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A SELF-EMULSIFYING SYSTEM FOR CO-DELIVERY OF DOXORUBICIN AND Β-CARYOPHYLLENE-RICH *PINUS WALLICHIANA* OIL: FORMULATION, PHYSICOCHEMICAL CHARACTERISATION AND IN VITRO ACTIVITY

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Abstract

Doxorubicin (DOX) is an effective anticancer drug, but its use is limited by dose-related toxicity, while β-caryophyllene (BCP), a dietary sesquiterpene, has poor water solubility and low oral bioavailability. This study prepared a self-emulsifying drug delivery system (SEDDS) that carries both agents and evaluated its physical and in vitro biological properties. Essential oil was obtained from the spines and bark of Pinus wallichiana by steam distillation, with a yield of 2.00 ± 0.03% (w/w, n = 3). GC-MS gave a β-caryophyllene peak at 25.6 ± 0.1 min with a peak-area purity of 94.0 ± 0.3%. The oil was dissolved in a lipid phase of medium-chain fatty acids, Tween 80 and vitamin E, mixed with DOX in dimethyl sulfoxide (DMSO), and homogenised at 10,000 rpm. On 1:100 dilution in water, the system formed droplets of 180.6 ± 5.0 nm with a polydispersity index of 0.29 ± 0.01 and a zeta potential of −38.7 ± 1.0 mV. FTIR showed no new or lost bands, which suggests the components did not react with each other. SEM showed spherical droplets when BCP was present and irregular particles without it. The formulation showed no phase separation after centrifugation, three freeze–thaw cycles or 30 days at room temperature. It gave 56.8 ± 1.9 mg GAE/g total phenolics, 33.4 ± 1.3 mg QE/g total flavonoids and concentration-dependent DPPH scavenging (35.2–88.6% at 20–100 μg/mL). In the MTT assay, the IC_{50} fell from 10.1 ± 0.7 μg/mL at 24 h to 5.2 ± 0.4 μg/mL at 48 h. These results show that the two agents can be held together in a stable nanosized lipid system. Comparison with single-agent and blank controls is still needed before any synergy can be claimed.

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INTRODUCTION

Doxorubicin (DOX) is an anthracycline that remains in wide use against breast, lung, ovarian and haematological cancers. It acts mainly by inserting between DNA base pairs and blocking topoisomerase II, and it also produces reactive oxygen species through redox cycling of its quinone ring (Kciuk et al., 2023). The same mechanisms damage healthy tissue. The most serious problem is cumulative, dose-dependent heart damage that can progress to cardiomyopathy and heart failure, and bone marrow suppression is the usual dose-limiting effect in the short term (Carvalho et al., 2009; Belger et al., 2024; Bhutani et al., 2025). Resistance through drug-efflux pumps such as P-glycoprotein (P-gp) further reduces its value in advanced disease (Kciuk et al., 2023).

One way to reduce the dose of DOX is to combine it with a natural compound that adds its own anticancer activity or makes cancer cells more sensitive to the drug (Choudhari et al., 2020; Gao et al., 2022). β-Caryophyllene (BCP) is a bicyclic sesquiterpene found in the essential oils of many plants (Maffei, 2020). It is a selective agonist of the cannabinoid type 2 (CB2) receptor and does not act on the CB1 receptor, so it has no psychoactive effect (Fidyt et al., 2016). BCP and its oxide (BCPO) have shown anticancer activity in several cell models (Fidyt et al., 2016; Dahham et al., 2021; Mannino et al., 2021). Both compounds have also increased the cytotoxicity of DOX in human cancer cells, apparently by limiting P-gp-mediated efflux (Di Giacomo et al., 2017; Di Sotto et al., 2020). BCP has low acute and subchronic toxicity in rodents (Schmitt et al., 2016; da Silva Oliveira et al., 2018).

The practical problem is delivery. BCP is volatile, poorly soluble in water and easily oxidised, and its oral bioavailability is low (Francomano et al., 2019). Self-emulsifying drug delivery systems (SEDDS) are isotropic mixtures of oil, surfactant and sometimes co-solvent that form fine droplets when they meet an aqueous medium. In healthy volunteers, a SEDDS based on the VESIsorb® technology raised the Cmax of BCP 3.6-fold and its AUC_{0-24}h 2.0-fold compared with BCP in plain oil (Mödinger et al., 2022). The same platform increased the Cmax of cannabidiol 4.4-fold compared with a medium-chain triglyceride reference (Knaub et al., 2019).

Pinus wallichiana A.B. Jacks. (blue pine) is a Himalayan conifer whose essential oil has been studied for its composition and antiproliferative activity (Yousuf Dar et al., 2012; Sinha, 2019; Sharma et al., 2019). To our knowledge, no study has placed DOX and BCP from this source in one self-emulsifying carrier. The aims of this work were (i) to extract and identify BCP-rich oil from the spines and bark of *P. wallichiana*, (ii) to prepare a SEDDS containing this oil and DOX, (iii) to characterise the system physically, and (iv) to measure its antioxidant and in vitro cytotoxic activity.

2. Materials and methods

2.1. Chemicals

Doxorubicin hydrochloride (analytical grade), β-caryophyllene standard, Tween 80, vitamin E, medium-chain fatty acids (MCFA), dimethyl sulfoxide (DMSO), methanol and anhydrous sodium sulfate were used. [AUTHOR TO ADD: supplier and purity of each chemical, and the reagents for the antioxidant and MTT assays — Folin–Ciocalteu reagent, gallic acid, quercetin, aluminium chloride, sodium carbonate, DPPH, MTT, culture media, FBS, antibiotics.]

2.2. Plant material

Spines and bark of Pinus wallichiana were collected on 20 September 2024 from [AUTHOR TO ADD: exact locality in Azad Jammu and Kashmir, Pakistan, altitude and GPS coordinates]. The species, which grows at about 1800–4300 m above sea level, was identified by [AUTHOR TO ADD: name of taxonomist], and a voucher specimen was deposited at [AUTHOR TO ADD: herbarium and voucher number]. The material was washed with distilled water (Naeem et al., 2010), dried in the shade at room temperature for about two weeks, ground to a fine powder and stored in airtight containers until extraction.

2.3. Extraction of essential oil

The oil was obtained by steam distillation in a Clevenger-type apparatus (Guleria et al., 2012).

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Powdered material (200 g) was placed in a round-bottom flask with about 2.2 L of distilled water and heated on a mantle. Distillation continued for 3–4 h at a condensate return rate of 2–3 mL/min, or until the oil volume in the graduated arm stopped increasing. The oil layer was separated, dried over anhydrous sodium sulfate and stored at [AUTHOR TO ADD: storage temperature] until use. Three independent batches were prepared. Yield was calculated as:
Yield (%) = (mass of oil, g / mass of dried plant material, g) × 100

2.4. GC-MS analysis

The oil was analysed on a Varian GC-MS 4000 system (Varian, USA) with a CP-Sil 8 CB column (30 m × 0.32 mm i.d., 1 µm film). The injector was set at 230 °C. The oven was held at 60 °C for 5 min and then raised to 250 °C at 3 °C/min. Helium was the carrier gas at 1.0 mL/min, and the injection volume was 0.20 µL. Mass spectra were recorded with electron-impact ionisation at 70 eV over m/z 40–500. BCP was identified by matching its retention time and mass spectrum with those of the authentic standard (Sharma et al., 2019). [AUTHOR TO ADD: split ratio; library used for spectral matching; whether linear retention indices were calculated against an n-alkane series.] Purity was expressed as the relative peak area:
Purity (%) = (area of BCP peak / total area of all peaks) × 100

2.5. Preparation of the SEDDS

The lipid phase was prepared following the composition approach reported for VESIsorb-type systems (Knaub et al., 2019). MCFA (6.5 g) served as the oil phase, Tween 80 (14 g) as the non-ionic surfactant and vitamin E (3 g) as an antioxidant. The BCP-rich oil (0.12 g) [AUTHOR TO CONFIRM: whether the extracted oil or the commercial BCP standard was used here] was added and the mixture was stirred until a clear, uniform phase formed.
Separately, DOX hydrochloride (1.5 g) was dissolved in DMSO (15 mL). This DOX solution was added dropwise to the lipid phase under constant magnetic stirring. The mixture was then homogenised at 10,000 rpm for 10 min with a high-speed homogeniser (Wiggens D-500) (Natsheh et al., 2025). The product was inspected visually for clarity, uniformity and phase separation. The composition is summarised in Table 1.

Table 1. Composition of the DOX–BCP SEDDS.

Component	Amount	Approx. % w/w*	Function
Medium-chain fatty acids	6.5 g	15.6	Oil phase
Tween 80	14 g	33.6	Surfactant
Vitamin E	3 g	7.2	Antioxidant
BCP-rich oil	0.12 g	0.29	Active phytoconstituent
Doxorubicin HCl	1.5 g	3.6	Cytotoxic drug
DMSO	15 mL	39.6	Co-solvent for DOX

*Calculated by the authors of this manuscript from the amounts above, taking the density of DMSO as 1.10 g/mL. [AUTHOR TO CHECK]

2.6. Physicochemical characterisation

Stability. The formulation was centrifuged at 4000 rpm for 30 min, subjected to three freeze–thaw cycles [AUTHOR TO ADD: temperatures and hold times] and stored at room temperature for 30

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days. Samples were checked for phase separation, turbidity and precipitation.

FTIR. Spectra of BCP, DOX, a physical mixture and the final formulation were recorded as KBr pellets at 4 cm^{-1} resolution with 16 scans per sample (Helal & Jaradat, 2024). [AUTHOR TO CONFIRM: (a) the instrument — the thesis names both a PerkinElmer Spectrum Two and an Agilent Cary 630; (b) the scan range — the thesis states 2000–4000 cm^{-1}, but bands at 1605–1710 cm^{-1} are reported, so the range must have extended below 2000 cm^{-1} (normally 4000–400 cm^{-1}).]

SEM. A drop of the formulation was placed on an aluminium stub, dried at room temperature and coated with platinum in a Quorum SC7620 sputter coater. Images were taken with a JEOL JSM-7610F field-emission SEM at 10–15 kV and magnifications of 10,000–50,000×. A formulation without BCP was imaged for comparison. [AUTHOR TO CONFIRM: SEM instrument — the thesis instrument table lists a different model.]

Droplet size, PDI and zeta potential. These were measured by dynamic light scattering on a Malvern Zetasizer Nano ZS90 at 25 °C after 1:100 dilution in double-distilled water (Lunardi et al., 2021). Each of three independent batches was measured in triplicate. A PDI below 0.3 was taken to indicate a narrow size distribution, and an absolute zeta potential above 30 mV to indicate good electrostatic stability.

2.7. Antioxidant assays

Total phenolic content (TPC). TPC was measured with the Folin–Ciocalteu method (Thusoo et al., 2014). The sample was mixed with diluted Folin–Ciocalteu reagent and, after 5 min, with sodium carbonate solution. After 30 min in the dark at room temperature, absorbance was read at 765 nm. Results were expressed as mg gallic acid equivalents per g of formulation (mg GAE/g) from a gallic acid calibration curve (10–50 µg/mL).

Total flavonoid content (TFC). TFC was measured by the aluminium chloride method (Jia et al., 1999). The sample was mixed with methanolic aluminium chloride and kept in the dark for 30 min, and absorbance was read at 415 nm. Results were expressed as mg quercetin equivalents per g (mg QE/g) from a quercetin calibration curve (10–50 µg/mL).

DPPH radical scavenging. The sample (20–100 µg/mL) was mixed with a methanolic DPPH solution and kept in the dark for 30 min, and absorbance was read at 517 nm (Bozin et al., 2006). Inhibition was calculated as:

Inhibition (%) = $[(A_0 - A_1) / A_0] \times 100$

where A_0 is the absorbance of the control and A_1 that of the sample.

[AUTHOR TO ADD: sample concentration used for TPC/TFC; whether a sample colour blank was subtracted (DOX is coloured); and the positive control for DPPH, e.g. ascorbic acid or BHT, if one was run.]

2.8. Cytotoxicity (MTT assay)

[AUTHOR TO ADD: name, source and authentication of the cell line(s) used. Journals will not accept "human cancer cell lines" without the name.] Cells were kept in RPMI-1640 or DMEM with 10% fetal bovine serum, penicillin and streptomycin at 37 °C in a humidified 5% CO_2 incubator. They were seeded in 96-well plates at 5000 cells per well and left to attach overnight. The formulation was then added at 1, 5, 10, 20 and 50 µg/mL [AUTHOR TO CONFIRM: concentration of formulation or of DOX equivalent] and incubated for 24 h and 48 h. Untreated cells served as controls. After treatment, the medium was removed, 20 µL of MTT (5 mg/mL in PBS) was added, and the plates were incubated for 3–4 h at 37 °C. The formazan was dissolved in 150 µL DMSO and absorbance was read at 570 nm in a microplate reader (Di Sotto et al., 2020). Cell viability was calculated as:

Viability (%) = (absorbance of treated cells / absorbance of control cells) × 100

IC_{50} values were read from the concentration–viability curves. [AUTHOR TO ADD: fitting method and software.]

2.9. Statistical analysis

All experiments were done in triplicate, and results are given as mean ± standard deviation (SD). [AUTHOR TO ADD: any statistical tests

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used and the software. If none were used, remove words such as "significantly" from the results.]

3. Results

3.1. Oil yield and GC-MS identification

The three batches gave 2.03%, 1.97% and 2.00% oil, a mean of 2.00 ± 0.03% (Table 2). All three chromatograms showed a strong peak at 25.5–25.7 min (mean 25.6 ± 0.1 min) that matched the BCP standard. The relative peak area of BCP was 94.0 ± 0.3%, and minor constituents together made up 3.8 ± 0.1%.

Table 2. Essential oil yield and BCP content of three batches.

Batch	Oil yield (%)	BCP (% peak area)	Retention time (min)	Minor constituents (%)
1	2.03	93.7	25.5	3.9
2	1.97	94.1	25.6	3.7
3	2.00	94.2	25.7	3.8
Mean ± SD	2.00 ± 0.03	94.0 ± 0.3	25.6 ± 0.1	3.8 ± 0.1

3.2. Appearance and stability

The formulation was clear and uniform on visual inspection. It showed no phase separation after centrifugation at 4000 rpm for 30 min, no destabilisation after three freeze–thaw cycles, and no turbidity or precipitation after 30 days at room temperature.

3.3. FTIR

Pure BCP showed aliphatic C–H stretching near 2955 cm^{-1} and C=C stretching near 1640 cm^{-1}, which fits its sesquiterpene structure. The formulation showed a broad O–H/N–H band near 3400 cm^{-1}, a carbonyl (C=O) band near 1710 cm^{-1} and an aromatic C=C band near 1605 cm^{-1}. No new bands appeared and no characteristic band disappeared. Only slight broadening was seen, which is consistent with physical interactions such as hydrogen bonding rather than chemical reaction between the components. [AUTHOR TO ADD: DOX spectrum and physical-mixture spectrum, if recorded, so that DOX bands can be traced in the formulation.]

3.4. Morphology

SEM showed nearly spherical droplets with smooth surfaces and no visible aggregation when BCP was present. The formulation without BCP gave irregularly shaped particles with a wider spread of sizes.

3.5. Droplet size, PDI and zeta potential

The mean droplet size was 180.6 ± 5.0 nm, the PDI 0.29 ± 0.01 and the zeta potential −38.7 ± 1.0 mV (Table 3). Batch-to-batch variation was small for all three measures.

Table 3. Droplet size, PDI and zeta potential of three batches.

Batch	Droplet size (nm)	PDI	Zeta potential (mV)
1	175.1	0.28	−37.5
2	184.7	0.30	−39.2
3	182.0	0.29	−39.3
Mean ± SD	180.6 ± 5.0	0.29 ± 0.01	−38.7 ± 1.0

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3.6. Antioxidant activity

The TPC of the formulation was 56.8 ± 1.9 mg GAE/g. The quercetin standard curve was linear between 10 and 50 µg/mL (absorbance 0.125–0.620 at 415 nm). The formulation gave an absorbance of 0.415 ± 0.02, corresponding to a TFC of 33.4 ± 1.3 mg QE/g. DPPH scavenging increased with concentration: 35.2% at 20 µg/mL, 56.8% at 40 µg/mL, 72.4% at 60 µg/mL, 81.0% at 80 µg/mL and 88.6% at 100 µg/mL. Linear interpolation between 20 and 40 µg/mL gives an approximate IC_{50} of 34 µg/mL.

3.7. Cytotoxicity

Cell viability fell as concentration rose, and it was lower at 48 h than at 24 h at most concentrations. At 1 µg/mL viability stayed at about 80–90% at both times, and at 50 µg/mL it fell to about 10% or less. The IC_{50} was 10.1 ± 0.7 µg/mL at 24 h and 5.2 ± 0.4 µg/mL at 48 h. Based on the composition in Table 1, these values correspond to roughly 0.36 and 0.19 µg/mL of DOX, respectively. [AUTHOR TO CHECK this conversion, and to report the IC_{50} of free DOX, free BCP and the blank SEDDS if these arms were tested.]

4. Discussion

4.1. Yield and composition of the oil

The oil yield of 2.0% is higher than the 1.2% reported for hydrodistilled needle oil of the same species (Sharma et al., 2019). The low spread across three batches shows that the extraction was reproducible.

The composition result needs careful handling. Earlier studies of P. wallichiana oil found monoterpenes to dominate. Sharma et al. (2019) reported α-pinene (48.6%), β-pinene (45.6%) and limonene (5.6%) in needle oil, with no BCP listed. Yousuf Dar et al. (2012), as summarised by Sinha (2019), reported β-pinene (46.8%), α-pinene (25.2%) and myrcene (9.5%), with trans-caryophyllene at 1.8%. A relative peak area of 94% for BCP is therefore well outside the published range for this species. Plant part (spines and bark rather than needles), distillation method, altitude and season can all change essential oil composition, and BCP is the main component of some other pine oils (Hamzaoui et al., 2024). Still, the size of the difference means the identification has to be shown in full: the complete chromatogram, a table of every peak with its retention index and library match, and co-injection with the authentic standard. Until then, the oil is best described as "BCP-rich" rather than as purified BCP.

4.2. Formulation and physical properties

The system combined an MCFA oil phase, a high proportion of Tween 80 and vitamin E, a composition similar in concept to the VESIsorb-type SEDDS used in human bioavailability studies (Knaub et al., 2019; Mödinger et al., 2022). DOX hydrochloride is water-soluble, so it was carried in DMSO as a co-solvent and mixed into the lipid phase. The product is therefore a water-free pre-concentrate that forms droplets on dilution, which is the defining behaviour of a SEDDS.

On 1:100 dilution the droplets were about 180 nm across. This is larger than the 40–50 nm droplets of the commercial VESIsorb BCP product (Mödinger et al., 2022). It is still in the nanometre range, and the PDI of 0.29 shows a fairly narrow distribution. The zeta potential of −38.7 mV is above the usual 30 mV threshold for electrostatic stability (Lunardi et al., 2021). Because Tween 80 carries no charge, the negative surface charge most likely comes from ionised carboxyl groups of the free fatty acids at the droplet surface. The absence of phase separation after centrifugation, freeze–thaw cycling and 30 days of storage agrees with these measurements. Thirty days is a short period, however, and longer storage under ICH conditions, with DOX content measured over time, would give a firmer picture of shelf life.

FTIR showed no new bands and no loss of the characteristic BCP bands, which suggests that the components were physically mixed rather than chemically changed. The bands near 3400, 1710 and 1605 cm^{-1} in the formulation are consistent with hydroxyl, amine, carbonyl and aromatic groups of DOX and the excipients. A DOX reference spectrum would make this assignment more secure.

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SEM showed that adding BCP changed the dried formulation from irregular particles to smooth spheres. BCP may act at the oil–surfactant interface, but SEM of dried liquid systems has known limits: drying can distort droplets and cause artefacts. Transmission electron microscopy (TEM) with negative staining, or cryo-TEM, would show droplet shape in the hydrated state more reliably.

4.3. Antioxidant activity

The formulation gave measurable TPC, TFC and DPPH activity, with an approximate DPPH IC_{50} of 34 µg/mL. These values should be read with the composition in mind. BCP is a hydrocarbon with no phenolic hydroxyl group, so it would not be expected to react directly in the Folin–Ciocalteu or aluminium chloride assays. Vitamin E, which makes up about 7% of the formulation, is a phenolic antioxidant and is a likely main contributor to the TPC and DPPH results. DOX has phenolic hydroxyl groups and is strongly coloured, so it may also contribute, both chemically and through its own absorbance near the assay wavelengths. The antioxidant results therefore describe the formulation as a whole. They cannot be attributed to BCP without testing a blank formulation and a formulation without BCP under the same conditions, with colour blanks for each.

4.4. Cytotoxicity

The formulation reduced cell viability in a concentration- and time-dependent way, and the IC_{50} roughly halved between 24 h and 48 h. This fits the known action of DOX, whose DNA damage builds up with exposure time (Kciuk et al., 2023). Earlier studies give a reason to expect BCP to help. In Caco-2, CCRF/CEM and CEM/ADR5000 cells, both BCP and BCPO had little toxicity alone but increased DOX cytotoxicity, and they raised intracellular rhodamine 123, which points to inhibition of efflux pumps (Di Giacomo et al., 2017). In HepG2 cells, both compounds strengthened the effect of low-dose DOX, increased intracellular DOX and reduced P-gp expression (Di Sotto et al., 2020). BCPO alone also increased DOX toxicity in HepG2 cells by about 20% (Helal & Jaradat, 2024). However, BCPO did not improve the effect of DOX in fully resistant CEM/ADR cells (Ambrož et al., 2017), so the benefit depends on the cell type.

In the present study, the MTT data come from the combined formulation only. Without IC_{50} values for free DOX, free BCP and the blank SEDDS tested on the same cells, it is not possible to say whether the combination is synergistic, additive or simply equal to DOX alone. A full checkerboard design with combination index analysis would answer this. The BCP:DOX mass ratio in this formulation was about 1:12.5, and other ratios may behave differently.

4.5. Limitations

This work has several limitations. The cancer cell line used was not specified, and no normal cell line was tested, so selectivity cannot be judged. Drug loading, encapsulation efficiency and in vitro release were not measured, and DOX and BCP were not quantified in the final product. HPLC was planned for this purpose but not carried out. The time needed for self-emulsification and the effect of dilution in simulated gastric and intestinal fluids were not tested. The formulation contains about 40% DMSO, which may need to be reduced for oral or in vivo use. Finally, no statistical tests were applied beyond mean ± SD.

5. Conclusions

BCP-rich oil was extracted from the spines and bark of Pinus wallichiana with a reproducible yield of 2.0%. Combined with DOX in an MCFA–Tween 80–vitamin E system, it formed nanosized droplets (about 180 nm) with a narrow size distribution, a strongly negative surface charge and good physical stability over 30 days. The formulation showed antioxidant activity and concentration- and time-dependent cytotoxicity in vitro. These results support the idea that DOX and BCP can be carried together in one self-emulsifying system. The next steps are to confirm the oil composition in full, measure drug loading and release, compare the formulation with free DOX, free BCP and blank controls on named

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cancer and normal cell lines, and then test pharmacokinetics and heart safety in vivo.

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Conflicts of interest: The authors declare no conflict of interest. [CONFIRM]

Data availability: The data supporting this study are available from the corresponding author on reasonable request. [CONFIRM]

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