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Antitumour Activity of the Microencapsulation of *Annona vepretorum* Essential Oil

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Abstract: Annona vepretorum Mart. (Annonaceae), popularly known as 'bruteira', has nutritional and medicinal uses. This study investigated the chemical composition and antitumour potential of the essential oil of *A. vepretorum* leaf alone and complexed with β-cyclodextrin in a microencapsulation. The essential oil was obtained by hydrodistillation using a Clevenger-type apparatus and analysed using GC-MS and GC-FID. *In vitro* cytotoxicity of the essential oil and some of its major constituents in tumour cell lines from different histotypes was evaluated using the alamar blue assay. Furthermore, the *in vivo* efficacy of essential oil was demonstrated in mice inoculated with B16-F10 mouse melanoma. The essential oil included bicyclogermacrene (35.71%), spathulenol (18.89%), (*E*)-β-ocimene (12.46%), α-phellandrene (8.08%), *o*-cymene (6.24%), germacrene D (3.27%) and α-pinene (2.18%) as major constituents. The essential oil and spathulenol exhibited promising cytotoxicity. *In vivo* tumour growth was inhibited by the treatment with the essential oil (inhibition of 34.46%). Importantly, microencapsulation of the essential oil increased *in vivo* tumour growth inhibition (inhibition of 62.66%).

Annona vepretorum Mart. belongs to the family Annonaceae, and it is native to the Brazilian biome Caatinga [1]. This plant is popularly known as 'bruteira' in the Brazilian north-east, and it has nutritional and medicinal uses. The fruits are consumed raw or in juice form for nutritional value [2]. The roots are used medicinally to soften bee and snake bites, inflammatory conditions and heart pains. The leaves are used in a decoction in baths to treat allergies, skin diseases and yeast and bacterial infections [3].

The chemical and biological effects of *A. vepretorum* were investigated previously. The ethanolic extract of *A. vepretorum* leaves has antinociceptive, anti-inflammatory, sedative, antioxidant, antimicrobial and cytotoxic activities, which are attributed to the presence of phenols, steroids, terpenoids and flavonoids [4–6]. Several cytotoxic *ent*-kaurane diterpenes, including *ent*-3β-hydroxy-kaur-16-en-19-al, *ent*-3β-hydroxy-kaur-16-eno and kaurenoic acid, were isolated in *A. vepretorum* stem bark [3]. Four aporphine alkaloids, liriodenine, oxonantenine, lanuginosine and lysicamine, as well as 1,3,6,6-tetramethyl-5,6,7,8-tetrahydro-isoquinolin-8-one and vomifoliol, were also found in *A. vepretorum* leaves [7]. Additionally, the leaf essential oil shows trypanocidal, antimalarial, antifungal and antioxidant properties, and it primarily includes the presence of sesquiterpene and monoterpene compounds [8–10]. The chemical composition and antitumour potential of essential oil of *A. vepretorum* leaf alone (free EO) and complexed with β-cyclodextrin (EO complexed) were investigated in this study.

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Materials and Methods

Cells. Tumour cell lines B16-F10 (mouse melanoma), HepG2 (human hepatocellular carcinoma), K562 (human chronic myelocytic leukaemia) and HL-60 (human promyelocytic leukaemia) were donated by Hospital A.C. Camargo, São Paulo, SP, Brazil. Cells were maintained in Roswell Park Memorial Institute-1640 (RPMI-1640; Gibco-BRL) medium supplemented with 10% foetal bovine serum (Cultilab), 2 mM L-glutamine (Vetec Química Fina) and 50 μg/mL gentamycin (Novafarma). Adherent cells were harvested by treatment with 0.25% trypsin/EDTA solution (Gibco-BRL). All cell lines were cultured in cell culture flasks at 37°C in 5% CO_2 and subcultured every 3–4 days to maintain exponential growth. All experiments were conducted with cells in the exponential growth phase. All cell lines were tested for mycoplasma using a Lookout® Mycoplasma qPCR detection kit (Sigma-Aldrich, St Louis, MO, USA), and all cells were free from contamination.

Heparinized blood (from healthy, 20- to 35-year-old, non-smoker donors who had not taken any drugs for at least 15 days prior to sampling) was collected, and peripheral blood mononuclear cells (PBMCs) were isolated by a standard protocol using a Ficoll density gradient in a GE Ficoll-Paque Plus. PBMCs were washed and resuspended at a concentration of 0.3×10^6 cells/mL in RPMI-1640 medium supplemented with 20% foetal bovine serum, 2 mM glutamine and 50 μg/mL gentamycin at 37°C with 5% CO_2. Concanavalin A (ConA; Sigma Chemical Co. St Louis, MO, USA) was used as a mitogen to trigger cell division in T lymphocytes. ConA (10 μg/mL) was added at the beginning of culture, and cells were treated with the test drugs after 24 hr.

The Research Ethics Committee of the Oswaldo Cruz Foundation (Salvador, Bahia, Brazil) approved the experimental protocol (number 031019/2013). All participants signed a written informed consent to participate in the study.

Cell viability was examined using trypan blue exclusion assay for all experiments. Over 90% of the cells were viable at the beginning of the culture.

Animals. A total of 43 specific pathogen-free C57BL/6 mice (males, 25–30 g) were obtained and maintained at the animal facilities of the Gonçalo Moniz Research Center-FIOCRUZ (Salvador, Bahia, Brazil). The animals were housed in cages with free access to food and water. All animals were kept under a 12:12 hr light–dark cycle (lights on at 6:00 a.m.). The animals were treated according to the ethical principles for animal experimentation of SBCAL (Brazilian Association of Laboratory Animal Science), Brazil. The Animal Ethics Committee of the Oswaldo Cruz Foundation (Salvador, Bahia, Brazil) approved the experimental protocol (number 01/2013).

Botanical material. A. vepretorum leaves were collected in April 2012 in the proximity of the city of Poço Redondo [coordinates: S 09° 48′ 19″ W 37° 41′ 03″] in Sergipe State, Brazil. Dr. Ana Paula do Nascimento Prata of the Department of Biology from Federal University of Sergipe (UFS), Brazil, confirmed the identity of the plant, and a voucher specimen (23158) was deposited in the Herbarium of UFS (ASE/UFS). The authors have authorization from the Chico Mendes Institute for Biodiversity Conservation from Brazilian Ministry of the Environment for plant collection (number 25637-1). This work was performed according to special authorization for access to genetic resources in Brazil # 010240/2013-6, issued by CNPq/MCTI.

Hydrodistillation of the essential oil. The essential oil (EO) of *A. vepretorum* (200 g) dried (for 24 hr) leaves was obtained by hydrodistillation for 3 hr using a Clevenger-type apparatus. The EO was dried over anhydrous sodium sulphate, and the percentage content was calculated on the basis of the dry weight of plant material. It was stored in a freezer until analysis. The hydrodistillation was performed in triplicate.

GC-FID and GC-MS analyses of the essential oil. GC-FID and GC-MS analyses were performed on a Shimadzu GC-2010 Plus GCMS-QP2010 Ultra GC-FID equipped with a Shimadzu AOC-20i auto-injector. Separation of the compounds was achieved on a RTx®-5 MS fused capillary chromatography column (30 m × 0.25 mm × 0.25 μm film thickness) coated with 5% diphenyl–95% dimethylpolysiloxane. Helium was the carrier gas at 1.2-mL/min flow rate. The column temperature programme was 40°C/4 min, at a rate of 4°C/min to 240°C, then 10°C/min to 280°C, and 280°C/2 min. The injector and detector temperatures were 250°C and 280°C, respectively. Samples (10 mg/mL in CH_2Cl_2) were injected at a 1:30 split ratio. Retention indices were generated using a standard solution of *n*-alkanes (C_8-C_{18}). Peak areas and retention times were measured using an electronic integrator. The relative amounts of individual compounds were computed from GC peak areas without an FID response factor correction. Mass spectra (MS) were taken at 70 eV with scan intervals of 0.5 sec. and fragments from 40 to 550 Da. EO components were identified by comparisons of their retention times (t_R) to standards, isolated compounds under identical conditions, the retention indices of a series of *n*-alkanes (C_8-C_{18}) according to Van den Dool and Kratz [11], their mass spectra with the NIST (05, 05 sec., 21 and 107) and Wiley eight mass spectral libraries, and published data in the literature [12]. Analyses of the EO were performed in triplicate.

Spathulenol isolation. One part of the EO (175.0 mg) was subjected to a preparative thin-layer chromatography (TLC) that was eluted with a mixture of *n*-hexane-ethyl acetate (95:05, v/v) to give 29.3 mg of spathulenol, which was submitted do GC-FID (purity 88.0%) and GC-MS analysis.

α-Phellandrene, α-pinene and o*-cymene obtention.* α-Phellandrene (purity ≥85.0%), α-pinene (purity 98.0%) and *o*-cymene (purity 99.0%) were obtained from Sigma-Aldrich, St Louis, MO, USA. These compounds were also analysed using GC-FID and GC-MS as described above to confirm the purity degree.

In vitro *cytotoxic activity assay.* Cell growth was quantified using the alamar blue assay, as previously described [13]. Cells were seeded in 96-well plates for all experiments (0.7×10^5 cells/mL for adherent cells or 0.3×10^6 cells/mL for suspended cells in 100 μL of medium). EO and some of its major constituents (0.19–25 μg/mL) were dissolved in dimethyl sulphoxide (DMSO; Sigma Chemical Co.) and the solution was added to each well and incubated for 72 hr. 5-Fluorouracil (5-FU, purity >99%; Sigma-Aldrich) was used as a positive control (0.19–25 μg/mL). Negative controls received the vehicle that was used for diluting the tested compound (0.5% DMSO). Four (for cell lines) or 24 (for PBMCs) hr before the end of the incubation, 20 μL of a stock solution (0.312 mg/mL) of the alamar blue (resazurin; Sigma-Aldrich Co.) was added to each well. Absorbance was measured using a SpectraMax 190 multiplate reader, and the drug effect was quantified as the percentage of control absorbance at 570 nm and 600 nm.

Annexin assay. Phosphatidylserine externalization was analysed by flow cytometry. A FITC Annexin V Apoptosis Detection Kit I (BD Biosciences, Franklin Lakes, New Jersey, USA) was used to determine cell viability (viable, early apoptotic, late apoptotic and necrotic cells). Cells were washed twice with saline and then resuspended in 100 μL of binding buffer with 5 μl of propidium iodide and 5 μL of FITC Annexin V. The cells were gently vortexed and incubated for 15 min at room temperature (20–25°C) in the dark. Afterwards, 400 μl of binding buffer was added to each tube and the cells were analysed by flow cytometry in a BD LSRFortessa™ cytometer using the BD FACSDiva software (BD Biosciences). Ten thousand events were evaluated per experiment, and cellular debris was omitted from the analysis.

β-Cyclodextrin complex obtention. Inclusion of a complex between β-cyclodextrin and EO was prepared using cosolubilization as previously described [14,15]. Briefly, a solution (solution A) of EO and β-cyclodextrin (Sigma-Aldrich Co.) was prepared using acetone as a solvent. A second solution (solution B) of polysorbate 80 (1 mL; Sigma-Aldrich Co.) was prepared in distilled water (100 mL). These solutions were stirred until complete solubilization occurred. Solution A was mixed with solution B and stirred at room temperature for 12 hr until completely solubilized. The final solution was concentrated on a rotary evaporator. The resulting solid was stored in a glass container for further use.

Measurements of dynamic light scattering (DLS) and zeta potential were performed with the EO complexed to verify its distribution, size and stability. DLS analysis was performed using a light scattering spectrometer (Brookhaven Instruments, BI-200 goniometer, AT digital correlator BI-900) with a He-Ne laser (λ0 = 632.8 nm) as the radiation source. A sample of the EO complexed was filtered through a 0.45-mm-porosity optical filter and added to cuvettes. The volume detected by a scattering detector in DLS experiments was minimized with an aperture of 0.4 mm, which was also used as an interference filter between the sample holder and signal detection in the photomultiplier tube. Scattered light was collected at a 90° angle between the incident radiation in the sample and the detected scattered radiation (i.e. light scattered by the particles). Time correlation functions were

obtained in a multi-τ mode using 66 channels, and data were analysed using the method of cumulants, as provided by the equipment manufacturer (Brookhaven Instruments software). Measurements of scattered light (Is) intensity *versus* temperature were performed using a coupled external bath, which varied the temperature from 15 to 75°C. Samples were heated from 15 to 75°C using a 1°C/min ramp at 10-min intervals of 2°C each. Zeta potential analysis was performed using Microtrac (Zetatrac). EO complexes were diluted 250x in purified water and analysed at 25°C in this experiment.

In vivo *antitumour activity assay.* The *in vivo* antitumour effect was evaluated in C57BL/6 mice inoculated with B16-F10 melanoma as previously described [16,17]. Tumour cells (2×10^6 cells per 500 μL) were implanted subcutaneously into the left hind groin of mice. Mice were divided into five groups at the beginning of the experiment: group 1: animals that received injections of a 5% DMSO solution (negative control, n = 9); group 2: animals that received injections of a β-cyclodextrin solution (negative control, n = 9); group 3: animals that received injections of EO dissolved in 5% DMSO (free EO, 50 mg/kg, n = 9); group 4: animals that received injections of EO complexed with β-cyclodextrin (EO complexed, 50 mg/kg, n = 8); and group 5: animals that received injections of dacarbazine (purity ≥98.0%, Sigma-Aldrich Co., 30 mg/kg, n = 8). The dosages were determined based on previous articles. Treatments were initiated 1 day after tumour injection. Animals were treated intraperitoneally (200 μL per animal) once daily for 11 consecutive days. Peripheral blood samples were collected from the retro-orbital plexus under anaesthesia for biochemical and haematological analysis on day 12, as described below. Animals were euthanized by cervical dislocation, and tumours were excised and weighed. Drug effects are expressed as the per cent inhibition of control.

Systemic toxicological evaluation. Systemic toxicological effects were investigated as previously described [16,17]. Mice were weighed at the beginning and end of the experiment. Animals were observed for signs of abnormalities throughout the study. Livers, kidneys, lungs and hearts were removed, weighed and examined for any signs of gross lesions or colour changes and haemorrhage. Biochemical analyses of serum samples were performed using a Vet-16 rotor and quantified using an Analyst bench-top clinical chemistry system (Hemagen Diagnostics Inc., Columbia, MD, USA). Haematological analyses were performed using light microscopy. After gross macroscopic examination, tumours, livers, kidneys, lungs and hearts were fixed in 4% formalin buffer and embedded in paraffin. Tissue sections were stained for haematoxylin and eosin chromogens, and a pathologist performed analyses under light microscopy.

Statistical analysis. Data are presented as means ± S.E.M./S.D. or inhibition concentration of 50% (IC_{50}) values and their 95% confidence intervals (CI 95%) obtained by nonlinear regression. Differences between experimental groups were compared using analysis of variance (ANOVA) followed by the Student–Newman–Keuls test ($p < 0.05$). All statistical analyses were performed using GraphPad (Intuitive Software for Science, San Diego, CA, USA).

Results and discussion. Hydrodistillation of *A. vepretorum* leaf yielded 0.59 ± 0.02% of EO (light yellow) in relation to the dry weight of plant material. Table 1 shows that 26 compounds were identified. The EO was predominantly composed of sesquiterpene (67.41%) and monoterpene (30.18%) compounds. Chemical composition of EO of *A. vepretorum* leaf has been previously reported with some different constituents [8–10]. These variations may be related to detector sensitivity, sample dilution of the major constituents of *A. vepretorum* and differences in the contents of all components as a result of soil and climate conditions, water stress, collection place, nutrition and other abiotic factors.

Table 1.
Chemical composition of the essential oil of *Annona vepretorum* leaf.

Compound		RI[1]	RI[2]	Leaf Oil %
1	α-Pinene	931	932	2.18 ± 0.23
2	Myrcene	989	988	0.35 ± 0.02
3	α-Phellandrene	1005	1002	8.08 ± 0.63
4	*o*-Cymene	1023	1022	6.24 ± 0.26
5	Limonene	1028	1024	0.38 ± 0.02
6	(*Z*)-β-Ocimene	1036	1032	0.34 ± 0.02
7	(*E*)-β-Ocimene	1047	1044	12.46 ± 0.64
8	Linalool	1099	1095	0.16 ± 0.01
9	δ-Elemene	1332	1335	0.48 ± 0.02
10	β-Elemene	1390	1389	1.08 ± 0.03
11	α-Gurjunene	1408	1409	0.26 ± 0.01
12	(*E*)-Caryophyllene	1421	1417	1.06 ± 0.05
13	*Trans*-α-bergamotene	1433	1432	0.14 ± 0.01
14	Aromadendrene	1440	1439	0.53 ± 0.02
15	*Allo*-aromadendrene	1453	1458	0.36 ± 0.02
16	*Dehydro*-aromadendrene	1462	1460	1.79 ± 0.10
17	Germacrene D	1483	1484	3.27 ± 0.10
18	Viridiflorene	1493	1496	0.71 ± 0.02
19	Bicyclogermacrene	1498	1500	35.71 ± 1.17
20	δ-Amorphene	1519	1511	0.40 ± 0.01
21	Epiglobulol	1572	1576[3]	0.23 ± 0.02
22	Spathulenol	1580	1577	18.89 ± 0.41
23	Caryophyllene oxide	1585	1582	0.87 ± 0.06
24	Globulol	1588	1590	0.86 ± 0.05
25	Viridiflorol	1597	1592	0.54 ± 0.04
26	α-Cadinol	1656	1652	0.22 ± 0.03
	Monoterpene identified			30.18 ± 1.60
	Sesquiterpene identified			67.41 ± 1.65
	Total identified			97.59 ± 0.16

Data are expressed as the means ± S.D. of three analyses. RI (retention indices): [1]calculated on an RTx®-5 MS column according to Van den Dool and Kratz [11], based on a homologous series of normal alkanes; [2]according to Adams [12]; [3]according to Ferreira *et al.* [22].

Several tumour cell lines were treated with increasing concentrations of EO and some of its major constituents (spathulenol, α-phellandrene, α-pinene and *o*-cymene) for 72 hr and analysed using the alamar blue assay. Table 2 presents the results found. Our pre-clinical anticancer drug screening programme, which is based in the United States National Cancer Institute programme, demonstrated that extracts/oils with IC_{50} values below 30 μg/mL and pure compounds with IC_{50} values below 4 μg/mL in tumour cell line assays are promising [16–18]. Therefore, the EO obtained of *A. vepretorum* leaf presents promising results. However, only the constituent spathulenol showed promising results. These results suggested that spathulenol contributed to the cytotoxic effect of the EO. Moreover, the major and minor constituents must also be associated. The cytotoxic effect was also evaluated in non-tumour cells (PBMCs). The selectivity indices (SIs) were 3.7 and 1.3 for the EO and spathulenol, respectively (the SI was calculated using the following formula: SI = IC_{50}[PBMC]/IC_{50}[HL-60]). 5-FU, which was used as the positive control, showed a SI of 93.3.

In addition, phosphatidylserine externalization was measured in EO-treated B16-F10 melanoma cells after 24-hr incubation. A slight increase of the phosphatidylserine exposure was observed (fig. 1), without affecting the cell membrane integrity, suggesting apoptotic cell death.

In the genus *Annona*, the EO of *A. senegalensis* leaf showed moderate cytotoxicity in tumour cell lines (cytotoxicity at concentrations lower than 10 μg/mL) [19]. Herein, the EO of *A. vepretorum* leaf showed promising cytotoxic activity, which can be attributed, at least

Table 2.
In vitro cytotoxic activity the essential oil of *Annona vepretorum* leaf (EO) and some of its major constituents.

Compounds	IC_{50} (μg/mL)[1]				
	B16-F10	HepG2	K562	HL-60	PBMCs
EO	9.90 7.81–12.55	10.60 8.55–13.26	8.43 5.48–12.97	6.14 4.15–9.12	22.82 19.18–27.15
Spathulenol	11.67 9.76–13.96	11.19 9.58–13.07	3.79 1.48–9.70	11.38 8.46–15.31	15.59 13.12–18.53
α-Phellandrene	15.44 6.54–36.42	17.30 13.89–21.55	>25	20.18 16.91–24.08	>25
o-Cymene	>25	>25	>25	>25	>25
α-Pinene	11.46 5.46–24.04	13.05 9.79–17.38	14.00 10.56–18.55	14.96 12.25–18.26	>25
5-FU[2]	0.68 0.21–1.45	0.04 0.01–1.22	0.15 0.01–1.86	0.29 0.21–0.38	14.00 9.83–23.31

[1]Data are presented as IC_{50} values in μg/mL, and their 95% confidence interval obtained by nonlinear regression from three independent experiments performed in duplicate, measured using alamar blue assay after 72-hr incubation. Tumour cells: B16-F10 (mouse melanoma), HepG2 (human hepatocellular carcinoma), HL-60 (human promyelocytic leukaemia) and K562 (human chronic myelocytic leukaemia). Non-tumour cells: PBMCs (human peripheral blood mononuclear cells activated with concanavalin A – human lymphoblast). [2]5-Fluorouracil (5-FU) was used as the positive control.

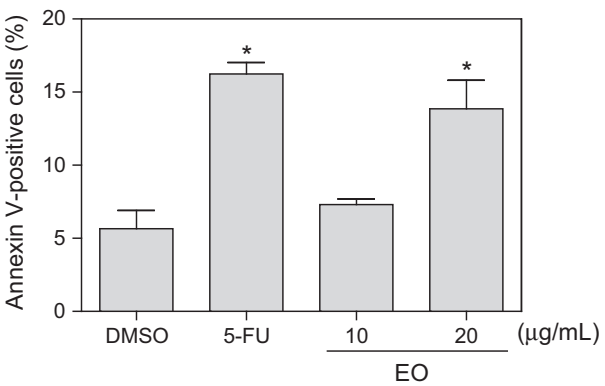

Fig. 1. Effect of the essential oil of *Annona vepretorum* leaf (EO) on cell viability of B16-F10 melanoma cells determined by flow cytometric using Annexin V–PI. Data are presented as mean values ± S.E.M. from three independent experiments performed in duplicate after 24-hr incubation. Negative control was treated with the vehicle (0.2% DMSO) used for diluting the tested substance. 5-Fluorouracil (1 μg/mL) was used as the positive control. For flow cytometric analysis, ten thousand events were analysed in each experiment. $*p < 0.05$ compared to negative control by ANOVA followed by Student–Newman–Keuls test.

in part, to its pro-apoptotic action. In relation to spathulenol, Areche *et al.* [20] verified cytotoxic activity against human gastric adenocarcinoma (AGS) cells with an IC_{50} value of 23 μM (~5 μg/mL) and human normal lung fibroblast (MRC-5) cells with an IC_{50} value of 32 μM (~7 μg/mL).

β-Cyclodextrin is a cyclic carbohydrate derived from starch, which contains seven glucopyranose units. It is used as a micro/nanoencapsulating agent because of its ability to establish specific interactions with several compounds through the formation of non-covalently bonded entities. Moreover, β-cyclodextrin can form inclusion complexes with essential oils to improve its characteristics (e.g. stabilizing volatile compounds by reducing or eliminating losses through evaporation) [21]. The present work prepared an inclusion complex between β-cyclodextrin and EO using co-solubilization. Zeta potential and DLS measurements demonstrated that the EO complex showed a zeta potential of -6.67 ± 3 mV, and 97.98% of its particles were smaller than 1 μm (microscale; fig. 2). A zeta potential value close to 0 mV provides little protection to ion particles because the absence of a surface charge may facilitate interactions between the particles to promote aggregation or flocculation processes. However, the presence of a surfactant in the formulation of encapsulated systems contributes to particle suspension in another system, which is self-repellent because of the surface charge and surfactant.

C57BL/6 mice were subcutaneously inoculated with B16-F10 melanoma cells and treated (intraperitoneally) daily for 11 consecutive days with the EO to investigate the *in vivo* antitumour activity (fig. 3). *In vivo* tumour growth was inhibited by the treatment with the EO (inhibition of 34.46%). Importantly, microencapsulation of the EO increased *in vivo* tumour growth inhibition (inhibition of 62.66%). Histopathological analyses of the tumours extirpated from all experimental groups exhibited intense pleomorphism and atypical mitoses. Focal areas of necrosis were also observed.

Systemic toxicological parameters were also examined in EO-treated mice. No significant changes in body or organ (liver, kidney, lung and heart) weight were observed in the EO-treated groups ($p > 0.05$, data not shown). No significant changes in peripheral blood biochemistry or haematological parameters were observed in the EO-treated groups ($p > 0.05$, data not shown). Histopathological analyses of livers revealed hydropic degeneration, focal areas of coagulation necrosis and focal inflammation in all experimental groups. β-Cyclodextrin- and EO complex-treated animals also exhibited vascular congestion and focal haemorrhage. Micro-metastatic nodules were observed in the liver parenchyma of groups treated with free EO and dacarbazine. Hydropic degeneration, vascular congestion, focal haemorrhage and areas of necrosis were observed in kidneys of all groups. Atelectasis, focal haemorrhage and vascular congestion were observed in the lungs of animals treated with β-cyclodextrin, EO complexed and dacarbazine, but not in 5% DMSO and EO groups. Focal areas of inflammation were also observed in β-cyclodextrin-treated mice. Metastatic emboli were observed in animals treated with complexed EO, and micro-metastatic nodules were observed in the alveoli of groups treated with dacarbazine. Histopathological analyses of hearts did not show alterations in any group. The histopathological features in this study (hydropic change, vascular congestion and focal areas of inflammation) are acute cellular responses to non-lethal treatment-related stimulus, and the injured cells can return to a homoeostatic state when the aggression ends.

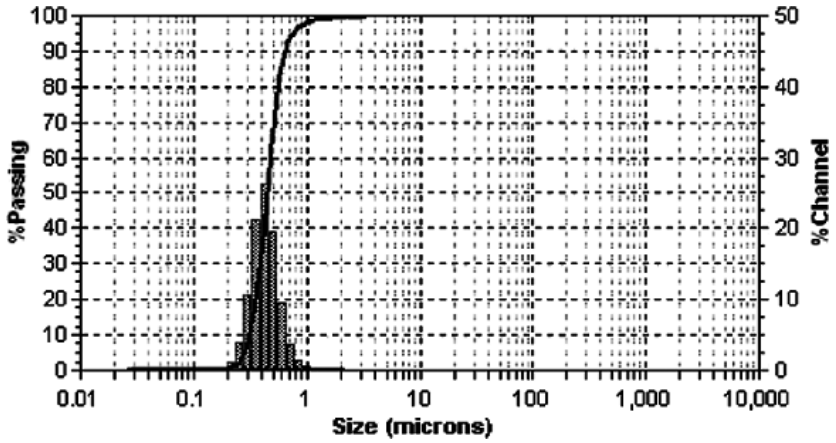

Fig. 2. Particle size distributions of the essential oil of *Annona vepretorum* leaf complexed with β-cyclodextrin analysed by dynamic light scattering using a light scattering spectrometer with a He-Ne laser (λ0 = 632.8 nm) as the radiation source. %Passing are represented by the line and %Channel are represented by the bars.

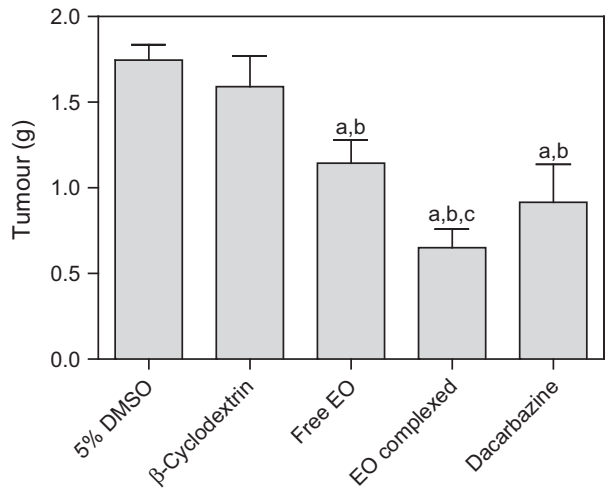

Fig. 3. *In vivo* antitumour effect of the essential oil of *Annona vepretorum* leaf alone (free EO, 50 mg/kg) and complexed with β-cyclodextrin (EO complexed, 50 mg/kg). Mice were injected with B16-F10 melanoma (2.0×10^6 cells/animal, s.c.). The animals received intraperitoneal drug administration for 11 consecutive days, beginning 1 day after tumour implantation. Dacarbazine (30 mg/kg) was used as a positive control. Negative controls were treated with the vehicles used for diluting the tested substance (5% DMSO or β-cyclodextrin). Data are presented as the means ± S.E.M. of 8–9 animals. ^{a}P <0.05 compared to the 5% DMSO group using ANOVA followed by Student–Newman–Keuls test. ^{b}P <0.05 compared to the β-cyclodextrin group using ANOVA followed by Student–Newman–Keuls test. ^{c}P <0.05 compared to the free EO group using ANOVA followed by Student–Newman–Keuls test.

Conclusion

The EO of *A. vepretorum* leaf presents predominantly sesquiterpene and monoterpene compounds. Both the EO and its constituent spathulenol exhibit promising cytotoxicity. In addition, *in vivo* tumour growth can be inhibited by the treatment with the EO, which is enhanced by microencapsulation.

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Conflict of Interest

The authors declare no conflict of interests.

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