire, belonging to family Zingiberaceae, is widely distributed in south-west China. Its dried fruit is a well-known and commercially important spice in south-east Asia (Zhang, Lu, & Jiang, 2014). Water extract of *A. tsaoko* has a strong anti-hepatitis B virus effect. *A. tsaoko* intake displayed hypoglycaemic effect and could lower the triacylglycerol concentrations of plasma and liver in mice (Yu et al., 2010). Recently, a series of studies have described the isolation and identification of the chemical composition from the dried fruit of *A. tsaoko* using hydrodistillation, microwave assisted extraction, and supersonic solvent extraction (Feng, Jiang, Wang, & Li, 2010; Li, Wang, Masami, & Lu, 2011). The essential oil from *A. tsaoko* could serve as a new medicinal source

* *Corresponding author.* College of Food and Bioengineering, South China University of Technology, Guangzhou, 510640, China. Tel.: +86 20 87113849; fax: +86 20 87113843.
E-mail address: jgjiang@scut.edu.cn (J.-G. Jiang).

Abbreviations: DPPH, 2,2-diphenyl-1-picryl-hydrazil radical; MTT, 3-(4,5-dimethylthiazol-z-yl)-2,5-diphenyl tetrazolium bromide; V_C, ascorbic acid; HPLC, high-performance liquid chromatography; ESI-MS, electrospray ionization ion trap multiple mass spectrometry; FBS, foetal bovine serum; PBS, phosphate buffer solution; DEPT, distortionless enhancement by polarization transfer; ^{1}H-^{1}H COSY, H-H correlation spectroscopy; HSQC, heteronuclear single-quantum coherence; HMBC, heteronuclear multiple-bond correlation; TMS, tetramethylsilane; TLC, thin-layer chromatography; PE, petroleum ether fraction; EA, ethyl acetate fraction; W, water fraction; OD, optical density

http://dx.doi.org/10.1016/j.jff.2015.08.005
1756-4646/

for antibacterial and as an antifungal agent (Li et al., 2011; Li, Zou, Zha, Zheng, & Huang, 1999; Qiu, Shou, Chen, Dai, & Liu, 1999). The volatile oil of *A. tsaoko* could improve the function of stomach and increase the percutaneous permeation rate of rutondine that has sedative, analgesic and hypnotic effects (Ma & Bai, 2006). However, all these studies on *A. tsaoko* have not been subjected to detailed chemical constitution analysis, and bioactivity studies were restricted to its crude extracts.

In the present research, the antioxidant activity and anti-tumour activity of crude extracts of *A. tsaoko* on cervical cancer cell Hela, hepatoma cells HepG-2 and SMMC-7721, and lung cancer cell A549 were investigated in order to isolate and identify the active compounds responsible for these biological activities.

2. Materials and methods

2.1. Plant material

The dried *A. tsaoko*, derived from Yunnan province (China), was purchased from Qingping market of Chinese medicinal material in Guangzhou, China. A voucher specimen was deposited in the department of Natural Products Studies, school of Light Chemistry and Food Science, South China University of Technology. Samples were pulverized to a powder and the dried materials were stored at room temperature until use.

2.2. Chemicals

2,2-Diphenyl-1-picryl-hydrazyl (DPPH) radical, 3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-H-tetrazolium bromide (MTT), 5-fluorouracil, ascorbic acid (V_C) and high-performance liquid chromatography (HPLC)-grade MeOH were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Acetone and MeOH employed for the electrospray ionization (ESI)-MS analyses were of HPLC supergradient quality. Foetal bovine serum (FBS) was purchased from Biochrome Co. (Germany). Cell culture DMEM media, trypsin-EDTA (ethylenediaminetetraacetic acid), penicillin, streptomycin and phosphate buffer solution (PBS, pH 7.4) were bought from Thermo Fisher Scientific Inc. (Waltham, MA, USA). All the other chemicals were of analytical grade.

2.3. General experimental procedure

A Bruker DRX-400 NMR spectrometer (Bruker Biospin Co., Karlsruhe, Germany), operating at 400 MHz for ^{1}H and at 101 MHz for ^{13}C, using the UXNMR software package, was used for NMR analysis. Distortionless enhancement by polarization transfer (DEPT), ^{13}C, H-H correlation spectroscopy (^{1}H-^{1}H COSY), heteronuclear single-quantum coherence (HSQC), and heteronuclear multiple-bond correlation (HMBC) NMR experiments were carried out using the conventional pulse sequences. Electrospray ionization ion trap multiple mass spectrometry (ESI-MS) was performed on an MDS SCIEX API 2000 LC/MS apparatus (MDS Sciex, Ontario, Canada). Chemical shifts were expressed in δ (ppm) downfield from tetramethylsilane (TMS) as an internal standard and coupling constants were reported in Hz. Silica gel (100–200, 200–300 mesh), ODS and Sephadex LH-20 were used for column chromatography. Thin-layer chromatography (TLC) was performed on a precoated silica gel HSGF254 plate. The spots on TLC plates were detected under UV light or vapour of iodine, and visualized by spraying with vanilin-H_2SO_4 after heating. HPLC separations were performed on Ultimate XB-C18 column (4.6 × 250 mm, 5 μm) equipped with a UV/Vis detector at 35 °C.

2.4. Isolation and identification of the compounds from the fruit of A. tsaoko

The dried powder (3 kg) of *A. tsaoko* was exhaustively extracted through refluxing with 95% ethanol (3 × 10 L) for 3h. The ethanolic extract was filtered and then concentrated under vacuum to yield a viscous residue. The residue was suspended in water and partitioned against petroleum ether and ethyl acetate successively. The solvents were evaporated under vacuum using rotary evaporator at lower temperature to yield petroleum ether fraction (PE), ethyl acetate fraction (EA) and water fraction (W), respectively (Hu, He, & Jiang, 2014). In the antioxidant and anti-tumour activities screening tests, ethyl acetate fraction showed better activity among the three fractions. Therefore, the ethyl acetate fraction was fractioned on a silica gel column (200–300 mesh) using a gradient of chloroform-methanol (100:0, 98:2, 95:5, 90:10, 80:20, 50:50, 0:100, v/v, each 10 L). The eluates were pooled into 14 fractions. Fraction EA-3 was further chromatographed on Sephadex LH-20 and eluted with chloroform-methanol (v/v, 1:1), followed by semi-preparative HPLC to yield compound I. Monitored by TLC and HPLC, fraction EA-4 and fraction EA-5 were merged together. The mixed sample was further purified by ODS column and eluted with a mixture of methanol and water (30, 60, 90, and 100%, v/v, each 2.5 L), followed by semi-preparative HPLC and Sephadex LH-20 column chromatography (eluent, methanol) to obtain compound II. Fraction EA-6 and fraction EA-7 were also merged together based on the results of TLC and HPLC. Compound III and compound IV were obtained by ODS column (eluent, methanol-water, v/v, gradient elution), semi-preparative HPLC and Sephadex LH-20 column chromatography (eluent, methanol) successively.

2.5. Antioxidant activity assays (DPPH radical-scavenging activity)

The DPPH radical-scavenging activity was estimated by the method of Schreiber, Bozell, Hayes, and Zivanovic (2013) with some modifications (Zhang & Jiang, 2015; Zhang, Yang, & Jiang, 2015a; Zhang et al., 2015). Briefly, the various fractions and four compounds obtained from *A. tsaoko* were dissolved in methanol to form sample solution in a series of concentrations. The DPPH stock solution (150 μmol/L) was diluted with MeOH to an absorbance of 1.0 at 517 nm before 3 mL of the diluted DPPH solution were mixed with 1 mL of various concentrations of sample. The reaction mixture was shaken well and incubated for 30 min at room temperature in the dark. The absorbance of the resulting solution was read with a spectrophotometer at 517 nm against a blank. Lower absorbance of the reaction mixture indicates higher free radical-scavenging activity. The radical scavenging activity of DPPH was calculated according to the following equation:

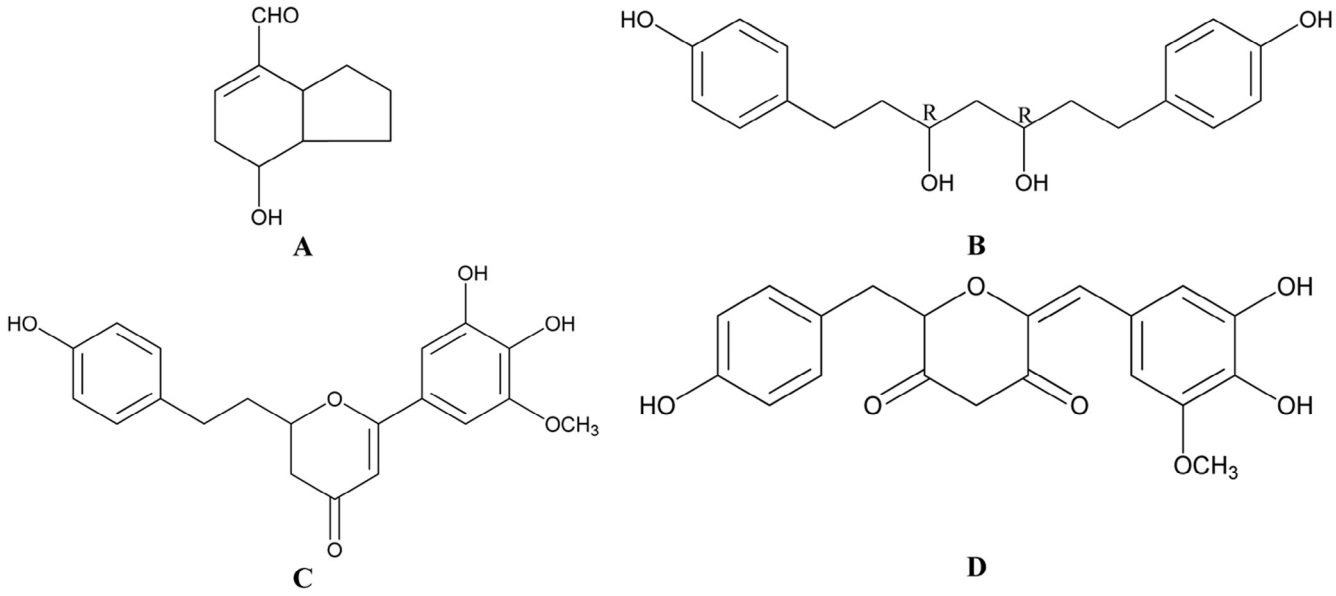

Fig. 1 – Compounds and their chemical structures isolated from *A. tsaoko* (A) isotsaokoin; (B) hannokinol; (C) 2,3-dihydro-2-(4′-hydroxy-phenylethyl)-6-[(3″,4″-dihydroxy-5″-methoxy)phenyl]-4-pyrone; (D) 4-dihydro-2-(4′-hydroxy-phenylmethyl)-6-[(3″,4″-dihydroxy-5″-methoxyphenyl)methylene]-pyran-3,5-dione.

$$\text{Scavenging activity}\,(\%) = \frac{Ac - As}{Ac} \times 100$$

where Ac is the absorbance of the DPPH radical solution without sample and As is the absorbance of the DPPH radical solution with tested samples. Ascorbic acid was used as a reference compound.

2.6. Cell culture

Human hepatocellular carcinoma cell lines HepG-2, SMMC-7721 and human cervix carcinoma cell line Hela, human lung cancer cell line A549 were purchased from the cell bank of Shanghai of Chinese Academy of Sciences. Cells were cultivated in DMEM supplied with 10% FBS and antibiotics (100 IU/mL penicillin and 100 μg/mL streptomycin) at 37 °C in a humidified atmosphere containing 5% CO_2 (Chen, Chen, Fu, & Liu, 2015).

2.7. Cell proliferation assay

The anti-tumour activity against HepG-2, SMMC-7721, Hela and A549 human cancer cells was detected using the MTT assay (Liao et al., 2015; Zhang & Jiang, 2015; Zhang, Xu, Jiang, & Jiang, 2013; Zhang, Yang, & Jiang, 2015b). Stock solutions of various fractions and four compounds obtained from *A. tsaoko* were prepared in dimethyl sulphoxide (DMSO) at concentrations of 10 mg/mL and diluted by cell culture medium to various working concentration. MTT was dissolved (5 mg/mL) in PBS (pH 7.2) and filtered through Millipore filter, 0.22 μm, before use. Cells were plated in 96-well plates (0.5×10^5 cells/well) and incubated to allow cell adhesion or equilibration. 24 hours later, the medium was removed, and 200 mL of new growth medium containing various concentrations of the samples was added. The cells were grown for another 24 h. Then, the medium was removed, and 20 μL of MTT assay stock solution (5 mg/mL) was added into each well. After incubation for an additional 4 h, the medium containing unreacted dye was removed carefully and 200 μL of DMSO was added to dissolve the purple formazan crystals. The plate was shaken for 20 min on a plate shaker to ensure complete dissolution. The optical density (OD) value was measured at a wavelength of 492 nm. Positive control was 5-fluorouracil and cells without sample were used as a control. Cell proliferation inhibition rate was calculated using the following formula:

$$\text{Cell proliferation inhibition}\,(\%) = \left(1 - \frac{OD_{sample}}{OD_{control}}\right) \times 100\%$$

2.8. Statistical analysis

Each experiment was performed in triplicate, and mean values were calculated. The data were expressed as mean ± SD. The significance of differences between groups was assessed by one-way analyses of variance performed using SPSS 11.5 software. $P < 0.05$ indicated the presence of a statistically significant difference and $P < 0.01$ was considered highly significant.

3. Results and discussion

3.1. Structure identification of the purified compounds

Results of active screening showed that EA fraction had better antioxidant and anti-tumour activities among the three fractions. Therefore, the bioactivity-guided separation was designed to investigate the chemical constituents of *A. tsaoko*; four compounds were obtained from EA fraction after repeated isolation and purification.

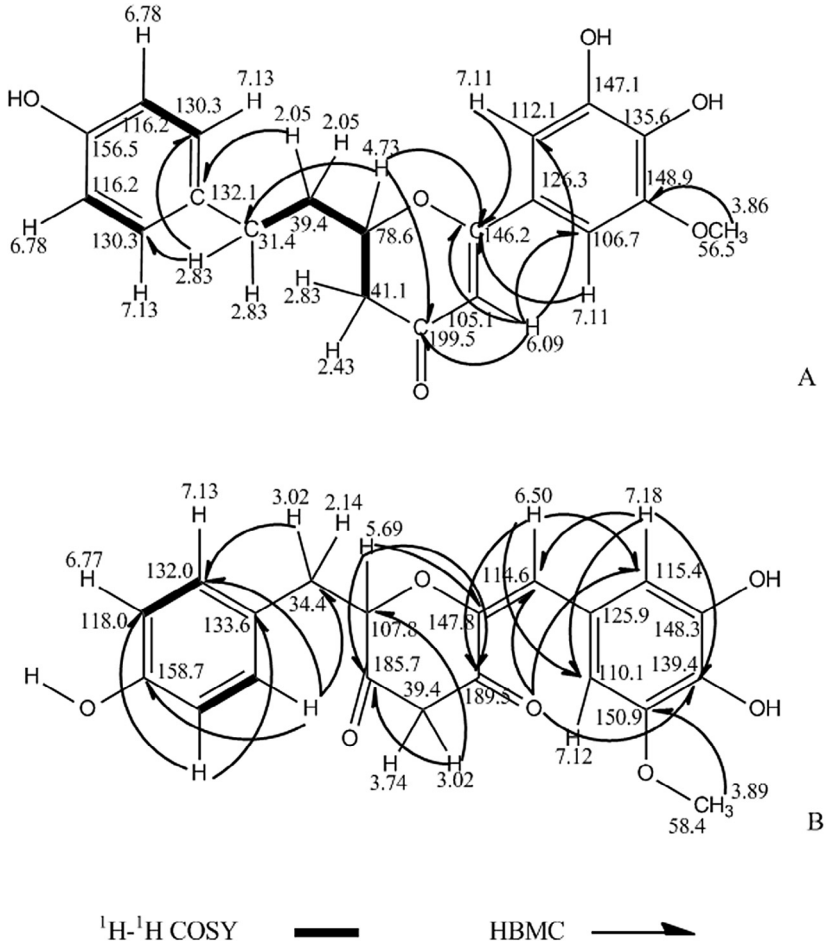

Fig. 2 – The key correlations in compounds III and IV. (A) 2,3-dihydro-2-(4′-hydroxy-phenylethyl)-6-[(3″,4″-dihydroxy-5″-methoxy)phenyl]-4-pyrone; (B) 4-dihydro-2-(4′-hydroxy-phenylmethyl)-6-[(3″,4″-dihydroxy-5″-methoxyphenyl)methylene]-pyran-3,5-dione.

On the basis of spectroscopic analysis (MS, ^{1}H-NMR, ^{13}C-NMR, DEPT135, HSQC, ^{1}H-^{1}H COSY and HMBC spectra), and comparison with the previously reported spectral data (Martin, Kikuzaki, Hisamoto, & Nakatani, 2000; Moon, Lee, & Cho, 2004), the structures of these compounds were identified as isotsaokoin (compound I), hannokinol (compound II), 2,3-dihydro-2-(4′-hydroxyl-phenylethyl)-6-[(3″,4″-dihydroxy-5″-methoxy)phenyl]-4-pyrone (compound III) and 4-dihydro-2-(4′-hydroxyl-phenylmethyl)-6-[(3″,4″-dihydroxy-5″-methoxyphenyl) methylene]-pyran-3,5-dione (compound IV). Chemical structures of the four compounds were shown in Fig. 1. Compound III and compound IV were considered as new compounds. Spectroscopic data of these four compounds are listed below.

Compound I was obtained as a colourless oil. The spot on TLC plates was purple by spraying with vanilin-H_2SO_4 after heating. ^{1}H-NMR and ^{13}C-NMR spectroscopic data suggested the occurrence of an aldehyde group and a double bond in the structure of compound I. Comparison of its ^{1}H-NMR and ^{13}C-NMR spectrums with those presented in the literature for isotsaokoin showed that these spectra were similar, then compoud 1 was identified as isotsaokoin (Moon et al., 2004).

Compound II was obtained as a white powder. ^{1}H-NMR and ^{13}C-NMR spectroscopic data suggested the occurrence of two phenyl groups and two oxygenated tertiary carbons in the structure of compound II. Compoud II was identified as hannokinol by comparison of its ^{1}H-NMR and ^{13}C-NMR spectrums with those presented in the literature (Martin et al., 2000).

Compound III was obtained as a yellowish powder. ESI-MS: + MS: *m/z* 378.6 $[M + Na]^+$; 356.6 $[M + H]^+$; –MS: *m/z* 354.5 $[M–H]^-$, suggesting that the molecular weight of compound III was 356. It could be seen from ^{1}H-NMR (CD_3COCD_3, 400 MHz) spectrum that five unsaturated hydrogen signals were detected in low-field region. Specifically, δ 6.78 (2H, d, J = 8.8 Hz) and δ 7.12 (2H, d, J = 8.8 Hz) suggested the occurrence of substituted benzene group. δ 3.86 (3H, s) suggested the occurrence of methoxyl group. Analysis of the ^{13}C NMR spectrum together with DEPT experiments (a total of twenty carbons, containing two substituted benzene groups, one methoxyl group, and one carbonyl carbon), HQSC spectrum, the ^{1}H-^{1}H COSY spectrum (cross-peaks: δ_H 4.73 with δ_H 2.05, δ_H 2.43 and δ_H 2.83; δ_H 2.05 and δ_H 2.83; δ_H 7.12 and δ_H 6.78), and HMBC correlations (δ_H 7.12 to δ_C 156.5, δ_C 130.3, and δ_C 31.4; δ_H 7.11 to δ_C 148.9, δ_C 146.2, δ_C 135.6, δ_C 112.1, δ_C 106.7 and δ_C 105.2; δ_H 6.78 to δ_C 156.5, δ_C 132.1, and δ_C 116.2; δ_H 6.09 to δ_C 199.5, δ_C 146.2,

δ_C 112.1, and δ_C 106.7; δ_H 4.73 to δ_C 199.5, δ_C 146.2, and δ_C 31.4; δ_H 3.86 to δ_C 148.9; δ_H 2.83 to δ_C 199.5, δ_C 132.1, δ_C 130.3, δ_C 78.6 and δ_C 39.4; δ_H 2.43 to δ_C 199.5, δ_C 78.6, δ_C 39.4; δ_H 2.05 to δ_C 132.1, δ_C 78.6, δ_C 41.1) determined the structure of compound III as 2,3-dihydro-2-(4′-hydroxy-phenylethyl)-6-[(3″,4″-dihydroxy-5″-methoxy)phenyl]-4-pyrone (Fig. 2A). There have been no related reports about the structure of compound III in SciFinder scholar, suggesting that the compound may be a new compound.

Compound IV was isolated from the same fraction with compound III. ESI-MS: +MS: *m/z* 392.7 $[M + Na]^+$; 371.2 $[M + H]^+$, suggesting that the molecular weight of compound IV was 370. It could be seen from ^{1}H-NMR (CD_3COCD_3, 400 MHz) spectrum that five unsaturated hydrogen signals were detected in low-field region; specifically, δ 6.77 (2H, d, *J* = 8.0 Hz) and δ 7.13 (2H, d, *J* = 8.0 Hz) suggested the occurrence of substituted benzene group. δ 3.89 (3H, s) suggested the occurrence of methoxy group. In the analysis of the ^{13}C NMR spectrum together with DEPT experiments, twenty carbons could be speculated in the structure of compound IV. Compared with compound III, similar structural fragments (two substituted benzene groups and one methoxyl group) presented in the structure of compound IV. Slight differences were that compound IV showed the two carbonyl group signals, and only two secondary carbon signals were detected in high-field region. Interpretation of the HQSC spectrum, the ^{1}H-^{1}H COSY spectrum and the HMBC spectrum afforded the gross structure of compound IV as 4-dihydro-2-(4′-hydroxy-phenylmethyl)-6-[(3″,4″-dihydroxy-5″-methoxyphenyl)methylene]-pyran-3,5-dione (Fig. 2B). There have been no related reports about the structure of compound IV in SciFinder scholar, suggesting that the compound may be a new compound.

3.2. *Antioxidant activity of the extracts and compounds*

DPPH assay has been widely used to measure radical scavengers because it is stabilized, and the reaction system only covers the direct reaction between the radical and antioxidant (Kassim et al., 2013; Sun et al., 2015). The DPPH radical exhibits a strong absorption maximum at 517 nm, resulting in a purple colour (Bellik et al., 2013). Different chemical constituents extracted from *A. tsaoko*, including 95% ethanol extract, petroleum ether fraction, ethyl acetate fraction, water fraction and compounds I-IV, were tested for their antioxidant capacities using DPPH free radical scavenging assay.

As shown in Fig. 3A, all extracts exhibited DPPH radical scavenging activity in a dose-dependent manner. The ethyl acetate fraction exhibited excellent DPPH radical-scavenging activity at the concentration of 200 μg/mL (DPPH radicals inhibition rate >90%), which was very close to Vc at the same concentration. 95% ethanol extract and petroleum ether fraction also had good DPPH radical-scavenging activities; both of the scavenging activities were more than 75% when the concentration were up to 200 μg/mL. The activity of water fraction was weaker. These results indicated that the active antioxidant ingredients were concentrated on ethyl acetate fraction. The new compound IV exhibited a high scavenging activity, at 80 μg/mL, its inhibition exceeded 60%, and the inhibition reached 79.04% at the concentration of 100 μg/mL (Fig. 3B). New compound III also showed a certain inhibition effect; its inhibition rate (58.55%) reached 69.7% of V_C (83.97%) at the concentration of 100 μg/mL. The antioxidant activities of known compounds I and II were lower; their scavenging inhibition rates were 39.23 and 28.65%, respectively (Fig. 3B). In previous phytochemical investigations of *A. tsaoko*, isotsaokoin and hannokinol had been separated and identified, but rarely reported about their activities. Isotsaokoin showed antifungal activity against *Tricophyton mentagrophytes* (Moon et al., 2004). Isotsaokoin and hannokinol both significantly inhibited lipopolysaccharide-induced nitric oxide production in BV2 microglial cells at concentrations ranging from 1 mu M to 100 mu M (Lee, Kim, Sung, & Kim, 2008). It was noted that new compounds III and IV exhibited higher antioxidant activities, and this may be also the cause of phenolic hydroxyl groups (Rebelo, Rego, Ferreira, & Oliveira, 2013; Santos, Ponte, Boonme, Silva, & Souto, 2013).

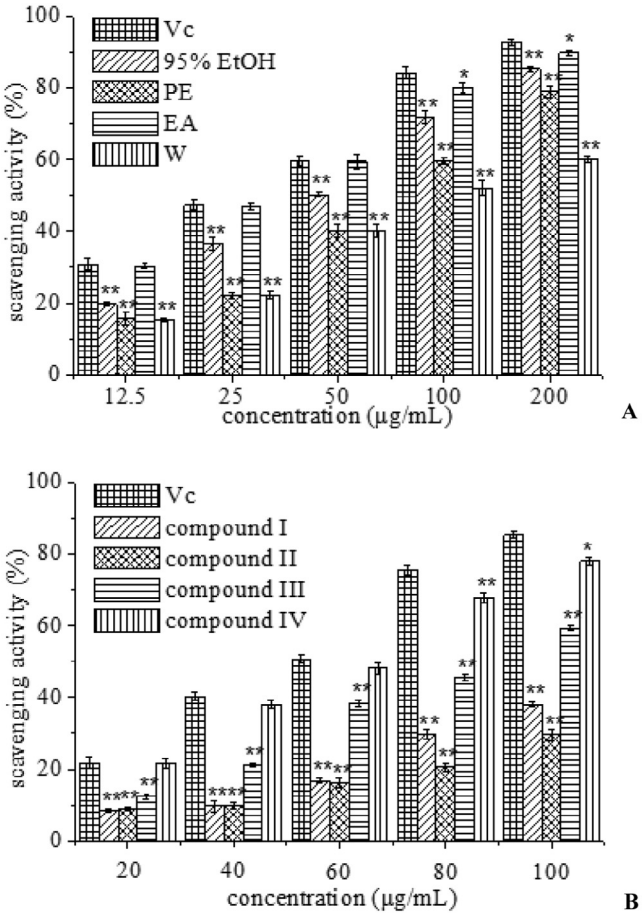

Fig. 3 – DPPH free radical scavenging ability. (A) 95% ethanol extract (95% EtOH), petroleum ether fraction (PE), ethyl acetate fraction (EA), and water fraction (W) extracted from *A. tsaoko*; (B) isolated compounds: isotsaokoin (compound I), hannokinol (compound II), 2,3-dihydro-2-(4′-hydroxy-phenylethyl)-6-[(3″,4″-dihydroxy-5″-methoxy)phenyl]-4-pyrone (compound III) and 4-dihydro-2-(4′-hydroxy-phenylmethyl)-6-[(3″,4″-dihydroxy-5″-methoxyphenyl)methylene]-pyran-3,5-dione (compound IV). Results are mean ± SD. *P < 0.05, **P < 0.01, statistically significant in comparison with control.

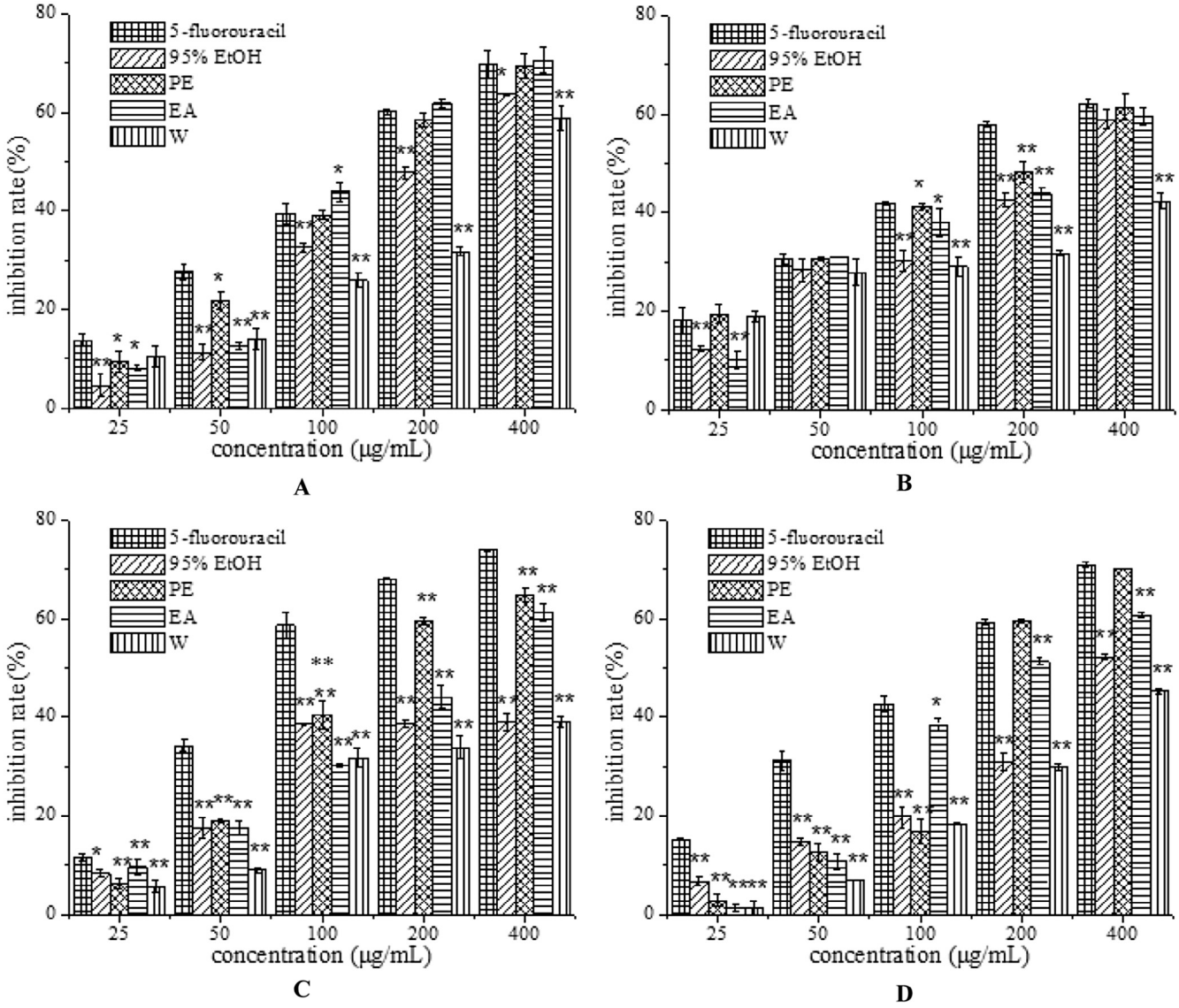

Fig. 4 – Cell proliferation inhibition rate of 95% ethanol extract (95% EtOH), petroleum ether fraction (PE), ethyl acetate fraction (EA), and water fraction (W) against SMMC-7721 (A), HepG-2 (B), Hela (C) and A549 (D) human cancer cell lines. Results are mean ± SD. *P < 0.05, **P < 0.01, statistically significant in comparison with control.

3.3. *Evaluation of anti-tumour activity*

Oxidative stress is among the main causes of cancer-related death, and the chemoprevention is defined as the use of natural or synthetic antioxidants to prevent cancer formation or cancer progress (Sugamura & Keaney, 2011; Valko et al., 2007). Dietary and medicinal plants are major sources of phytochemicals, which play important roles in the treatment of cancers (Grothaus, Cragg, & Newman, 2010). Phytochemicals from dietary and medicinal plants have emerged as very promising sources of potential anticancer agents with increasing anticancer mechanistic evidences, coupled with considerations of safety and efficacy (Shu, Cheung, Khor, Chen, & Kong, 2010).

In this investigation, the 95% ethanol extract, fractions and isolated compounds I–IV from *A. tsaoko* were evaluated for their antiproliferative activities against HepG-2, SMMC-7721, Hela and A549 human cancer cell lines using the MTT bioassay. The 95% ethanol extract exhibited moderate anti-tumour activity on these four cancer cell lines in a dose-dependent manner (Fig. 4). Ethyl acetate fraction exhibited the strongest anti-tumour activity against SMMC-7721 human cancer cell lines, and the inhibition rate reached 71.4% at the concentration of 400 μg/mL, which exceeded that of 5-fluorouracil (Fig. 4A). Petroleum ether fraction and ethyl acetate fraction showed higher inhibition rates against HepG-2, Hela and A549 human cancer cell lines than 95% ethanol extract and water fraction, which were close to that of 5-fluorouracil (Fig. 4). This result indicated that, after the 95% ethanol extract was partitioned into three fractions, active compounds were concentrated into petroleum ether fraction and ethyl acetate fraction. Many previous literatures showed that methanol/ethanol extracts or fractions had good anti-tumour activity. It was reported that the methanol extracts from *Salvia menthifolia* exhibited the

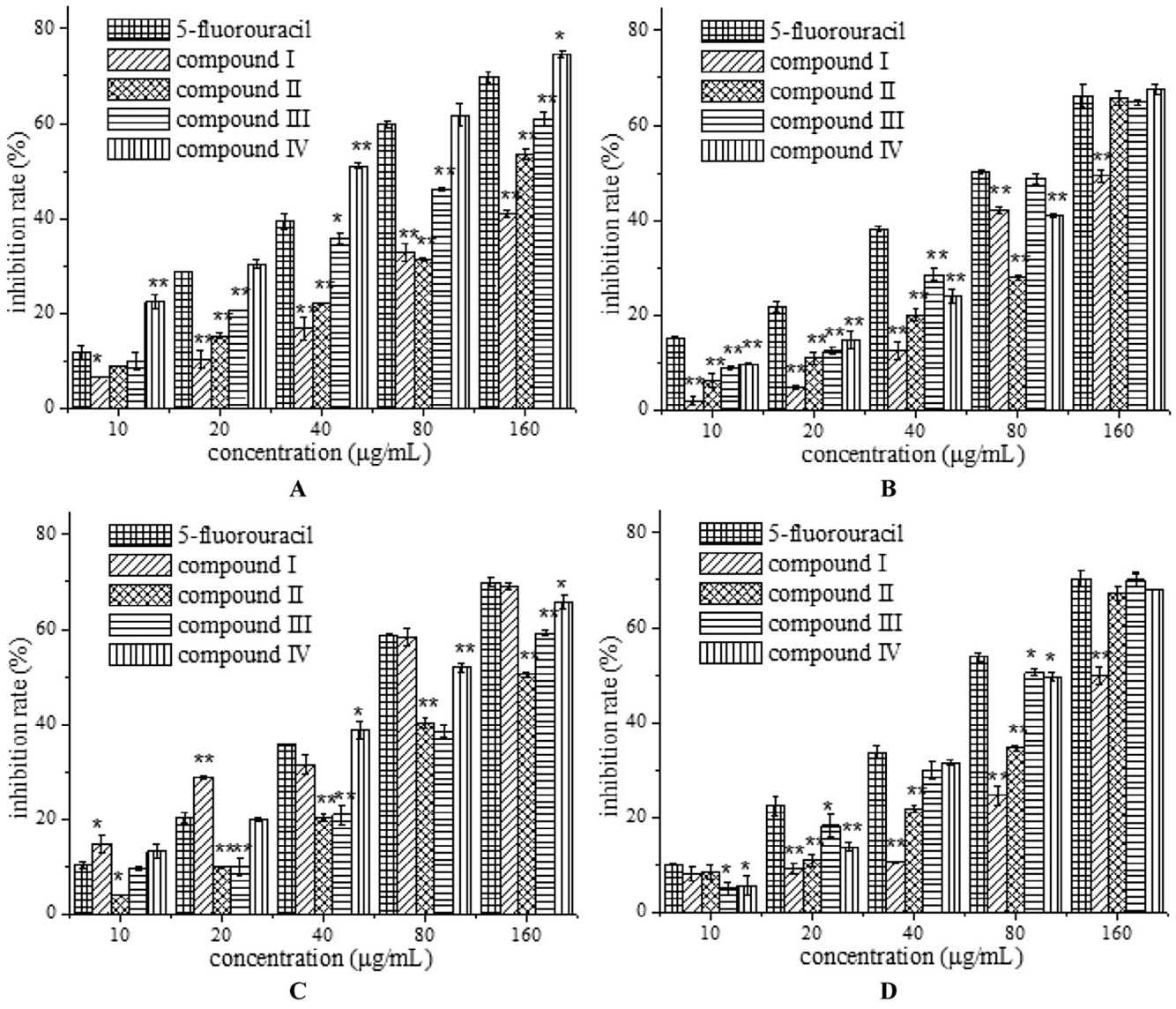

Fig. 5 – Cell proliferation inhibition rate of isolated compounds: isotsaokoin (compound I), hannokinol (compound II), 2,3-dihydro-2-(4′-hydroxy-phenylethyl)-6-[(3″,4″-dihydroxy-5″-methoxy)phenyl]-4-pyrone (compound III) and 4-dihydro-2-(4′-hydroxy-phenylmethyl)-6-[(3″,4″-dihydroxy-5″-methoxyphenyl)methylene]-pyran-3,5-dione (compound IV) against SMMC-7721 (A), HepG-2 (B), Hela (C) and A549 (D) human cancer cell lines. Results are mean ± SD. *P < 0.05, **P < 0.01, statistically significant in comparison with control.

anti-proliferative activity against human glioblastoma cell line DBTRG-05MG (Fiore, Massarelli, Sajeva, & Franchi, 2012). Ethyl acetate fraction from *Polytrichum commune* L.ex Hedw also displayed higher anti-tumour effect against L1210 cells than that of chloroform and butanol fractions (Cheng et al., 2012). Therefore, the bioactivity-guided separation of *A. tsaoko* was designed and four active compounds were isolated from the ethyl acetate fraction.

Compound I exhibited the strong anti-tumour activity against Hela cell lines, which was significantly higher than the other three compounds and very close to that of 5-fluorouracil (Fig. 5C). The Hela cell proliferation inhibition rate of compound I was higher than 50% at the concentration ≥80 μg/mL, while the inhibitory rates were less than 50% for the other three cell lines (HepG-2, SMMC-7721, and A549). Compound II showed a certain inhibition effect, the rates could reach 65.9% and 66.7% against A549 and HepG-2, respectively. Compound III exhibited a strong anti-tumour activity against A549 cell lines, its inhibition rate reached 70.03% at the concentration of 160 μg/mL, which was significantly higher than the other three compounds (Fig. 5D). Compound III also had a certain inhibition against HepG-2 cell lines, its rate was highest in the four isolated compounds when the concentration was ≤80 μg/mL (Fig. 5B). Compound IV showed the best cytotoxicities against SMMC-7721 and HepG-2 cancer cell lines, and the inhibition rate reached 73.4 and 68.3% at the concentration of 160 μg/mL, which exceeded those of 5-fluorouracil. In order to better compare the anticancer activity of each compound, IC50 was calculated and shown in Table 1. Tsapkoarylone, a diarylheptanoid, had been separated and identified from

Table 1 – IC50 of compounds isolated from *A. tsaoko* on SMMC-7721, HepG-2, Hela and A549 human cancer cell lines.

Compounds	IC50 (μg/mL)			
	SMMC-7721	HepG-2	Hela	A549
5-fluorouracil	59.83 ± 0.38	73.89 ± 2.9	65.89 ± 0.61	72.29 ± 1.55
Compound I	>160	>160	72.14 ± 1.44	>160
Compound II	150.55 ± 1.64	117.44 ± 1.74	143.19 ± 1.63	105.61 ± 1.56
Compound III	91.23 ± 3.8	89.08 ± 1.05	117.83 ± 2.42	79.77 ± 2.07
Compound IV	44.66 ± 2.1	97.18 ± 1.15	71.71 ± 1.40	80.95 ± 0.85

A. tsaoko and exhibited strong anti-tumour activity against human lung cancer cell A549 and human melanoma SK-Mel-2 (Moon, Cho, & Lee, 2005). Compound II, III and IV were all belonging to the family of diarylheptanoid; their high proliferation inhibition rates may be related to the similar structure of Tsaokoarylone.

Fig. 6 showed the cell morphology changes of HepG-2 cell lines with the treatment of 2,3-dihydro-2-(4′-hydroxy-phenylethyl)-6-[(3″,4″-dihydroxy-5″-methoxy)phenyl]-4-pyrone (compound III) for 24 h, 48 h, and 72 h at the concentration of 40 μg/mL. Their activities increased with time. After treatment for 24 h, the shape of some cells began to become round. At 48 h, almost all cells had changed to be spherical and the cell membrane became rough. At 72 h, a large number of shedding cells suspended in culture medium.

4. Conclusion

An efficient method for bioassay-guided preparation and isolation was used to identify the antioxidant and anti-tumour constituents in *A. tsaoko*. Chromatographic separation of the ethyl acetate fraction of 95% ethanol extract resulted in the isolation and identification of four compounds, isotsaokoin (compound I), hannokinol (compound II), 2,3-dihydro-2-(4′-hydroxy-phenylethyl)-6-[(3″,4″-dihydroxy-5″-methoxy)phenyl]-4-pyrone (compound III) and 4-dihydro-2-(4′-hydroxy-phenylmethyl)-6-[(3″,4″-dihydroxy-5″-methoxyphenyl)methylene]-pyran-3,5-dione (compound IV), among which compounds III and IV are new compounds. Compounds III and IV showed strong antioxidant activity. Compound II, III and IV exhibited good

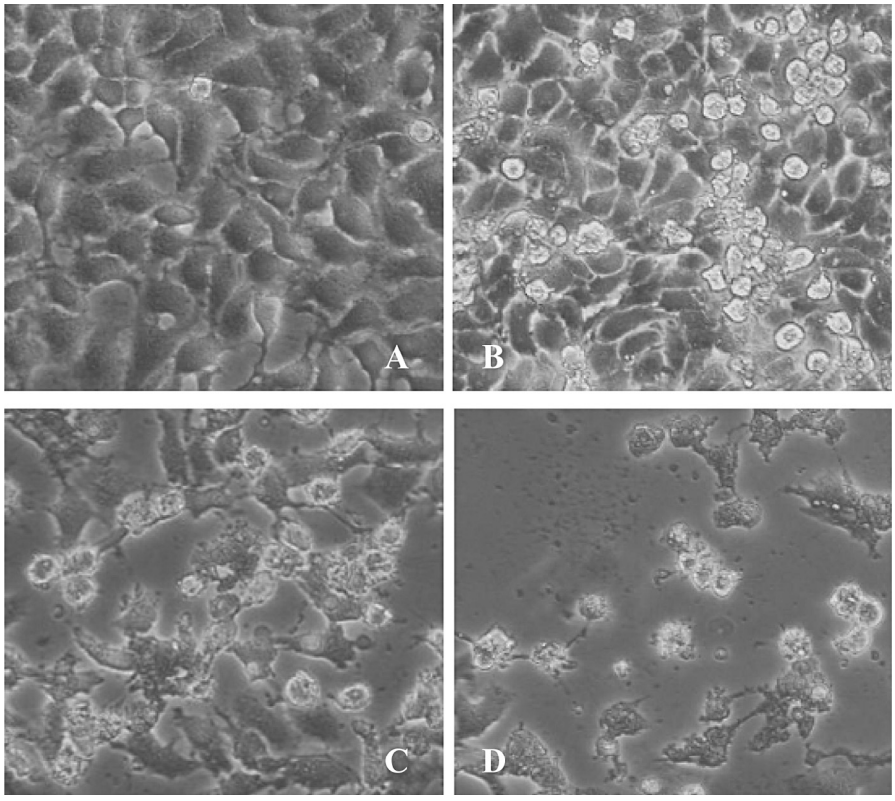

Fig. 6 – Cell morphology of HepG-2 with the treatment of 2,3-dihydro-2-(4′-hydroxy-phenylethyl)-6-[(3″,4″-dihydroxy-5″-methoxy)phenyl]-4-pyrone (compound III) for different periods at the concentration of 40 μg/mL. (A) Untreated group; (B) treatment for 24 h; (C) treatment for 48 h; (D) treatment for 72 h.

anti-tumour activity against tested tumour cell lines (HepG-2, SMMC-7721, Hela and A549). Compound I only showed the strong anti-tumour activity against Hela cell lines; its inhibitory rates on other three cell lines were less than 50%. The strong anti-oxidant activity and anti-tumour activity against tested tumour cell lines suggest that the new compounds III and IV warrant further testing as potential effective nutraceutical compounds and chemotherapeutic drugs.

REFERENCES

Bellik, Y., Benabdesselam, F., Ayad, A., Dahmani, Z., Boukraa, L., Nemmar, A., & Iguer-Ouada, M. (2013). Antioxidant activity of the essential oil and oleoresin of *Zingiber officinale* roscoe as affected by chemical environment. *International Journal of Food Properties*, *16*, 1304–1313.

Chen, Y. S., Chen, G., Fu, X., & Liu, R. H. (2015). Phytochemical profiles and antioxidant activity of different varieties of adinandra tea (*Adinandra jack*). *Journal of Agricultural and Food Chemistry*, *63*, 169–176.

Cheng, X. X., Xiao, Y. P., Wang, X. B., Wang, P., Li, H. X., Yan, H., & Liu, Q. H. (2012). Anti-tumor and pro-apoptotic activity of ethanolic extract and its various fractions from *Polytrichum commune* L.ex Hedw in L1210 cells. *Journal of Ethnopharmacology*, *143*, 49–56.

Feng, X., Jiang, Z. T., Wang, Y., & Li, R. (2010). Composition comparison of essential oils extracted by hydrodistillation and microwave-assisted hydrodistillation from *Amomum tsao-ko* in China. *Journal of Essential oil Bearing Plants*, *13*, 286–291.

Fiore, G., Massarelli, P., Sajeva, M., & Franchi, G. G. (2012). Anti-tumor activity of the methanolic extracts of Salvia menthifolia. Revista Brasileira de Farmacognosia-brazilian. *Journal of Pharmacognosy*, *22*, 381–388.

Grothaus, P. G., Cragg, G. M., & Newman, D. J. (2010). Plant natural products in anticancer drug discovery. *Current Organic Chemistry*, *14*, 1781–1791.

Hu, T., He, X. W., & Jiang, J. G. (2014). Functional analyses on antioxidant, anti-inflammatory, and antiproliferative effects of extracts and compounds from *Ilex latifolia* Thunb., a Chinese bitter tea. *Journal of Agricultural and Food Chemistry*, *62*, 8608–8615.

Kassim, N. K., Rahmani, M., Ismail, A., Sukari, M. A., Ee, G. C. L., Nasir, N. M., & Awang, K. (2013). Antioxidant activity-guided separation of coumarins and lignan from *Melicope glabra* (Rutaceae). *Food Chemistry*, *139*, 87–92.

Lee, K. Y., Kim, S. H., Sung, S. H., & Kim, Y. C. (2008). Inhibitory constituents of lipopolysaccharide-induced nitric oxide production in BV2 microglia isolated from *Amomum tsao-ko*. *Planta Medica*, *74*, 867–869.

Li, W., Wang, P. J., Masami, S., & Lu, Z. G. (2011). Chemical composition and antimicrobial activity of essential oil from *Amomum tsao-ko* cultivated in Yunnan area. *Environmental Biotechnology and Materials Engineering*, *183*, 910–914.

Li, W., Zou, Z. Y., Zha, G., Zheng, M. S., & Huang, X. T. (1999). Experimental research on anti-HBV activity of 170 kinds of Chinese herbs. *World Chinese Journal of Digestology*, *7*, 89–90.

Liao, W. Z., Lu, Y. J., Fu, J. N., Ning, Z. X., Yang, J. G., & Ren, J. Y. (2015). Preparation and characterization of Dictyophora indusiata polysaccharide-zinc complex and its augmented antiproliferative activity on human cancer cells. *Journal of Agricultural and Food Chemistry*, doi:10.1021/acs.jafc.5b00614.

Ma, Y. S., & Bai, Y. C. (2006). Effects of volatile oil of fructus *tsaoko* on the percutaneous permeation of rutondine *in vitro*. *Yunnan Journal of Traditional Chinese Medic Material Medicine*, *27*, 40–41.

Martin, T. S., Kikuzaki, H., Hisamoto, M., & Nakatani, N. (2000). Constituents of *Amomum tsao-ko* and their radical scavenging and antioxidant activities. *Journal of the American Oil Chemists Society*, *77*, 667–673.

Moon, S. S., Cho, S. C., & Lee, J. Y. (2005). Tsaokoarylone, a cytotoxic diarylheptanoid from *Amomum tsaoko* fruits. *Bulletin of the Korean Chemical Society*, *26*, 447–450.

Moon, S. S., Lee, J. Y., & Cho, S. C. (2004). Isotsaokoin, an antifungal agent from *Amomum tsao-ko*. *Journal of Natural Products*, *67*, 889–891.

Qiu, S. H., Shou, D. W., Chen, L. F., Dai, H. Y., & Liu, K. L. (1999). Pharmacological comparison between volatile oil and water extract. *China Journal of Chinese Material Medicine*, *24*, 297–299.

Rebelo, M. J., Rego, R., Ferreira, M., & Oliveira, M. C. (2013). Comparative study of the antioxidant capacity and polyphenol content of Douro wines by chemical and electrochemical methods. *Food Chemistry*, *141*, 566–573.

Santos, I. S., Ponte, B. M., Boonme, P., Silva, A. M., & Souto, E. B. (2013). Nanoencapsulation of ommano 514–523.

Schreiber, S. B., Bozell, J. J., Hayes, D. G., & Zivanovic, S. (2013). Introduction of primary antioxidant activity to chitosan for application as a multifunctional food packaging material. *Food Hydrocolloids*, *33*, 207–214.

Shu, L., Cheung, K. L., Khor, T. O., Chen, C., & Kong, A. N. (2010). Phytochemicals: Cancer chemoprevention and suppression of tumor onset and metastasis. *Cancer and Metastasis Reviews*, *29*, 483–502.

Sugamura, K., & Keaney, J. F. (2011). Reactive oxygen species in cardiovascular disease. *Free Radical Biology and Medicine*, *51*, 978–992.

Sun, J. B., Wang, X. Z., Wang, P., Li, L. Z., Qu, W., & Liang, J. Y. (2015). Antimicrobial, antioxidant and cytotoxic properties of essential oil from *Dictamnus angustifolius*. *Journal of Ethnopharmacology*, *159*, 296–300.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T., Mazur, M., & Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. *The International Journal of Biochemistry & Cell Biology*, *39*, 44–84.

Yu, L. Q., Shirai, N., Suzuki, H., Sugane, N., Hosono, T., Nakajima, Y., Kajiwara, M., & Takatori, K. (2010). The effect of methanol extracts of *tsao-ko* (*Amomum tsao-ko* Crevost et Lemaire) on digestive enzyme and antioxidant activity *in vitro*, and plasma lipids and glucose and liver lipids in mice. *Journal of Nutritional Science and Vitaminology*, *56*, 171–176.

Zhang, T. T., & Jiang, J. G. (2015). Analyses on essential oil components from the unripe fruits of *Rubus chingii* Hu by different methods and their comparative cytotoxic and anti-complement activities. *Food Analytical Methods*, *8*, 937–944.

Zhang, T. T., Lu, C. L., & Jiang, J. G. (2014). Bioactivity evaluation of ingredients identified from the fruits of *Amomum tsaoko* Crevost et Lemaire, a Chinese spice. *Food Function*, *5*, 1747–1754.

Zhang, T. T., Lu, C. L., Jiang, J. G., Wang, M., Wang, D. M., & Zhu, W. (2015). Bioactivities and extraction optimization of crude polysaccharides from the fruits and leaves of *Rubus chingii* Hu. *Carbohydrate Polymers*, *130*, 307–315.

Zhang, T. T., Xu, X. L., Jiang, M. H., & Jiang, J. G. (2013). Hepatoprotective function of *Penthorum chinense* Pursh. *Food Function*, *4*, 1581–1585.

Zhang, T. T., Yang, L., & Jiang, J. G. (2015a). Bioactive comparison of main components from unripe fruits of *Rubus chingii* Hu and identification of the effective component. *Food Function*, doi:10.1039/c5fo00406c.

Zhang, T. T., Yang, L., & Jiang, J. G. (2015b). Effects of thonningianin A in natural foods on apoptosis and cell cycle arrest of HepG-2 human hepatocellular carcinoma cells. *Food Function*, doi:10.1039/c5fo00388a.