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Research Paper

Chemical composition and biological activity of the essential oil obtained from *Bupleurum marginatum* (Apiaceae)

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Abstract

Objectives *Bupleurum marginatum* is a herb indigenous to the southern and southwestern part of China. It is widely used in many Chinese prescriptions. We aimed to investigate the chemical composition, antioxidant, anti-inflammatory, antimicrobial and in-vitro cytotoxic activity of the hydrodistilled and extracted essential oil from *B. marginatum* to validate some of its ethnopharmacologial uses.

Methods The essential oil of the aerial parts of *B. marginatum* was analysed by capillary gas chromatography (GLC/FID) and gas chromatography–mass spectrometry (GLC/MS). The ability of the oil to reduce diphenylpicrylhydrazine (DPPH$^{\bullet}$) and to prevent the degradation of deoxyribose were used to evaluate the antioxidant activity. Inhibition of both prostaglandin E_2 production and lipoxygenase were used to assess the anti-inflammatory activity. Antimicrobial activity was studied *in vitro* against a range of bacteria and fungi. The in-vitro cytotoxicity of the essential oil on six human cancer cell lines (HepG2, Caco-2, CCRF-CEM, HeLa, MiaPaCa-2 and MCF-7) was examined using the MTT assay.

Key findings Seventy-two components, comprising almost 94.29% of the total peak area, were identified in the analysis. The main components were tridecane (13.18%), undecane (10.42%), pentadecane (8.71%), β-caryophyllene (5.53%) and β-caryophyllene oxide (5.29%). The ability of the oil to reduce diphenylpicrylhydrazine (DPPH$^{\bullet}$) and to prevent the degradation of deoxyribose were used to evaluate the antioxidant activity and the corresponding IC50 values (drug concentration which resulted in a 50% reduction in inhibition of the activity) were found to be 3.66 mg/ml and 17.4 μg/ml, respectively. Inhibition of both prostaglandin E_2 production and lipoxygenase were used to assess the anti-inflammatory activity (IC50 of 63.64 μg/ml for lipoxygenase, 26.04% inhibition of prostaglandin E_2 at 25 μg/ml dose). The oil also showed a significant in-vitro antimicrobial activity against Gram positive pathogens (*Streptococcus pyogenes* and *Streptococcus agalactiae*) with minimum inhibitory concentration (MIC) values ranging from 0.125 up to 4.00 mg/ml. The in-vitro cytotoxicity of the essential oil on six human cancer cell lines (HepG2, Caco-2, CCRF-CEM, HeLa, MiaPaCa-2 and MCF-7) examined using the MTT assay revealed the highest activity to be in the CCRF-CEM cell line with an IC50 (concentration which resulted in a 50% reduction in cell viability) of 46.01 μg/ml after 24 h treatment.

Conclusions The essential oil of *B. marginatum* exhibited a promising anti-inflammatory activity along with strong cytotoxicity against many cancer cells (CCRF-CEM and HepG2) mediated through induction of apoptosis, and this in-vitro activity make its local traditional uses rational. However, its limited antimicrobial activity indicates that a combination with other drugs is essential for effective use. Further selectivity testing is required to evaluate the effect of the oil against normal cells.

Keywords anti-inflammatory activity; antimicrobial activity; *Bupleurum marginatum* essential oil; chemical composition; cytotoxicity

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Introduction

Two hundred species of the genus *Bupleurum* (Apiaceae) are widely distributed in the northern hemisphere and commonly used in Eurasia for their interesting medicinal

output.[1] The roots of several *Bupleurum* species have been included either alone or in combination with other ingredients in many pharmaceutical preparations, based upon traditional Chinese medicine (TCM) for the treatment of common cold,[2] inflammation,[3] hepatitis, cancer[4] and fever associated with malaria.[5]

Bupleurum marginatum Wall. ex DC. is a perennial, rarely annual or biennial, herb indigenous to the southern and southwestern part of China. The herb normally reaches a height up to 60 cm and the leaves are oblanceolate with yellow umbel flowers. The herb is widely used in many Chinese prescriptions as a representative of *bupleuri* herb under the name of 'Chaihu' in Yunnan district (China), although many other *Bupleurum* species could be used under the same name.[1]

Though the chemical composition of different essential oils from *Bupleurum* species has been reported,[6–8] only three species (*B. fruticosum*, *B. fruticescens* and *B. gibraltaricum*) have been studied for their medicinal uses.[9–11] These showed in-vivo anti-inflammatory activity but, to our knowledge, nothing could be traced regarding the chemical composition and the biological activity of the essential oil obtained from *Bupleurum marginatum*.

In this study, the chemical composition, antioxidant, anti-inflammatory, antimicrobial and in-vitro cytotoxic activity of the hydrodistilled and extracted essential oil from *B. marginatum* were investigated to validate some of its ethnopharmacologial uses in the aforementioned disorders.

Materials and Methods

Plant material

The aerial part of *Bupleurum marginatum* was commercially obtained by one of us (T.E.). The identity of the plant was ascertained in our laboratory and confirmed at the Botanical Garden, University of Heidelberg. Voucher specimens of the plant material are deposited at the Department of Biology, Institute of Pharmacy and Molecular Biotechnology, University of Heidelberg under the number of P7367.

Chemicals

Chemicals were purchased from AppliChem (Darmstadt, Germany), Fluka (Buchs, Switzerland) and Sigma (Sigma Aldrich GmbH, Sternheim, Germany). Media and supplements for cell cultures were obtained from Gibco (Invitrogen; Karlsruhe, Germany).

Essential oil isolation

The essential oil of *Bupleurum marginatum* was obtained by hydrodistillation of two portions (500 g each) of air-dried aerial parts of the plant for 6 h and 24 h using a Clevenger-type apparatus; the yields were 0.053% and 0.094% dry weight, respectively. In addition, 200 g plant material was macerated with n-hexane–diethyl ether (1 : 1 v/v) for three days and the yield was 3.08% of the dried plant material. The oil and extract were dried over anhydrous sodium sulfate and kept in separated sealed vials at −30°C for analyses.

GLC/FID analysis

The GLC analyses were carried out on a Varian 3400 equipped with an OV-1 fused bonded column (30 m × 0.25 mm × 0.25 μm) (Ohio Valley, Marietta, USA) and FID detector; carrier gas was helium (2 ml/min); the operating conditions were: initial temperature 45°C, 2 min isothermal, 300°C, 4°C/min 300°C, then 20 min isothermal. Detector and injector temperatures were 300 and 250°C, respectively. The split ratio was 1 : 20. PeakSimple 2000 chromatography data system (SRI Instruments, Torrance, USA) was used for recording and integrating of the chromatograms. Average areas under the peaks of three independent chromatographic runs were used for calculating the % composition of each component.

GLC/MS analysis

The analyses were carried out on a Hewlett-Packard gas chromatograph (GC 5890 II; Hewlett-Packard GmbH, Bad Homburg, Germany) equipped with the same column and conditions as for the GLC/FID. The capillary column was directly 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 the following conditions: filament emission current, 100 mA; electron energy, 70 eV; ion source, 175°C; diluted samples (0.5% v/v) were injected with split mode (split ratio, 1 : 15).

Compounds were identified by comparison of their spectral data and retention indices with Wiley Registry of Mass Spectral Data 8th edition, NIST Mass Spectral Library (December 2005), our own laboratory database and the literature.[12–14]

Antioxidant activity

Radical scavenging activity

The radical scavenging activity of the essential oil and extract were evaluated according to Brandwilliams *et al.*[15] using diphenyl picryl hydrazyl (DPPH$^{\bullet}$). Equal volumes of sample solutions containing 0.02–40 mg/ml of the oils or 0.005–15 mg/ml of the extract and 0.2 mM methanolic solution of DPPH$^{\bullet}$ 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 compared with DPPH$^{\bullet}$ control after background subtraction. Quercetin was used as a positive control. The percent inhibition was calculated from three different experiments using the following equation:

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

Deoxyribose degradation assay

The ability of the tested samples to prevent the degradation of deoxyribose was determined according to Halliwell *et al.* and Houghton *et al.*[16,17] A mixture of freshly prepared 28 mM 2-deoxy-2-ribose (2-DR) in phosphate buffer pH 7.1, 1.04 mM EDTA and 200 μM $FeCl_3$, 1.0 mM H_2O_2 and 1.0 mM ascorbic acid was mixed with an equal volume of various sample concentrations (0.002–6 mg/ml of the oils or

0.001–10 mg/ml of the extract) and kept at 37°C for 1 h. A mixture of thiobarbituric acid and 2.8% trichloroacetic acid (1 : 1 v/v) was added to the reaction mixture and incubated at 100°C for 20 min. Absorbance was measured at 532 nm against blank using a Tecan Safire II Reader. Quercetin was used as a positive control. Inhibition of deoxyribose degradation was measured in triplicate at 532 nm as above.

Anti-inflammatory activity

Prostaglandin E_2 assay

The effect of the tested samples on prostaglandin E_2 (PGE_2) production was assessed using a Monoclonal EIA Kit (Cayman Chemicals, Ann Arbor, USA). Briefly, MiaPaCa-2 cells were treated with 50 μl sample solutions (25 μg/ml), incubated at room temperature and, 24 h later, cells were stimulated with 30 μM arachidonic acid and the culture supernatants were collected (after 15 min) then centrifuged to remove debris. Prostaglandin levels were determined in three independent experiments by a competitive enzyme immunoassay.[18] Inhibition of the PGE_2 level was calculated relative to the blank control and NS-398 (3.14 μg/ml) was used as a positive control.

5-Lipoxygenase inhibition assay

Inhibition of soybean lipoxygenase by the tested samples was determined spectrophotometrically[19] with minor modifications. One milliliter of 0.1 M phosphate buffer pH 9.0 containing 10 μl enzyme (7.9 U/ml) and 20 μl of 10 different concentrations of the tested samples (20–420 μg/ml oils and 5–160 μg/ml extract) were incubated at room temperature for 10 min. The reaction was initiated by adding 25 μl of 62.5 μM sodium linoleate and the reaction kinetics was monitored at 234 nm at 10-s intervals using an LKB Biochrom spectrophotometer. The initial reaction rates were determined from the slope of the straight-line portion of the curve and inhibition of the enzyme activity was calculated from three independent experiments by comparison with the control (ethanol). Norhydroguaretic acid (NDGA) was used as a positive control (IC50 0.24 μg/ml).

Antimicrobial activity

Test microorganisms

The antimicrobial activity was evaluated using standard strains which included the Gram-positive bacteria *Bacillus subtilis* (ATCC 6051), *Staphylococcus aureus* (ATCC 29213), *Staphylococcus epidermidis* (ATCC 14990), *Streptococcus pyogenes* (ATCC 12344), *Streptococcus agalactiae* (ATCC 27956) and methicillin-resistant *Staphylococcus aureus* (NTCC 10442); the Gram-negative bacteria, *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853); and the fungi *Candida albicans* (ATCC 90028) and *Candida glabrata* (ATCC MYA 2950). Clinical isolates from patients, *MRSA* 818014 and *MRSA* 818081 were also used. All of the microorganism cultures were supplied by Medical Microbiology Lab., Hygiene Institute, University of Heidelberg, Germany.

Inocula preparation

Before the test, bacterial and fungal cultures were prepared as follows: the bacterial cultures were sub-cultured in Columbia medium with 5% sheep blood (BD, Heidelberg, Germany) and incubated at 37°C for 24 h while the cultures of fungi were sub-cultured in CHROMagar Candida (BD) and incubated at 25°C for 48 h.

Diffusion method

Suspensions of microorganisms were prepared in a saline solution and adjusted with 0.5 McFarland Standard to a final concentration of approximately 1×10^6 colony forming units (CFU)/ml as recommended by NCCLS (2006).[20] Mueller Hinton agar (Biomerieux, Marcy l'Étoile, France) was inoculated with pathogens. Paper disks with a diameter of 6 mm were loaded with essential oils or extract (3.2 mg/disc) and applied on the agar surface. Dimethyl sulfoxide (DMSO), ampicillin, vancomycin and nystatin were used as controls. The diameters of the growth inhibition zones were measured in triplicate after incubation at 37°C for 24 h (bacteria) or 48 h (yeast).

Determination of minimum inhibitory concentration and minimum microbicidal concentration

The minimum inhibitory concentration (MIC) was determined by the micro-broth dilution method according to NCCLS (2006).[21] Various sample concentrations (0.05–25 μg) were dissolved in 5% DMSO and placed in a 96-well plate (Greiner Bio-one, Frickenhausen, Germany). The final concentration of the microorganism suspension in Mueller Hinton broth (Fluka, Buchs, Switzerland) and Sabouraud Dextrose broth (Oxoid, Hampshire, UK) for bacteria and yeast, respectively, was adjusted to approximately 5×10^5 CFU/ml. Plates were incubated at 37°C for 24 h and 48°C for yeast. To determine the minimum microbicidal concentration (MMC), 3 μl of suspension from each well was spread out on medium and then incubated at 37°C for 24 h or at 48°C for yeast. The MMC was defined as the lowest concentration of oils that killed the microorganism completely. Each test was performed in duplicate. Ampicillin, vancomycin and nystatin were used as positive controls.

Cytotoxicity assay

Cell culture

HepG2 (hepatic cancer), MiaPaCa-2 (pancreatic cancer), MCF-7 (breast cancer), HeLa (cervical cancer) and Caco-2 (colon cancer) cell lines were maintained in DMEM complete media (L-glutamine supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 U/ml streptomycin) in addition to 1 mM sodium pyruvate and 10 mM non-essential amino acids in cultivation of Caco2. Human CCRF-CEM (leukaemia cells) were maintained in RPMI complete medium. Cells were grown at 37°C in a humidified atmosphere of 5% CO_2. All experiments were performed with cells in the logarithmic growth phase.

Cytotoxicity and cell proliferation MTT assay

Sensitivity to drugs was determined in triplicate using the MTT cell viability assay.[22,23] Cells (5×10^4 cells/well of CCRF-CEM and 2×10^4 cell/well of exponentially growing cells of each individual HepG2, Caco2, HeLa, MiaPaCa-2 and MCF-7 cell lines) were seeded in a 96-well plate (Greiner Labortechnik, Frickenhausen, Germany), the cells

were cultivated for 24 h and then incubated with various concentrations of the serially diluted tested samples (stock solution 1 mg/ml) at 37°C for 24 h and 48 h and then with 0.5 mg/ml MTT for 4 h. The formed formazan was dissolved in 200 μl DMSO. The absorbance was detected at 570 nm with a Tecan Safire II Reader, Tecan, Crailsheim, Germany. The cell viability rate (%) of three independent experiments was calculated by the following formula: cell viability rate (%) = (OD of treated cells)/(OD of control cells) × 100%.

Apoptosis assay

Caspase-Glo 3/7 Assay (Promega Mannheim, Germany) was used to detect Caspase 3/7 activities on MiaPaCa-2 cancer cells triggered by samples under investigation (62.5–1000 μg/ml). This test provides a proluminescent caspase-3/7 substrate, which contains the caspase-3 specific tetrapeptide sequence DEVD in a reagent optimized for cell lysis and determination of caspases. Cells cultured in DMEM were seeded in 96-well plates and treated with the tested samples (62.5 μg to 1 mg). At 6 h and 24 h after treatment, 100 μl of caspase 3/7 reagent was added to each well, mixed and incubated for 30 min at room temperature according to Reimer *et al.*[24] Luminescence was measured using Mithras LB 940 instrument (Berthold Technologies, Bad Wildbad, Germany). Cellular apoptosis was expressed in folds relative to the untreated medium control of three independent experiments.

Statistical analysis

All experiments were carried out three times unless mentioned in the procedure. Continuous variables were presented as mean ± SE. The IC50 was determined as the drug concentration which resulted in a 50% reduction in cell viability or inhibition of the biological activity. IC50 values were calculated using a four parameter logistic curve (SigmaPlot 11.0) and all the data were statistically evaluated using Student's *t*-test or the Kruskal–Wallis test (GraphPad Prism 5.01; GraphPad Software, Inc., San Diego, USA) followed by Dunn's post-hoc multiple comparison test when the significance value is < 0.05 using the same significance level. The criterion for statistical significance was taken as $P < 0.05$.

Results

GLC and GLC/MS analyses

Essential oils of *Bupleurum marginatum* showed a yellow and dark yellow colour. Both of them had a characteristic unpleasant odour with specific gravities of 0.860 and 0.845. The hexane–ether extract had the same odour but it was dark green. Seventy-two compounds, accounting for 94.20% of the essential oil, were unambiguously identified by direct comparison (mass fragmentation, retention index) with published data as well as computer library search. Quantitative and qualitative analytical results are shown in Table 1. The essential oil after 24 h distillation showed a higher percentage of fatty acids while the n-hexane–diethyl ether extract also showed some sterols, lignans and flavonoid aglycones.

Table 1 Chemical composition of the *Bupleurum marginatum* essential oil after 6 h hydrodistillation

Name	Retention index OV-1 (RI)	Composition[a] (%)
Hexanal[b]	812	0.55
Heptanal[b]	884	3.64
Nonane	900	<0.10
α-Thujene	913	0.22
Heptanol	956	0.36
6-Methyl-5-heptene-2-one	963	0.15
Octan-2-one	968	<0.10
Pentylfuran	977	0.39
Octanal	983	0.21
Limonene[b,c]	1017	1.03
cis-Linalool oxide[b]	1057	0.33
P-Cymenene	1078	0.18
β-Linalool[c]	1085	0.72
n-Undecane	1100	**10.42**
trans-Pinocarveol	1119	0.17
DL-Menthone	1129	0.92
(E)-2-Nonenal[b]	1134	1.06
Isomenthone	1139	0.14
Borneol[c]	1144	<0.10
α-Terpineol[b,c]	1168	1.08
Myrtenol[b,c]	1173	0.21
β-Cyclocitral	1190	<0.10
cis-Carveol[b]	1193	0.32
Pulegone	1208	0.22
Piperitone	1220	0.49
Neral[c]	1236	0.61
(E)-Anethole[c]	1263	0.20
Undecan-2-one	1270	0.20
Thymol[c]	1274	0.21
3-Undecanol	1286	<0.10
2E,4E Decadienal	1288	<0.10
2-Undecanol	1289	<0.10
n-Tridecane	1300	**13.18**
α-Cubebene[b]	1343	1.96
(+)Longicyclene	1358	<0.10
α-Copaene[b]	1368	0.52
α-Bourbonene	1369	<0.10
iso-Longifolene	1379	0.44
β-Elemene[b]	1382	0.34
β-Caryophyllene[b,c]	1408	**5.53**
β-Copaene	1417	0.62
β-Gurjunene[b]	1420	1.22
Geranyl acetone[b]	1427	0.15
α-Humulene[c]	1441	0.22
β-Farnesene[b]	1447	0.44
Germacrene D	1469	0.69
β-Selinene	1483	1.39
α-Muurolene	1489	0.18
α-Farnesene	1498	4.11
n-Pentadecane	1500	**8.71**
δ-Cadinene[b]	1512	1.69
α-Calacorene	1525	1.33
Spathulenol	1560	4.40
β-Caryophyllene oxide[c]	1564	**5.29**
Davanone	1567	0.68
Viridiflorol	1570	0.78
Salvial-4(14)-en-1-one	1584	1.00
Cubenol	1607	2.40

(*continued*)

Table 1 (*Continued*)

τ-Cadinol	1618	1.21
δ-Cadinol	1630	1.17
cis-α-Santalol	1647	0.70
(Z,E)-Farnesol[c]	1660	1.78
Heptadec-8-ene	1675	0.45
α-Cyperone	1688	1.48
n-Heptadecane	1700	0.51
Hexadecanal	1795	<0.10
Hexahydrofarnesyl acetone[b]	1828	1.99
Neophytadiene	1836	0.69
Hexadecanol	1864	0.44
Palmitic acid[b]	1953	1.35
Phytol	2091	1.01
Linoleic acid	2114	0.21
Total		94.29

[a]Average of three analyses; [b]previously reported from the plant;[8,25] [c]identified by co-injection. The major components are highlighted in bold.

Antioxidant activity

The oils and hexane–ether extract were able to reduce DPPH$^{\bullet}$ and to prevent the degradation of the deoxyribose sugar in a concentration-dependent manner. The tested samples were statistically different ($P < 0.05$, Kruskal–Wallis test) over the dose range 0.02–10 mg/ml and 0.004–2 mg/ml for the DPPH and the deoxyribose experiments, respectively.

The maximum scavenging activity of DPPH$^{\bullet}$ was produced with the hexane–ether extract with an IC50 of 313.79 μg/ml followed by the activity of the essential oils obtained after 6 h and 24 h of hydrodistillation with an IC50 of 3.66 mg/ml and 4.42 mg/ml, respectively (Table 2). Inhibition of deoxyribose degradation was more pronounced for the extract, with an IC50 of 7.19 μg/ml, as compared with the essential oils (17.40 μg/ml and 36.57 μg/ml for the 6 h and 24 h hydrodistillates, respectively).

Anti-inflammatory activity

The anti-inflammatory activity of the essential oils and the hexane–ether extract was clearly observed through their ability to suppress both production of PGE_2 formation in MiaPaCa-2 cells and soybean lipoxygenase (Figures 1 and 2, respectively). Inhibition of PGE_2 formation was seen in all the tested samples with ratios of 43.16% for the extract and 26.04% and 16.95% for the 6-h and 24-h oil, respectively. On the other hand, inhibition of the lipoxygenase was also observed with an IC50 of 19.21 μg/ml for the extract while the essential oils were also less active with IC50 of 63.64 and 70.98 μg/ml for the 6-h and 24-h essential oils, respectively. Statistical analysis revealed that the tested samples were significantly different with *P*-values of 0.0273 for inhibition of the PGE_2 production at 25 μg/ml dose and 0.0328 for inhibition of lipoxygenase at 20–160 μg/ml dose.

Table 2 IC50 values of scavenging the DPPH$^{\bullet}$ radical and prevention of the degradation of 2-deoxyribose (2-DR) by *B. marginatum*

	DPPH$^{\bullet}$ assay	2-DR assay
Essential oil (6 h)	3.66 ± 0.011 mg/ml	17.40 ± 3.13 μg/ml
Essential oil (24 h)	4.42 ± 0.021 mg/ml	36.57 ± 3.49 μg/ml
Hexane ether extract	313.2 ± 0.02 μg/ml	7.19 ± 2.72 μg/ml
Quercetin	3.42 ± 0.21 μg/ml	4.71 ± 1.34 μg/ml

IC50, drug concentration that resulted in a 50% reduction in inhibition of the activity.

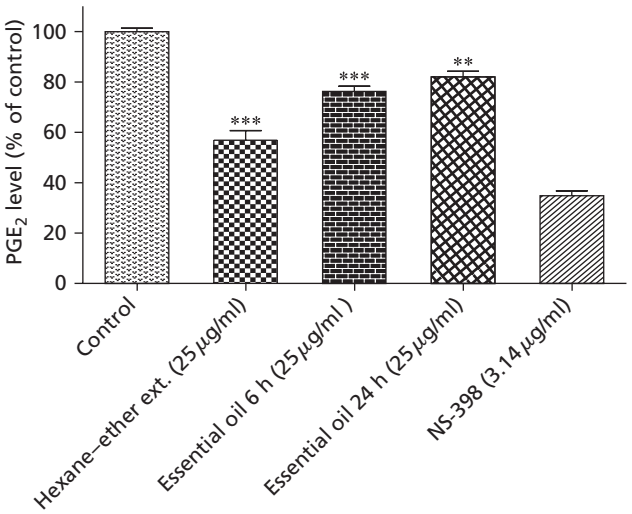

Figure 1 Inhibitory effect of the *B. marginatum* essential oils and hexane–ether extract on prostaglandin E_2 production in MiaPaCa-2 cells. Effect was studied using ELISA immunoassay. Data are presented as means ± SEM from three independent experiments. PGE_2, prostaglandin E_2. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the control.

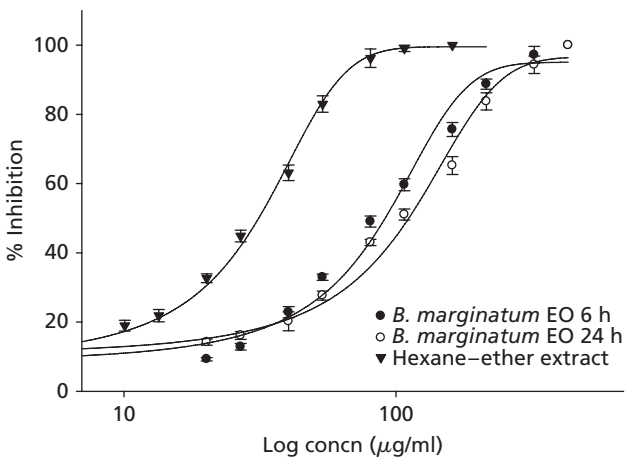

Figure 2 Inhibitory effects of the *B. marginatum* essential oils and hexane–ether extract on soybean 5-lipoxygenase. Data are presented as means ± SEM of three independent assays. Eo, essential oils.

Antimicrobial activity

The antimicrobial activity of the essential oil and extract is represented in Tables 3 and 4. The data demonstrated that there was a considerable antimicrobial activity against Gram-positive pathogens but lesser activity against Gram-negative pathogens and yeast. At the tested doses, *Streptococcus*

agalactiae ATCC 27956 and *Streptococcus pyogenes* ATCC 12344 were the most sensitive bacteria to both oil and extract with inhibition zones of 14–16.7 mm while *Bacillus subtilis* ATCC 6051 exhibited weak inhibition zones of 8–10.7 mm. The three drugs under investigation were significantly different ($P = 0.0072$) in their antimicrobial actions and in comparison with ampicillin and vancomycin ($P = 0.0027$).

The lowest MICs and MMCs were observed also for the essential oil (24 h) against Gram-positive pathogens, especially *Streptococcus pyogenes* and *Streptococcus agalactiae* (0.125 and 0.25 mg/ml, respectively), while the largest values were obtained in the pathogenic MRSA isolates, Gram-negative pathogens and yeast with values higher than 4.00 mg/ml.

Cytotoxicity and apoptosis

The cytotoxicity of the essential oil and the hexane–ether extract was evaluated using six different human cancer cell

Table 3 Mean inhibition zones of *B. marginatum* essential oils and hexane–ether extract against different pathogens using the agar diffusion method

Microorganism	Diameter of inhibition zone (mm)					
	Extract 3.2 mg/disc	EO 6 h 3.2 mg/disc	EO 24 h 3.2 mg/disc	Ampicillin 10 μg/disc	Vancomycin 10 μg/disc	Nystatin 10 μg/disc
G+ MRSA 818014	6.7 ± 0.6	13.7 ± 1.5	13 ± 2.0	7.3 ± 1.2	16.3 ± 0.6	NT
G+ MRSA 818081	7.3 ± 0.6	10.3 ± 1.2	15 ± 0.0	9.6 ± 0.6	16.3 ± 0.6	NT
G+ MRSA *S. aureus* NTCC 104421	0.3 ± 0.6	14.0 ± 1.0	13.7 ± 0.6	NA	18.0 ± 0.0	NT
G+ *Staphylococcus aureus* ATCC 29213	9.3 ± 0.6	12.6 ± 1.2	10.3 ± 1.5	22.0 ± 0.0	16.7 ± 0.6	NT
G+ *Staphylococcus epidermidis* ATCC 14990	9.3 ± 1.2	15.0 ± 1.0	17.7 ± 0.6	25.3 ± 0.0	18.0 ± 0.0	NT
G+ *Streptococcus pyogenes ATCC* 12344	16.7 ± 0.6	15.0 ± 0.0	15 ± 0.0	30.0 ± 0.0	17.3 ± 0.6	NT
G+ *Streptococcus agalactiae* ATCC 27956	14.0 ± 0.0	16.0 ± 0.0	16.6 ± 0.6	30.0 ± 0.0	20.0 ± 0.0	NT
G+ *Bacillus subtilis* ATCC 6051	8.0 ± 0.0	8.7 ± 1.2	10.7 ± 0.6	29.7 ± 0.6	20.7 ± 1.2	NT
G– *Pseudomonas aeruginosa* ATCC 27853	7.6 ± 2.0	7.3 ± 2.3	8.0 ± 1.7	NA	NA	NT
G– *Escherichia coli* ATCC 25922	8.3 ± 0.6	8.6 ± 1.2	10.0 ± 1.7	10.7 ± 0.6	NA	NT
Yeast *Candida albicans* ATCC 90028	6.3 ± 0.6	17.6 ± 2.9	12.3 ± 2.5	NT	NT	17.0 ± 2.6
Yeast *Candida glabrata* ATCC MYA 2950	7.7 ± 1.2	12.3 ± 2.3	12.3 ± 2.5	NT	NT	12.7 ± 0.6

Presented data are means ± SEM, $n = 3$. EO, essential oil, G+, Gram-positive; G–, Gram-negative; NA, not active; NT, not tested.

Table 4 Minimum inhibitory concentrations and minimum microbicidal concentrations of *B. marginatum* essential oils and hexane–ether extract against different pathogens using the broth micro-dilution method

Microorganism	Extract (mg/ml)		EO 6 h (mg/ml)		EO 24 h (mg/ml)		Ampicillin (μg/ml)		Vancomycin (μg/ml)		Nystatin (μg/ml)	
	MIC	MMC	MIC	MMC	MIC	MMC	MIC	MMC	MIC	MMC	MIC	MMC
G+ MRSA 818014	4	>4	2	4	4	4	6.2	6.2	0.8	0.8	NT	NT
G+ MRSA 818081	>4	4	4	4	4	4	6.2	50	0.8	0.8	NT	NT
G+ MRSA *S. aureus* NTCC 10442	4	>4	2	2	4	4	6.2	12.5	0.8	1.6	NT	NT
G+ *Staphylococcus aureus* ATCC 29213	4	>4	2	>4	2	4	0.4	3.1	0.4	0.8	NT	NT
G+ *Staphylococcus epidermidis* ATCC 14990	>4	>4	1	>4	2	>4	0.4	0.8	0.8	1.6	NT	NT
G+ *Streptococcus pyogenes* ATCC 12344	0.125	0.25	0.125	0.25	0.063	0.063	0.1	0.1	0.1	0.2	NT	NT
G+ *Streptococcus agalactiae* ATCC 27956	0.5	>4	0.25	0.25	0.125	0.125	0.1	0.2	0.4	0.4	NT	NT
G+ *Bacillus subtilis* ATCC 6051	0.5	1	0.5	1	1	2	0.1	0.8	0.2	0.8	NT	NT
G– *Pseudomonas aeruginosa* ATCC 27853	>4	>4	>4	>4	>4	>4	NA	NA	NA	NA	NT	NT
G– *Escherichia coli* ATCC 25922	>4	>4	>4	>4	>4	>4	6.2	12.5	NA	NA	NT	NT
Yeast *Candida albicans* ATCC 90028	4	>4	4	4	4	>4	NT	NT	NT	NT	1.6	1.6
Yeast *Candida glabrata* ATCC MYA 2950	>4	>4	4	>4	4	>4	NT	NT	NT	NT	1.6	1.6

EO, essential oil; MIC, minimum inhibitory concentration; MMC, minimum microbicidal concentration; NA, not active; NT, not tested.

lines, namely HepG2, MiaPaCa-2, MCF-7, HeLa, Caco-2 and CCRF-CEM after 24 h and 48 h incubation. The respective IC50 values are represented in Table 5.

The cytotoxicity of the essential oils were in the range 46.01–341.74 μg/ml. No significant difference was observed between the 6-h and 24-h essential oils (except in HepG2 and MCF-7 cell lines for 24 h incubation). The hexane–ether extract showed a significantly higher activity in all the cell lines tested (31.4–263 μg/ml). CCRF-CEM, followed by HepG2 cells, were the most susceptible to the cytotoxic effects of all tested samples whereas Caco2 cells were the most insensitive. Caspases 3/7 activity was used to measure the ability of the tested samples to initiate the apoptotic cascade. All the tested samples caused a significant increase in activation of caspase 3/7 in a dose-dependent manner and this activation was time dependent (Table 6).

Discussion

Chemical analysis of the essential oil from *Bupleurum marginatum* Wall. ex DC. revealed that almost 33% of its total composition constitutes long-chain aliphatic hydrocarbons (C9–C17), which could be considered as characteristic constituents of the Chinese species.[25] The essential oil also contains β-caryophyllene (5.53%), β-caryophyllene oxide (5.29%), spathulenol (4.40%) and α-farnesene (4.11 %). The presence of a high content of alkanal and other aliphatic aldehydes (≈6%) is responsible for the inverted U-shape formation of the curve in the deoxyribose degradation assay at concentrations higher than 2 μl/ml. These alkanals, alkenals and other aldehydes reacted with the reagent thiobarbituric acid and formed yellow, orange and pink-coloured products, which increase the absorbance at 532 nm.[26] The same effect was also observed with other essential oils.[27,28]

The inflammatory cycle involves the formation of prostaglandins and leukotrienes by cyclooxygenase and lipoxygenase followed by the liberation of neutrophils and production of reactive oxygen species. The potential radical-scavenging activity of the essential oil and the hexane–ether extract and their significant inhibition of both cyclooxygenase and lipoxygenase are the possible mechanisms by which they exert their anti-inflammatory activity. This anti-inflammatory activity of the essential oil could be attributed to specific components, like β-caryophyllene and α-humulene, which inhibit cyclooxygenase-2.[29] The alkanal content may interact with the free amino groups of the enzymes, forming a covalent modification (Schiff's base) and prolonged inactivation of many enzymes.[30] The activity of the extract is higher due to the presence of flavonoid aglycones and lignans, which form noncovalent (hydrogen and ionic) bonds with many enzymes.[31] Moreover, sterols are known inhibitors of the classical complement pathway[32] and lignans inhibit platelet activating factor and prevent the granulation of the neutrophils.[33] These actions might be considered to be the main reasons for the anti-inflammatory activity.

The previous findings, and taking into consideration that the genus *Bupleurum* is well known for its high yield of saikosaponin triterpenoids (known for high therapeutic potential as an anti-inflammatory agent),[3] make the local traditional use of *B. marginatum* in the management of various inflammatory disorders possible.

Table 5 Cytotoxic effect of *B. marginatum* essential oils and hexane–ether extract on growth of different cell lines as determined by MTT assay

	Hexane–ether extract		Essential oil 6 h		Essential oil 24 h	
	24 h	48 h	24 h	48 h	24 h	48 h
CCRF-CEM	33.48 ± 2.47	15.94 ± 0.92	46.01 ± 4.94	22.43 ± 5.93	49.26 ± 6.36	18.85 ± 1.99
HepG2	31.40 ± 4.10	19.57 ± 2.24	120.46 ± 2.42	90.51 ± 5.87	166.98 ± 22.69	73.14 ± 4.33
Caco2	263.00 ± 24.56	98.56 ± 15.82	267.10 ± 12.72	183.19 ± 29.07	290.18 ± 67.64	170.12 ± 15.94
HeLa	143.27 ± 13.26	69.76 ± 6.46	303.51 ± 11.91	129.21 ± 5.35	312.93 ± 11.79	184.88 ± 15.43
MiaPaCa-2	245.93 ± 12.54	3.36 ± 0.16	341.74 ± 6.35	36.68 ± 5.10	308.61 ± 23.00	32.70 ± 3.50
MCF-7	168.37 ± 26.76	35.00 ± 5.78	319.38 ± 4.23	124.74 ± 9.57	161.24 ± 12.45	105.93 ± 20.13

IC50 values (μg/ml) of the cytotoxicity effects after 24 h and 48 h incubation of *B. marginatum* essential oils and hexane–ether extract on growth of different cell lines wee determined by MTT assay. The data of three independent experiments was represented as mean ± SEM.

Table 6 Caspase-3/7 activity in MiaPaCa-2 cells after incubation with *B. marginatum* essential oils and extract

	Hexane–ether extract		Essential oil 6 h		Essential oil 24 h	
	6 h	24 h	6 h	24 h	6 h	24 h
Control	1.00	1.00	1.00	1.00	1.00	1.00
62.5 μg/ml	1.98 ± 0.09	2.92 ± 0.15	2.47 ± 0.23	2.52 ± 0.14	2.66 ± 0.26	2.75 ± 0.22
125 μg/ml	3.71 ± 0.18	4.58 ± 0.15	3.64 ± 0.25	4.26 ± 0.18	4.37 ± 0.09	4.43 ± 0.15
250 μg/ml	5.62 ± 0.15	8.21 ± 0.32	6.80 ± 0.51	7.68 ± 0.13	5.52 ± 0.13	8.71 ± 0.24
500 μg/ml	9.29 ± 0.18	10.52 ± 0.47	9.88 ± 0.12	12.42 ± 0.26	10.02 ± 0.53	12.24 ± 0.32
1000 μg/ml	14.34 ± 0.30	21.77 ± 1.04	13.06 ± 0.46	22.36 ± 0.42	14.19 ± 0.13	21.73 ± 0.62

Activity is shown relative to control (set to be 1.00). The data of three independent experiments was represented as means ± SEM.

The presence of a high amount of phenolic components in essential oils usually contributes to their antimicrobial activity against fungi and Gram-positive bacteria. A lack of potent antimicrobial components like eugenol, 1,8-cineole and camphor in the *B. marginatum* essential oil could be the reason for its weak antimicrobial activity.[34]

The cytotoxicity of essential oil could be attributed to some specific components found in the oil. β-caryophyllene and β-caryophyllene oxide were reported to show a potent cytotoxic activity over a wide range of cell lines.[35] Limonene has anti-tumour activity against rodent mammary, liver, lung, stomach and skin cancers.[36] Farnesol is strongly active against pancreatic tumour cell lines.[37] β-Elemene and α-humulene show also strong cytotoxicity against many cell lines.[38,39] The synergistic effects of these active chemicals with other constituents of both essential oil and hexane–ether extract should be considered in validating its use as an anti-cancer drug. This cytotoxicity could be explained by the ability of the oil and other lipophilic terpenoids to trigger apoptosis via either the caspase pathway, as indicated by the increase in the caspase-3/7 level,[40] or through their influence on the fluidity of the biomemberane.[30,41]

Conclusion

The essential oil of *B. marginatum* exhibited a promising anti-inflammatory activity along with strong cytotoxicity against many cancer cells (CCRF-CEM and HepG2) mediated through induction of apoptosis, and this in-vitro activity makes its local traditional uses rational. The limited antimicrobial activity of the oil indicated that a combination with other drugs is essential for effective use; however, further selectivity testing is required to evaluate the effect of the oil against normal cells.

Declarations

Conflict of interest

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

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