JPP Journal of Pharmacy and Pharmacology

Royal Pharmaceutical Society of Great Britain

JPP Journal of Pharmacy and Pharmacology

Research Paper

JPP 2010, 62: 1037–1044

Received September 09, 2009
Accepted April 15, 2010
DOI 10.1111/j.2042-7158.2010.01119.x
ISSN 0022-3573

Biological activity of the essential oil of *Kadsura longipedunculata* (Schisandraceae) and its major components

Sri Mulyaningsih[a], Mahmoud Youns[b], Mahmoud Z. El-Readi[a], Mohamed L. Ashour[a], Endalkachew Nibret[a], Frank Sporer[a], Florian Herrmann[a], Jürgen Reichling[a] and Michael Wink[a]

[a]Institute of Pharmacy and Molecular Biotechnology, Heidelberg University and [b]Department of Functional Genome Analysis, German Cancer Research Center (DKFZ), Heidelberg, Germany

Abstract

Objectives The aim was to determine the chemical composition of the essential oil of *Kadsura longipedunculata* and the biological activity of the oil and its major components.
Methods The essential oil from stem bark of *Kadsura longipedunculata* was analysed by capillary gas chromatography (GLC/FID) and gas chromatography–mass spectrometry (GLC/MS). The ability of the oil to reduce diphenylpicrylhydrazine (DPPH•) was used to evaluate the antioxidant activity. Inhibition of both lipoxygenase and prostaglandin E_2 was used to assess the anti-inflammatory activity. Antimicrobial activity was studied *in vitro* against a range of bacteria and fungi using diffusion and microdilution methods. Inhibition of trypanosome proliferation was assessed using resazurin as vital stain. The in-vitro cytotoxicity of the essential oil on six human cancer cell lines (HepG2, MIA PaCa-2, HeLa, HL-60, MDA-MB-231 and SW-480) was examined using the MTT assay.
Key findings Fifty compounds, representing 97.63% of total oil, were identified. δ-Cadinene (21.79%), camphene (7.27%), borneol (6.05%), cubenol (5.12%) and δ-cadinol (5.11%) were found to be the major components of the oil. The oil exerted a good antimicrobial activity against all Gram-positive bacteria tested, including methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecalis*. *Streptococcus pyogenes* and *S. agalactiae* were the most sensitive bacteria with a minimal inhibitory concentration (MIC) of 60 μg/ml oil. The essential oil showed a moderate fungicidal activity against yeasts, but it did not show any activity against Gram-negative bacteria. The essential oil showed a good trypanocidal activity in *Trypanosoma b. brucei* with an IC50 value of 50.52 $\pm$ 0.029 μg/ml. Radical scavenging activity had an IC50 value of 3.06 $\pm$ 0.79 mg/ml. 5-Lipoxygenase inhibition (IC50 = 38.58 μg/ml) and prostaglandin E_2 production inhibition (28.82% at 25 μg/ml) accounted for anti-inflammatory activity of the oil. The oil exhibited some degree of cytotoxic activity against MIA PaCa-2, HepG-2 and SW-480 cell lines with IC50 values of 133.53, 136.96 and 136.62 μg/ml, respectively. The oil increased caspase 3/7 activity (an indicator of apoptosis) 2.5–4 fold in MIA Paca-2 cells. Camphene and borneol did not show antioxidant activity. However, both compounds exhibited some degree of antimicrobial, trypanocidal, anti-inflammatory and cytotoxic activity.
Conclusions This investigation provided evidence for, and confirmed the efficacy of, *K. longipedunculata*, a traditionally used Chinese medicinal plant for the treatment of inflammation and infection.
Keywords antimicrobial and anti-inflammatory; cytotoxicity; *Kadsura longipedunculata* essential oil; traditional Chinese medicine (TCM); trypanocidal

Introduction

Essential oils have been used in traditional medicine all over the world to treat infection and many diseases. Essential oils are a rich source of biologically active compounds and generally possess a strong and persistent odour, usually characteristic of the plant in which they are found. Essential oils have been shown to possess in-vitro antibacterial, antifungal, antioxidant, analgesic, anti-inflammatory, insecticidal and antiviral properties.[1–3] Some components of essential oils have also been shown to exert both in-vitro and in-vivo

Correspondence: Professor Dr Michael Wink, Institute für Pharmazie und Molekulare Biotechnologie, Universität Heidelberg, Im Neuenheimer Feld 364, 69120 Heidelberg, Germany.
E-mail: wink@uni-hd.de

anti-tumour activity against murine leukaemia, hepatoma and melanoma cells. Additionally, the chemotherapeutic values of some essential oil components against cancer cells are under evaluation in Phase I clinical trials.[4] Moreover, some essential oils are directly used in pharmaceutical, food and perfumery industries and are found in marketed products.

Kadsura longipedunculata Finet et Gagnep belongs to the medicinally important family Schisandraceae, which is widely distributed particularly in southern China. This plant is an evergreen scandent vine and is used in traditional Chinese medicine (TCM) for the treatment of rheumatoid arthritis as well as gastrointestinal diseases, such as gastric and duodenal ulcers, traumatic injury and ulcers with pyogenic infection.[5] Previous findings confirmed that a few lignans and triterpenoids possess some beneficial biological effects, including anti-inflammatory, antioxidant,[6] anti-hyperlipidaemic,[7] antitumour and antiviral activity.[8] A previous study also reported that the essential oil from the roots of *K. longipedunculata* has antimicrobial, antioxidant and cytotoxic activity and also found δ-cadinene to be the main compound of the oil.[9] However, the contribution of the main components of the essential oil towards its biological activity has not been investigated.

To our knowledge, no reports on the essential oil from stem bark have been published. Therefore, this study aimed to investigate the chemical composition of the essential oil of *K. longipedunculata* stem bark and also to examine its biological activity that is based on its ethnopharmacological use for the treatment of inflammation and infections. Additionally, we tested the potential of this plant as an anti-tumour drug. We report for the first time the contribution of two major compounds (camphene and borneol) of the essential oil to its biological properties.

Materials and Methods

Plant material

The stem bark of *K. longipedunculata* was commercially obtained from China. The plant material was purchased from a TCM plant shop in January 2007 by Wan Chuangxing. The identity of the plant was confirmed by DNA barcoding. DNA was isolated from the dried crude drug as well as reference plants in the Botanical Garden of Heidelberg. The chloroplast *rbc*L gene was amplified and sequenced. The drug and the authentic reference had identical sequences. Voucher specimens (P6879) of the plant material were deposited at the Department of Biology, Institute of Pharmacy and Molecular Biotechnology, Heidelberg University.

Isolation of essential oil

The dried powder of *K. longipedunculata* was subjected to hydrodistillation for 6 h using a Clevenger-type apparatus. After separation, the oil was kept in sealed vials at 4°C for further analysis. Camphene was purchased from Sigma Aldrich (St Louis, USA) and borneol from Fluka Chemika (Buchs, Switzerland).

GLC/FID

High resolution gas chromatography was carried out on a Varian 3400 equipped with FID detector and OV-1 column (30 m × 0.25 mm × 0.25 μm) (Ohio Valley, Marietta, USA). The operating conditions were as follows: carrier gas helium with a flow rate of 2 ml/min, split ratio 1 : 20. The oven temperature was programmed with an initial temperature 40°C, 2 min isothermal, 300°C, 4°C/min, then 10 min isothermal. Injector and detector temperatures were set at 250 and 300°C, respectively. PeakSimple 2000 chromatography data system (SRI Instruments, Torrance, USA) was used for recording and integrating of the chromatograms.

GLC/MS analysis

GLC/MS was carried out on a Hewlett-Packard gas chromatograph (GC 5890 II) equipped with the same column as GLC (see above). Samples (2 μl) were injected in a split mode (split ratio, 1 : 15) with the carrier gas helium at a flow rate of 2 ml/min. The capillary column was coupled to a quadrupole mass spectrometer (SSQ 7000; Thermo-Finnigan, Bremen, Germany). The injector temperature was 250°C. Helium carrier gas flow rate was 2 ml/min. All the mass spectra were recorded with electron energy of 70 eV and ion source of 175°C. The oil components were identified by their retention time, retention indices (RI) relative to C8–C28 *n*-alkanes, computer matching with the Wiley Registry of Mass Spectral Data 8th edition, NIST Mass Spectral Library (December 2005) and by comparison of their mass spectra with data already available in the literature.[10,11]

Antimicrobial assay

Microbial strains

The essential oil and its components were tested against Gram-positive bacteria (*Bacillus subtilis* ATCC 6051, *Staphylococcus aureus* ATCC 29213, *Staphylococcus epidermidis* ATCC 14990, *Staphylococcus saprophyticus* ATCC 15305, *Streptococcus pyogenes* ATCC 12344, *Streptococcus agalactiae* ATCC 27956, methicillin-resistant *Staphylococcus aureus* (MRSA) NCTC 10442, *Enterococcus faecalis* ATCC 29212, vancomycin-resistant *Enterococcus faecalis* (VRE) ATCC 51299), Gram-negative bacteria (*Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853) and fungi (*Candida albicans* ATCC 90028, *Candida glabrata* ATCC MYA 2950 and *Candida parasilopsis* ATCC 22019). The essential oil was also tested against clinical isolates from patients, MRSA MR818014 and MRSA MR818081. All of the microorganism cultures were obtained from the Medical Microbiology Laboratory, Hygiene Institute, University of Heidelberg, Germany. The strains were sub-cultured on an appropriate agar plate 24 h before any antimicrobial test.

Culture media

Mueller Hinton broth (MH) (Fluka, Switzerland) and Mueller Hinton agar (MH2) (bioMérieux, France) were used for MIC and MBC determination (*B. subtilis*, *S. aureus*, *S. epidermidis*, *S. saprophyticus*, MRSA, *E. coli* and *P. aeruginosa*). Brain Heart Infusion (BHI) (Merck, Germany) and MH2 supplemented with 5% sheep blood (Heipha, Germany) were used for MIC and MBC determination of *S. agalactiae*, *S. pyogenes*, *Enterococcus faecalis* and VRE. The antifungal activity test used the Sabouraud Dextrose broth (SDB) and Sabouraud Dextrose agar (SDA) with chloramphenicol (Becton Dickinson, Germany).

Diffusion test

The antimicrobial diffusion test was carried out as described by NCCLS (2006) using a cell suspension of about 1×10^6 CFU/ml.[12] The suspension was standardised with 0.5 McFarland standard using densitometry (DENSIMAT; bioMérieux, France). The wells with 6 mm diameter were punched out and filled with essential oil. Inhibition zones after 24 h (for bacteria) and 48 h (for fungi) incubation at 37°C were measured.

Determination of minimum inhibitory concentration and minimum bactericidal concentration

The MIC of the samples was determined by microdilution methods as recommended by NCCLS as described previously.[10] The samples were dissolved in dimethyl sulfoxide (DMSO) and added to the medium, then diluted two fold to obtain concentrations in the range 0.015–8 mg/ml. Inoculum suspension with a final concentration of 0.5×10^6 cfu/ml were added to a 96-well microplate. The MIC was determined as the lowest concentration without growth (turbidity, precipitation) after incubation at 37°C for 24 h. The MBC was determined by subcultivation of medium from clear wells with no growth. The experiments were performed in duplicate and repeated twice.

Anti-trypanosomal activity

Trypanosoma b. brucei TC221 bloodstream forms, the causative agent of Nagana, were grown in Baltz medium supplemented with 20% inactivated fetal bovine serum and 1% penicillin–streptomycin. The cells were incubated in a humidified atmosphere containing 5% CO_2 at 37°C. Trypanocidal activity was determined as described in a previous paper.[13] Briefly, the samples were serially diluted with the medium in a two-fold fashion to attain final concentrations in the range 250–3.91 μg/ml in 96-well plates. *T. b. brucei* cells were seeded into 96 wells at a density of 1×10^4 cells per 100 μl. The cells were incubated for a total of 48 h and the antitrypanosomal activity of the extracts was evaluated using resazurin as cell proliferation indicator dye with some modifications from the method that was used by Rolón.[14] Furthermore, 10 μl of resazurin was added to trypanosome culture and the culture was incubated with the resazurin for 24 h before measuring the plates after 48 h of incubation. The absorbance of the plates was read using Tecan Safire II Reader at dual wavelengths of 570 nm and 600 nm. Trypanocidal activity was tested in triplicate and repeated twice.

Antioxidant activity

The free radical scavenging capacity of the essential oil and its components were determined in three independent experiments using the diphenylpicrylhydrazyl (DPPH•) assay. Equal volumes of various sample solutions and 0.2 mM methanolic solution of DPPH• were mixed and the absorbance was measured against a blank at 517 nm using a Tecan Safire II Reader after incubation in the dark for 30 min at room temperature. Rutin was used as a positive control. The percentage inhibition was calculated using the following equation:

$$\text{Inhibition}\,(\%) = 100 \times [\text{A517}\,(\text{control}) - \text{A517}\,(\text{sample})/\text{A517}\,(\text{control})] \quad (1)$$

Anti-inflammatory effect

Prostaglandin E_2 inhibition assay

The ability of the essential oil and its components to inhibit prostaglandin E_2 (PGE_2) production was assessed using PGE2 Monoclonal EIA Kit (Cayman Chemicals). MIA PaCa-2 (pancreatic cancer cells) were treated with 50 μl of each sample (25 μg/ml) and incubated at 37°C with 5% CO_2. Twenty four hours later, cells were stimulated with 30 μM arachidonic acid. The culture supernatants were collected after 15 min then centrifuged to remove debris. Prostaglandin levels were determined from the supernatant by a competitive enzyme immunoassay. Inhibition of the prostaglandin E_2 level was calculated relative to the untreated control in three independent experiments; the selective cyclooxygenase (COX)-2 inhibitor NS-398 (Cayman) was used as a positive control.

5-Lipoxygenase inhibition

The reaction mixtures containing 10 μl enzyme (7.9 U/ml) and 20 μl of the tested samples in 0.1 M phosphate buffer pH 9.0 were incubated at room temperature for 10 min. The reaction was started by addition of the substrate sodium linoleate (Sigma) (62.5 μM) to the reaction mixture. The reaction was monitored for a 10-min period, by recording absorbance at 234 nm using a LKB Biochrom spectrophotometer.[15] The initial reaction rates were determined from the slope of the straight portion of the curve and inhibition of the enzyme activity was calculated from three independent experiments by comparison with the control (ethanol).

In-vitro cytotoxicity assay

HepG2 (human hepatocellular liver carcinoma), MIA PaCa-2 (human pancreatic carcinoma) and HeLa (human cervical carcinoma) cell lines were maintained in DMEM medium, whereas HL60 (human promyelocytic leukaemia), MDA-MB-231 (human breast adenocarcinoma) and SW-480 (human colon adenocarcinoma) cell lines were maintained in RPMI1640 medium. Both media were supplemented with L-glutamine, 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 U/ml streptomycin. Cells were grown at 37°C in a humidified atmosphere of 5% CO_2.

The cytotoxicity was determined with the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, based on cellular conversion of tetrazolium salt into a formazan product,[16] in three independent experiments. Briefly, 2×10^5 cells of each cell lines were seeded in a 96-well plate. Cells were cultivated for 24 h and then treated with various concentrations of the samples at 37°C for 24 h. Afterwards, 0.5 mg/ml MTT was added to each well and incubated for 4 h. The formed formazan was dissolved in 200 μl DMSO and the absorbance was recorded at 570 nm with a Tecan Safire II Reader. Cytotoxicity was expressed as the concentration of sample inhibiting cell viability by 50% (IC50).

Caspase-Glo 3/7 assay

The influence of the oil and its components on caspase 3/7 activity in Mia PaCa-2 cell line was detected using Caspase-

Glo 3/7 Assay kit (Promega, Mannheim, Germany). Cells cultured in DMEM were seeded in 96-well plates and treated with the samples (0.125–1 mg). After 24 h treatment, 100 μl of caspase 3/7 reagent were added to each well, mixed and incubated for 30 min at room temperature. Luminescence was measured using Mithras LB 940 instrument (Berthold Technologies, Bad Wildbad, Germany). Cellular apoptosis was expressed as percentage of the untreated medium control[17] from three independent experiments.

Statistical analysis

The IC50 value was determined from a four-parameter logistic curve (SigmaPlot 11.0). Statistical analysis of all data was performed using a Student's *t*-test or Kruskal–Wallis test followed by Dunn's post-hoc multiple comparison test (GraphPad Prism 5.01; GraphPad Software, Inc., CA, USA). $P < 0.05$ denoted significance in all cases.

Results

Chemical composition of the essential oil

The yield of essential oil from *K. longipedunculata* after hydrodistillation was 0.64%. The chemical composition of the essential oil is listed in Table 1. By GLC-MS, 50 components were identified, representing 97.63% of the total oil. The most abundant constituents were δ-cadinene (21.79%), camphene (7.27%), borneol (6.05%), cubenol (5.12%) and δ-cadinol (5.11%). The oil contained 22.63% monoterpenes (which include 10.25% oxygenated monoterpenes) and 75% sesquiterpenes (which include 23.77% oxygenated sesquiterpenes).

Antimicrobial assay

The antimicrobial activity of the essential oil and the two components (camphene and borneol) was determined with the agar diffusion method (Table 2). The crude oil produced inhibition zones in the range 8.0–13.0 mm, whereas isolated components of the oil displayed an antimicrobial effect with inhibition zones in the range 6.7–11.0 mm.

All Gram-positive bacteria were susceptible to the oil with MIC values of 0.06–2 mg/ml. *Streptococcus agalactiae* and *S. pyogenes* were the most susceptible bacteria with an MIC of 0.06 mg/ml (Table 3). The oil exhibited a substantial activity against *B. subtilis*, *S. saprophyticus*, *S. epidermidis*, *S. aureus* and *E. faecalis* with an MIC of 0.25–0.5 mg/ml, but did not show activity against Gram-negative bacteria, such as *E. coli* and *P. aeruginosa*, even at the highest concentration. The oil displayed a moderate activity against yeasts, such as *C. albicans*, *C. glabrata* and *C. parasilopsis*, with MIC values of 1–2 mg/ml. The oil exhibited a good activity against antibiotic-resistant strains, both MRSA and VRE, with an MIC value of 0.5–2 mg/ml. The statistical analysis revealed significant differences in antimicrobial activity between the oil and the isolated compounds (camphene and borneol) ($P < 0.01$).

Trypanocidal activity

The results of trypanocidal activity are summarised in Table 4. The essential oil showed moderate activity with an

Table 1 Chemical composition of *K. longipedunculata* essential oil

No.	Compound	RI (OV-1)	Percentage (%)[a]
Monoterpene hydrocarbons			
1	Tricycline	905	0.55
2	α-Thujene	913	0.18
3	α-Pinene	918	1.55
4	Camphene	931	7.27
5	β-Pinene	959	0.81
6	β-Myrcene	980	tr
7	α-Terpinene	1005	tr
8	P-Cymene	1009	0.13
9	Limonene	1017	1.56
10	γ-Terpinene	1046	0.18
11	Terpinolene	1076	tr
Oxygenated monoterpenes			
12	1,8-Cineole	1015	0.50
13	Camphor	1114	0.19
14	Borneol[b]	1145	6.05
15	Terpinen-4-ol[b]	1157	1.26
16	α-Tepineol	1168	0.21
17	Bornyl acetate	1266	2.04
Sesquiterpene hydrocarbons			
18	δ-Elemene	1331	4.03
19	α-Cubebene	1344	1.20
20	α-Copaene	1368	2.83
21	β-Elemene	1381	1.50
22	α-Gurjunene	1405	1.16
23	β-Caryophyllene	1408	0.19
24	β-Copaene	1418	0.11
25	(+)-Aromadendrene	1428	0.33
26	α-Humulene	1441	0.52
27	allo-Aromadendrene	1449	0.90
28	β-Chamigrene	1463	2.42
29	γ-Muurolene	1466	1.41
30	Germacrene D	1468	0.59
31	β-Selinene	1473	1.74
32	*epi*-Bicyclosesquiphellandrene	1477	2.04
33	Viridiflorene	1484	0.48
34	α-Muurolene[b]	1489	3.45
35	Calamenene	1504	1.39
36	δ-Cadinene[b]	1512	21.79
37	Cadina-1,4-diene[b]	1519	0.94
38	α-Calacorene	1523	1.37
39	Cadala-1(10),3,8-triene	1540	0.84
Oxygenated sesquiterpenes			
40	*trans*-Nerolidol[b]	1547	0.88
41	Spathulenol[b]	1555	3.07
42	β-Caryophyllene oxide	1559	0.67
43	Viridiflorol	1566	0.75
44	γ-Eudesmol	1604	2.00
45	Cubenol	1607	5.12
46	τ-Cadinol	1619	4.39
47	δ-Cadinol[b]	1623	5.11
48	τ-Muurolol	1630	1.10
49	Cadalene	1647	0.38
50	α-Bisabolol[b]	1664	0.30
Monoterpene hydrocarbons			12.38
Oxygenated monoterpenes			10.25
Sesquiterpene hydrocarbons			51.23
Oxygenated sesquiterpenes			23.77
Total			97.63

[a]Average of two analyses. [b]Previously reported.[9] tr, trace (<0.1%).

Table 2 In-vitro antimicrobial activity of *K. longipedunculata* oil, components and reference antibiotics determined with the diffusion method

No	Microorganism	Diameter of inhibition zone (mm)					
		Essential oil	Borneol	Camphene	Ampicillin	Vancomycin	Nystatin
Gram-positive bacteria							
1	*Bacillus subtilis* ATCC 6051	8.0 ± 0.0	7.3 ± 0.6	6.7 ± 0.6$^{\#}$	29.7 ± 0.6	20.8 ± 0.8	NT
2	*Staphylococcus saprophyticus* ATCC 15305	11.7 ± 0.6	8.3 ± 0.6*	9.7 ± 1.5	33.7 ± 0.6	17.0 ± 0.0	NT
3	*Staphylococcus epidermidis* ATCC 14990	11.3 ± 0.6	10.3 ± 1.5	9.7 ± 0.6$^{\#}$	25.3 ± 1.3	18.0 ± 0.0	NT
4	*Staphylococcus aureus* ATCC 29213	13.0 ± 1.0	NI***	NI$^{\#\#\#}$	22.0 ± 0.0	17.3 ± 0.8	NT
5	*Streptococcus agalactiae* ATCC 27956	9.0 ± 0.0	7.0 ± 1.0*	9.3 ± 1.2	30.0 ± 0.0	29.7 ± 0.6	NT
6	*Streptococcus pyogenes* ATCC 12344	9.7 ± 0.6	9.7 ± 0.6	9.7 ± 0.6	30.0 ± 0.0	17.3 ± 0.6	NT
7	*Enterococcus faecalis* ATCC 29212	9.0 ± 1.0	NI***	NI$^{\#\#\#}$	24.7 ± 0.6	16.3 ± 0.6	NT
8	VRE *E. faecalis* ATCC 51299	8.3 ± 1.2	NI***	NI$^{\#\#\#}$	25.0 ± 0.0	12.7 ± 0.6	NT
9	MRSA NCTC 10442	11.7 ± 0.6	NI***	NI$^{\#\#\#}$	0.0 ± 0.0	17.7 ± 0.0	NT
10	MRSA MR 818014 (clinical isolate)	11.3 ± 0.6	NI***	NI$^{\#\#\#}$	7.3 ± 1.2	17.3 ± 0.6	NT
11	MRSA MR 818081 (clinical isolate)	12.0 ± 1.0	NI***	NI$^{\#\#\#}$	9.6 ± 0.6	16.7 ± 0.6	NT
Gram-negative bacteria							
12	*Escherichia coli* ATCC 25922	NI	NI	NI	10.7 ± 0.6	NI	NT
13	*Pseudomonas aeruginosa* ATCC 27853	NI	NI	NI	NI	NI	NT
Yeasts							
14	*Candida albicans* ATCC 90028	8.6 ± 0.6	10.7 ± 1.2	10.0 ± 0.0	NT	NT	17.3 ± 0.6
15	*Candida glabrata* ATCC MYA 2950	10.3 ± 0.6	10.0 ± 1.1	10.0 ± 0.6	NT	NT	12.7 ± 0.6
16	*Candida parasilopsis* ATCC 22019	8.3 ± 0.6	11.0 ± 1.7	7.3 ± 0.6	NT	NT	12.3 ± 1.2

NI, no inhibition; NT, not tested. The dosage of essential oil and components were 3.2 mg, ampicillin and vancomycin were 10 μg, and nystatin 20 μg. Presented data are mean values ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, essential oil compared with borneol. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, essential oil compared with camphene.

Table 3 Minimal inhibitory concentration (MIC) and minimal biocidal concentration (MBC) of *K. longipedunculata* oil, components and reference antibiotics determined with microdilution method

No.	Microorganism	*K. longipedunculata*		Borneol		Camphene		Ampicillin		Vancomycin		Nystatin	
		MIC*	MBC	MIC*	MBC	MIC$^{\#}$	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Gram-positive bacteria													
1	*Bacillus subtilis* ATCC 6051	0.25	0.25	2	4	2	4	0.1	0.8	0.2	0.8	NT	NT
2	*Staphylococcus saprophyticus* ATCC 15305	0.25	2	1	4	1	4	0.2	0.4	1.6	3.1	NT	NT
3	*Staphylococcus epidermidis* ATCC 14990	0.5	2	1	4	2	4	0.4	0.8	0.8	1.6	NT	NT
4	*Staphylococcus aureus* ATCC 29213	0.5	2	4	8	4	>8	0.4	3.1	0.4	0.8	NT	NT
5	*Streptococcus agalactiae* ATCC 27956	0.06	0.06	1	2	1	1	0.1	0.2	0.4	0.4	NT	NT
6	*Streptococcus pyogenes* ATCC 12344	0.06	0.06	1	2	1	2	0.1	0.1	0.1	0.2	NT	NT
7	*Enterococcus faecalis* ATCC 29212	2	4	8	>8	>8	>8	0.4	0.8	1.6	3.1	NT	NT
8	VRE *E. faecalis* ATCC 51299	2	4	8	>8	>8	>8	0.4	0.8	25	50.0	NT	NT
9	MRSA NCTC 10442	0.5	2	4	8	8	>8	6.2	12.5	0.8	1.6	NT	NT
10	MRSA MR 818014 (clinical isolate)	1	2	8	8	>8	>8	6.2	6.2	0.8	0.8	NT	NT
11	MRSA MR 818081 (clinical isolate)	1	2	8	8	>8	>8	6.2	50	0.8	0.8	NT	NT
Gram-negative bacteria													
12	*Escherichia coli* ATCC 25922	NI	NI	4	8	8	>8	6.2	12.5	NI	NI	NT	NT
13	*Pseudomonas aeruginosa* ATCC 27853	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NT	NT
Yeasts													
14	*Candida albicans* ATCC 90028	2	4	4	8	8	8	NT	NT	NT	NT	1.6	1.6
15	*Candida glabrata* ATCC MYA 2950	2	4	4	4	4	4	NT	NT	NT	NT	1.6	1.6
16	*Candida parasilopsis* ATCC 22019	1	2	2	4	4	4	NT	NT	NT	NT	1.6	1.6

Units of oil and components are expressed as mg/ml, reference antibiotics are μg/ml. NI, no inhibition; NT, not tested. Presented data are mean values. *$P < 0.01$, oil compared with borneol; $^{\#}P < 0.01$ oil compared with camphene.

IC50 value of 50.52 ± 0.029 μg/ml, whereas camphene and borneol exhibited trypanocidal activity with a statistically significant IC50 value of 80.66 ± 0.87 and 70.00 ± 1.28 μg/ml, respectively ($P < 0.001$).

Antioxidant activity

As shown in Table 4, the essential oil was able to reduce the purple-coloured DPPH radical to the yellow-coloured diphe-

Table 4 Antitrypanosomal and radical scavenging activity of *K. longipedunculata* essential oil, camphene and borneol

Substance	Trypanocidal activity IC50 (μg/ml)	DPPH assay IC50
K. longipedunculata oil	50.52 ± 0.029	3.06 ± 0.79 mg/ml
Camphene	80.66 ± 0.87*	>10 mg/ml
Borneol	70.00 ± 1.28#	>10 mg/ml
Positive control	0.17 ± 0.03	4.10 ± 0.41 μg/ml

Presented data are means ± SD. *$P < 0.001$, oil compared with camphene; #$P < 0.001$, oil compared with borneol.

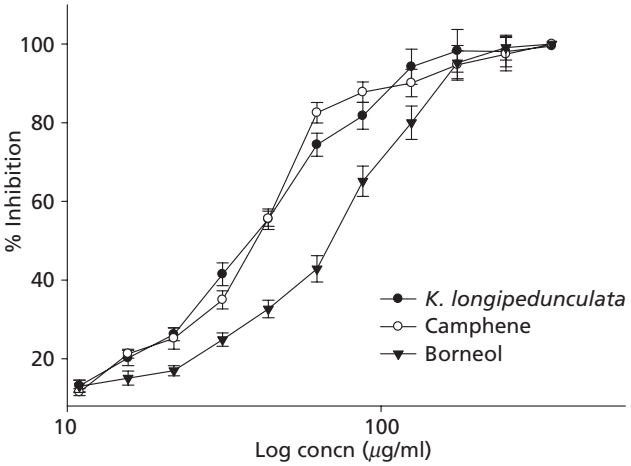

Figure 1 Inhibitory effect of *K. longipedunculata* essential oil, camphene and borneol on soybean 5-lipoxygenase. Data are from three independent experiments. There was a significant difference between the oil and borneol ($P < 0.001$).

nylpicrylhydrazine with an IC50 of 3.06 ± 0.79 mg/ml. On the other hand, camphene and borneol were hardly active; they showed a 10% inhibition up to a concentration of 10 mg/ml.

5-Lipoxygenase inhibition

The inhibition of 5-lipoxygenase by the essential oil, camphene and borneol is shown in Figure 1. The IC50 value of the oil was 38.58 ± 3.8 μg/ml, whereas the IC50 of camphene and borneol was 39.72 ± 2.16 and 69.22 ± 3.66 μg/ml, respectively. The inhibition of 5-lipoxygenase by the oil and camphene showed no significant difference ($P > 0.05$), although a significant difference was found between the oil and borneol ($P < 0.001$).

Inhibition of prostaglandin E_2 production

The amount of PGE_2 produced by MIA PaCa-2 cells was determined to assess a potential inhibitory effect of the essential oil and its components on cyclooxygenase activity. As shown in Figure 2, 25 μg/ml of the oil inhibited prostaglandin E_2 formation by 28.82%, whereas borneol and camphene displayed 33.74 and 45.78% inhibitory activity compared with untreated control, respectively. A concentration of 3.14 μg/ml of NS-398 was used as positive control and resulted in 64.11% inhibition of PGE_2 production. The oil and borneol showed no

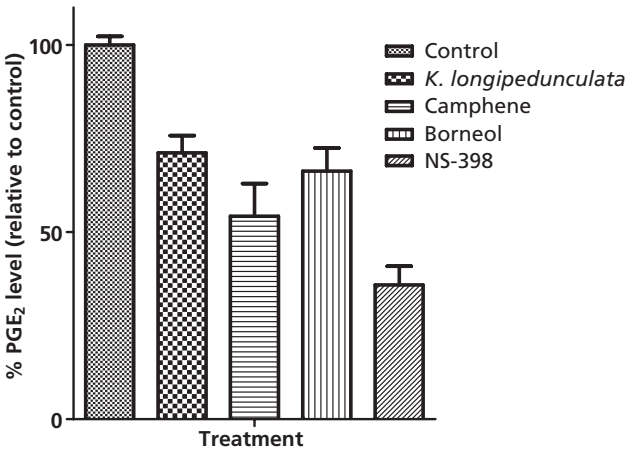

Figure 2 Inhibition of PGE_2 in MIA PaCa-2 cells with *K. longipedunculata* essential oil, camphene, borneol and NS-398. Oil, camphene and borneol concentration was 25 μg/ml; NS398 concentration was 3.14 μg/ml. Data are presented as means ± SD. There was a significant difference between the oil and camphene ($P < 0.05$).

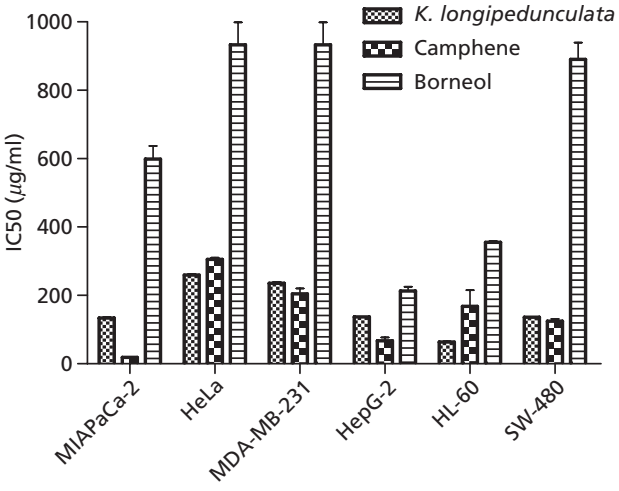

Figure 3 Cytotoxic activity of *K. longipedunculata* essential oil, camphene and borneol in mammalians cell lines. Data are shown as means ± S.D of IC50 values (μg/ml). There was a significant difference between the essential oil and borneol ($P < 0.01$).

significant difference in the inhibition of prostaglandin ($P > 0.05$), although a significant difference was observed between the oil and camphene ($P < 0.05$).

Cytotoxicity

The cytotoxicity of the essential oil, camphene and borneol in HepG2, MIA PaCa-2, HeLa, HL-60, MDA-MB-231 and SW-480 cell lines is illustrated in Figure 3. The oil exhibited some degree of cytotoxicity against MIA PaCa-2, HepG-2 and SW-480 cell lines (IC50 values were 133.53, 136.96 and 136.62 μg/ml, respectively). Camphene exerted a cytotoxic effect against SW-480 and HL-60 cells (IC50 values were 124.21 and 167.75 μg/ml, respectively). The statistical analysis revealed significant differences in cytotoxicity between the

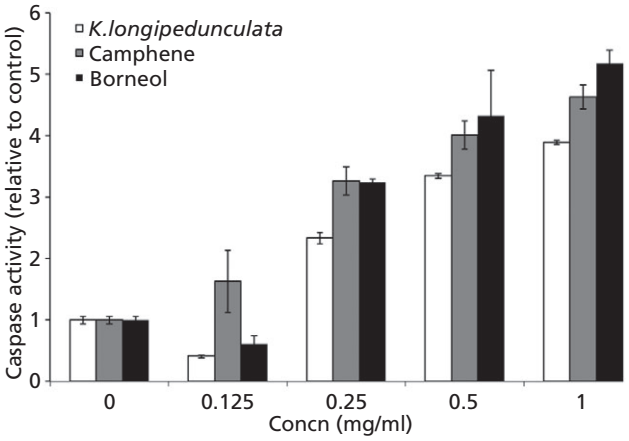

Figure 4 Caspase activity of *K. longipedunculata* essential oil, camphene and borneol. Data are shown as means ± SD from three independent experiments.

oil and borneol ($P < 0.01$) but no significant difference was observed between the oil and camphene ($P > 0.05$).

Caspase assay

To gain insight into the mechanism involved in the cytotoxicity induced by the essential oil and its components, caspase 3/7 activity was evaluated as an indicator and measure for apoptosis (Figure 4). After 24 h of incubation of MIA PaCa-2 cells with essential oil, the caspase 3/7 activity was increased 2.5–4 fold above the control. Camphene and borneol stimulated caspase activity in a dose-dependent manner, from 1.75 to 4.5 fold and 3 to 5 fold, respectively. A significant difference was observed between the oil and the two isolated compounds at a concentration of 0.25 mg/ml ($P < 0.01$).

Discussion

The essential oil exerted an inhibitory activity against all Gram-positive bacteria and against yeasts but no activity against Gram-negative bacteria. Previous investigations had also shown Gram-positive bacteria to be more susceptible to essential oils than Gram-negative bacteria.[18,19] The low susceptibility of Gram-negative bacteria, such as *E. coli* and *P. aeruginosa*, to the essential oil is probably due to the fact that these types of bacteria have an outer membrane consisting of a very thick lipopolysaccharide layer that serves as a barrier membrane to lipophilic substances.[19] Methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecalis* strains were sensitive to the oil with an MIC value twice as high as in non-resistant strains, indicating that the oil has a different mode of action to penicillin.[20]

The pronounced antimicrobial and trypanocidal activity of the oil could be attributed to camphene or borneol. Both the oil and the two isolated compounds are lipophilic substances and might interact with membrane lipids and proteins and this would result in cell disruption and finally cell death.[21,22]

The good radical scavenging activity and significant inhibition of both cyclooxygenase and lipoxygenase validated the anti-inflammatory activity of the essential oil of *K. longipedunculata*. 5-Lipoxygenase (5-LOX) converts arachidonic acid into leukotrienes, which are mediators of inflammatory and allergic reactions. Low IC50 values suggest the better inhibitory actions on 5-LOX and hence a greater anti-inflammatory activity. Additionally, PGE_2 is the major inflammatory mediator produced by COX from arachidonic acid. COX expression is induced by various stimuli, and its over-expression is closely related to the pathogenesis of some degenerative diseases, including cancer. Reduction of PGE_2 level is directly related to COX inhibition. Inhibition of both 5-LOX and COX by the oil would be very useful for relief of inflammation.

The cytotoxicity of the oil was highest in the HL-60 cell line whereas the other cell lines were less sensitive. The low cytotoxicity of the oil in HeLa cells may be due to the expression of ABCB7; ABC-transporters, which are detected in most organs, increase survival of endothelial cells since they can pump out toxins that have entered the cells by diffusion.[23] Insensitivity of MDA-MB-231 to essential oil may be related to the highly expressed ABCG2 (breast cancer resistance protein, BCRP) and ABCC11 (multidrug resistance protein 8, MRP8), which have been implicated as resistance factors in breast cancer.[24] These ABC-transporters play an important role in multiple drug resistance and cause a failure of cancer chemotherapy by increasing the efflux of cytotoxic agent and thereby decreasing its efficacy.[25]

Apoptosis can be deduced from activation of caspase 3/7 activity. The assay demonstrated that an increase of caspase activity corresponds to decrease in cell viability.[26] The essential oil, borneol and camphene stimulated caspase activity in MIA PaCa-2 cells at all tested concentrations.

Comparing caspase activity with the cytotoxicity against MIA PaCa-2 cells, camphene was the most active cytotoxic agent; however, the caspase induction was close to that of borneol. This suggests that camphene can induce cell death by more than one mechanism, such as cell cycle arrest or any other form of cell death beside apoptosis. On the other hand, borneol exhibited lower cytotoxicity but the caspase activity was relatively high, indicating that apoptosis is the main mechanism of action.

In contrast to the other biological actions, both camphene and borneol showed low radical scavenging activity. This result is in agreement with previous findings.[2] The crude oil was found to be more effective than the isolated compounds indicating possible synergistic interactions of the components of the oil.

Conclusions

The results of this investigation provided evidence for, and confirmed the efficacy of, the traditional use of *K. longipedunculata* against inflammation and infection. Moreover, the essential oil of *K. longipedunculata* could be a potential drug against trypanosomes. Our experiments indicated that camphene and borneol, the main compounds of the essential oil, contributed partially to the biological activity of the oil. Further investigation is warranted for possible involvement of other components of the essential oil of *K. longipedunculata*.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

Funding

This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

Acknowledgements

We are grateful to Prof. Dr K. Heeg and Dr med S. Zimmermann for providing us with access to the laboratory facilities of Medical Microbiology, Hygiene Institute, University of Heidelberg, Germany. We also thank Astrid Backhaus for performing parts of the GC experiments and Wan Chuangxing, Tarim University, P.R China, for his assistance in purchasing plant (*Kadsura longipedunculata*) materials.

References

1. Ngassoum MB *et al*. Chemical composition, insecticidal effect and repellent activity of essential oils of three aromatic plants, alone and in combination, towards *Sitophilus oryzae* L. (Coleoptera: Curculionidae). *Nat Prod Commun* 2007; 2: 1229–1232.
2. Vardar-Unlu G *et al*. Antimicrobial and antioxidant activity of the essential oil and methanol extracts of *Thymus pectinatus* Fisch. et Mey. var. pectinatus (Lamiaceae). *J Agric Food Chem* 2003; 51: 63–67.
3. Silva J *et al*. Analgesic and anti-inflammatory effects of essential oils of *Eucalyptus*. *J Ethnopharmacol* 2003; 89: 277–283.
4. Crowell PL. Prevention and therapy of cancer by dietary monoterpenes. *J Nutr* 1999; 129: 775S–778S.
5. Li L-N. Biologically active components from traditionaI Chinese medicines. *Pure Appl Chem* 1998; 70: 547–554.
6. Choi YW *et al*. Schisandrene, a dibenzocyclooctadiene lignan from *Schisandra chinensis*: structure-antioxidant activity relationships of dibenzocyclooctadiene lignans. *J Nat Prod* 2006; 69: 356–359.
7. Kangouri K *et al*. Cholesterol biosynthesis inhibitory activities of novel triterpenoid acids from *Kadsura heteroclita* and *K. longipedunculata*. *Agric Biol Chem* 1990; 54: 993–997.
8. Sun QZ *et al*. Three new lignans, longipedunins A-C, from *Kadsura longipedunculata* and their inhibitory activity against HIV-1 protease. *Chem Pharm Bull* 2006; 54: 129–132.
9. Song L *et al*. Compositions and biological activities of essential oils of *Kadsura longepedunculata* and *Schisandra sphenanthera*. *Am J Chin Med* 2007; 35: 353–364.
10. Ashour ML *et al*. Chemical composition and biological activity of the essential oil obtained from *Bupleurum marginatum* (Apiaceae). *J Pharm Pharmacol* 2009; 61: 1079–1087.
11. Adams RP. *Identification of Essential Oil Components by Gas Chromatography/Quadrupole Mass Spectroscopy*, 3rd edn. Carol Stream, Illinois: Allured Pub Corp, 2004.
12. National Committee for Clinical Laboratory Standards. *Performance Standards for Antimicrobial Disk Susceptibility Test, Approved Standard. In, M2-A9*. Wayne, Pennsylvania, USA: NCCLS, 2006.
13. Nibret E *et al*. Antitrypanosomal and cytotoxic activities of pyrrolizidine alkaloid-producing plants of Ethiopia. *J Pharm Pharmacol* 2009; 61: 801–808.
14. Rolón M *et al*. Development of resazurin microtiter assay for drug testing of *Trypanosoma cruzi* epimastigotes. *Parasitol Res* 2006; 99: 103–107.
15. Baylac S, Racine P. Inhibition of 5-lipoxygenase by essential oils and other natural fragrant extracts. *Int J Aromather* 2003; 13: 138–142.
16. Marks DC *et al*. The MTT cell viability assay for cytotoxicity testing in multidrug-resistant human leukemic cells. *Leuk Res* 1992; 16: 1165–1173.
17. Youns M *et al*. Gene expression profiling identifies novel key players involved in the cytotoxic effect of Artesunate on pancreatic cancer cells. *Biochem Pharmacol* 2009; 78: 273–283.
18. Smith-Palmer A *et al*. Antimicrobial properties of plant essential oils and essences against five important food-borne pathogens. *Lett Appl Microbiol* 1998; 26: 118–122.
19. Inouye S *et al*. Antibacterial activity of essential oils and their major constituents against respiratory tract pathogens by gaseous contact. *J Antimicrob Chemother* 2001; 4: 565–573.
20. Möller M *et al*. Antibacterial, antiviral, antiproliferative and apoptosis-inducing properties of *Brackenridgea zanguebarica* (Ochnaceae). *J Pharm Pharmacol* 2006; 58: 1131–1138.
21. Wink M. Bioprospecting: the search for bioactive lead structures from nature. In: Kayser OQW, ed. *Medicinal Plant Biotechnology: from Basic Research to Industrial Applications*. Weinheim: Wiley-VCH, 2007: 97–116.
22. Wink M. Importance of plant secondary metabolites for protection against insect and microbial infections. In: Rai, Carpinella, eds. *Naturally Occurring Bioactive Compounds*. Amsterdam: Elsevier, 2006: 251–268.
23. Cavadini P *et al*. RNA silencing of the mitochondrial ABCB7 transporter in HeLa cells causes an iron-deficient phenotype with mitochondrial iron overload. *Blood* 2007; 109: 3552–3559.
24. Kruh GD *et al*. ABCC10, ABCC11, and ABCC12. *Pflugers Arch* 2007; 453: 675–684.
25. Wink M. Evolutionary advantage and molecular modes of action of multi-component mixtures used in phytomedicine. *Curr Drug Metab* 2008; 9: 996–1009.
26. Gurtu V *et al*. Fluorometric and colorimetric detection of caspase activity associated with apoptosis. *Anal Biochem* 1997; 251: 98–102.