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β-bisabolene, a sesquiterpene from the essential oil extract of Opoponax (Commiphora guidottii) exhibits cytotoxicity in breast cancer cell lines

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Running title: Anti-tumour properties of β-bisabolene
Abstract

The essential oils from Commiphora species have for centuries been recognised to possess medicinal properties. Here we performed gas chromatography-mass spectrometry (GC-MS) on the essential oil from opoponax (Commiphora guidottii) and identified bisabolene isomers as the main constituents of this essential oil. Opoponax essential oil, a chemical component; β-bisabolene and an alcoholic analogue; α-bisabolol, were tested for their ability to selectively kill breast cancer cells. Only β-bisabolene, a sesquiterpene constituting 5% of the essential oil, exhibited selective cytotoxic activity for mouse cells (IC50 in normal Eph4: >200 µg/ml, MG1361: 65.49 µg/ml, 4T1: 48.99 µg/ml) and human breast cancer cells (IC50 in normal MCF10A: 114.3 µg/ml, MCF7: 66.91 µg/ml, MDA-MB-231: 98.39 µg/ml, SKBR3: 70.62 µg/ml and BT474: 74.3 µg/ml). This loss of viability was due to the induction of apoptosis as shown by AnnexinV-Propidium Iodide and Caspase3/7 activity assay. β-bisabolene was also effective in reducing the growth of transplanted 4T1 mammary tumours in vivo (37.5% reduction in volume by endpoint). In summary, we have identified an anti-cancer agent from the essential oil of opoponax that exhibits specific cytotoxicity to both human and murine mammary tumour cells in vitro and in vivo and this warrants further investigation into the use of β-bisabolene in the treatment of breast cancers.
Introduction

The medicinal properties of natural products have been appreciated since ancient times and they remain a rich source for anti-tumour drug discovery (Da Rocha et al., 2001). Derivatives of natural compounds currently used to treat breast cancer include chemotherapeutic agents such as paclitaxel, a microtubule stabilizing drug (Wani et al., 1971). However, there are unwanted side effects associated with chemotherapeutics and this warrants the search for targeted therapeutics which exhibit cytotoxicity that is more specific towards cancer cells. Several natural compounds from a class of molecules termed sesquiterpenes have shown promise by selectively inducing apoptosis in cancer cells. These include β-elemene which has been shown to exhibit differential anti-tumour properties against non-small-cell lung carcinoma (Wang et al., 2005) and cacalol which selectively kills breast cancer cell lines (Liu et al., 2011).

In a large scale screening study to ascertain the anti-tumour activity of 374 medicinal herbs from across the globe, it was found that extracts with tumouricidal properties were not segregated within particular families or genus of plants. Interestingly however, the biblical herbs, myrrh gum from Commiphora molmol and opoponax from Commiphora guidottii both exhibited potent cytotoxic properties against neuroblastoma cells. Accordingly, both of these herbs were categorized as natural products with strongest anti-tumour activity, characterized by EC_{50} values in the range of 0.019-0.528 mg/ml (Mazzio & Soliman, 2009). The efficacy of Myrrh gum extracts from Commiphora molmol in inducing cytotoxicity of Ehrlich solid tumours in mice has been previously reported (Al-Harbi et al., 1994). In the case of o Commiphora guidottii (also known as opoponax, scented myrrh, Sweet myrrh or habak haddi), some of its traditional applications involve treatment of wounds,
diarrhoea, stomach discomforts and removal of the placenta after childbirth (Thulin, 1999; Thulin & Claeson, 1991). The components of opoponax have also been reported to exhibit pharmacological properties such as smooth muscle relaxing effects (Andersson et al., 1997; Claeson et al., 1991) and anti-microbial properties (de Rapper et al., 2012).

In this study, we set out to investigate the cytotoxic properties of opoponax essential oil with regards to breast cancer. The constituents of opoponax essential oil have been identified for decades (Craveiro et al., 1983; Ishwar & Levi, 1966) and it is known to be rich in sesquiterpenes (Baser et al., 2003; Tian & Shi, 1996), naturally-occurring molecules known to exert anticancer effects (Ahn et al., 2015; Han et al., 2014; Martins et al., 2014; Pitchai et al., 2014). Among these is bisabolene, the main constituent of opoponax of which α, β, and γ isomers constitute more than one third of the essential oil. Noteworthily, the alcoholic analogue of the α-isomer of bisabolene, the main component of *Matricaria chamomilla* essential oil, has selective cytotoxicity against glioma cells (Cavaliere et al., 2004) and mammary tumours (Costarelli et al., 2010); yet the anti-tumour properties of the other chemically related isoforms of bisabolene have not been determined until recently. β-bisabolene was found to be the major component of the essential oil of leaves from *Duguetia gardneriana* which exhibited potent antitumor properties against melanoma, hepatocellular carcinoma and leukemia cells (Rodrigues et al., 2015). However, the efficacy of β-bisabolene against breast cancer cells and its potential as an anti-tumour agent *in vivo* has not been investigated. Hence, following primary analysis of the constituents of opoponax, the focus of this study was to assess the potential of β-bisabolene as a selective therapeutic agent against breast cancers.
Materials and Methods

Materials

Opoponax (C. guidottii) essential oil was purchased from Sigma-Aldrich (Dorset, UK). β-bisabolene was kindly provided by R C Treat limited (Suffolk, UK) and α-Bisabolol purchased from KIC Chemicals (New York, USA). All extracts were solubilized and diluted in ethanol to obtain working concentrations.

Gas chromatography- mass spectrometry (GC-MS) analysis

Opoponax extracts were analysed using a Finnegan GC 8000 gas chromatograph equipped with a MD 800 mass selective detector and an AS 800 Finnegan autosampler. For the capillary column, a DB-5 fused silica column (J&W Scientific) was used with the following dimensions: 30m x 0.32mm id. and 0.25µm film thickness. The temperature of the oven was programmed from 50°C to 240°C at a rate of 3°C min⁻¹ and maintained at this final temperature for two minutes. The helium carrier gas was set at a flow-rate set of 1ml min⁻¹, maintained under constant pressure while the injector and source temperatures were both set at 260°C. The mass detector was used in the positive electron impact ionisation mode (EI⁺) using an ionisation voltage of 70 eV. A scan range of 35 to 450 mass units in 0.45 seconds was used for acquiring mass spectra with an interscan time of 0.08 seconds.

Cell culture

4T1, EpH4 and MG1361 murine mammary cancer cell lines along with MCF-10A, MCF-7, MDA-MB-231, SKBR3 and BT474 human breast cancer cell lines were obtained from ATCC. 4T1, SKBR3 and BT474 cells were grown in RPMI 1640. EpH4, MCF-7 and MDA-MB-231 cells were maintained in DMEM while MG1361
cells were cultured in L-15 media. All of the respective culture media were supplemented with 10% FBS, 1% L-Glutamine and 20 units of Penicillin-Streptomycin (Invitrogen, UK). For MG1361 cells, media was also supplemented with non essential amino acids. MCF-10A cells were cultured in an equal mixture of DMEM/F12 media supplemented with 10% horse serum, 1% L-Glutamine, 20 units of Penicillin-Streptomycin, 10ug.ml insulin, 100ng/ml cholera toxin, 0.5ug/ml hydrocortisone and 20ng/ml EGF. The cell lines used were between passages 5 to 40 and maintained at 37°C with 5% CO₂.

Cell viability assay
For viability assays, murine cells were plated at a density of 10,000 cells/well and human cells were plated at 20,000 cells/well into a 96-well plate. Cells were treated 24 hours after seeding with respective agents prepared in media. The viability of cells in each well was then quantified using Cell Titer Blue (Promega, UK) according to manufacturer’s instructions 24 hours after treatment.

Apoptosis assays
Cell lysates after specified treatments were analysed with Caspase Glo 3/7 assay kit (Promega) according to manufacturer’s instructions. Annexin-V/PI analysis was performed using Dead Cell Apoptosis Kit (Invitrogen) according to manufacturer’s instructions and stained cells were analysed using a BD FACSCanto flow cytometer.

In vivo administration of β-bisabolene
1.12g/kg of the oily compound β-bisabolene, solubilised in corn oil, was administered intra-peritoneally, two times a week, for two weeks, did not result in any signs of
distress in all animals. Upon necropsy, the histology of liver, kidney, spleen and lung of these mice appeared normal (data not shown). When a dose of 2.24g/kg was administered, mice showed signs of morbidity after single treatment. Hence, we used a maximum non-lethal dose of 1.12g/kg β-bisabolene in our tumour growth studies. The treatment regime consisted of intra-peritoneal (i.p.) injections twice weekly, once palpable tumour were detected (at 2 weeks) and until mice have reached the endpoint of the experiment or showed signs of morbidity.

**Orthotopic cell transplants and tumour measurements**

All animal experiments were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986, under Home Office licence 30/2849. 4T1 cells were trypsinized and dissociated into single cell suspensions before transplantation into the abdominal mammary fat pads of wild type BALB/C mice which were between 8-12 weeks old. Mice were then checked for palpable tumours and the resulting tumours were measured using calipers three times weekly. At appropriate experimental endpoints, tumour, mammary gland, lung and liver tissues were harvested. Tissue samples were then fixed in 4% formaldehyde at 4°C overnight before processing for histological analysis.

**Immunohistology**

Fixed tissues were embedded in paraffin, sectioned into 5μm slices, mounted onto poly-L-lysine coated slides and stained with haematoxylin and eosin (H&E). Antibodies for cleaved caspase-3 (Cell Signaling Technologies) and Ki-67 (VectorLabs) were used for immunohistology according to manufacturer’s instructions.
Statistical analysis

Data were presented as means of at least three replicates with standard error of the mean. Significance was determined by a two-tailed t-test, where p<0.05 was taken as the threshold for significance.
Results

**GC-MS analysis of opoponax essential oil**

In order to better understand the molecular basis of the anti-tumour properties of Opoponax, we analyzed the composition of essential oil extracts by GC-MS to verify the nature of the chemical components present. As previously reported (Baser et al., 2003; Ishwar & Levi, 1966; Tian & Shi, 1996), we found that trans-β-ocimene, α-santalene and α-bisabolene were the main constituents in opoponax (Figure 1a). The bisabolenes with α, β and γ isomers, together comprised the major class of constituents (36% of the components present in opoponax essential oil extract) with α-bisabolene being the most abundant isomer. The alcoholic analogue α-bisabolol, had previously been shown to inhibit the formation of mammary tumours in a mouse model of HER2-positive breast cancer and more recently, β-bisabolene was reported to be the major component of *Duguetia gardneriana* essential oil which exhibits anti-tumour properties. In order to test the efficacy of β-bisabolene against breast cancer cells, we obtained a commercial fraction of this next most abundant bisabolene isomer in Opoponax, and confirmed its purity, chemical structure and its relationship to the original opoponax fraction by GC-MS (Figure 1). The β-bisabolene component identified in opoponax essential oil eluted at a retention of 34.4 minutes identical to that of the commercially available β-bisabolene standard. EI mass spectra of the identified β-bisabolene, in the essential oil extract and the commercially available standard were identical, revealing a base peak at m/z 69, a prominent signal at m/z 93 and a molecular ion at m/z 204, in good agreement with published literature (Figure 1b) (Craveiro et al., 1983).
β-bisabolene treatment decreases the viability of mammary cancer cell lines

In order to gauge the anti-cancer properties of β-bisabolene, we compared it with the crude parental essential oil extract, opoponax and the previously studied analogue, α-bisabolol (Cavalieri et al., 2004; Costarelli et al., 2010). We treated a tumourigenic (4T1) and a non-tumourigenic (EpH4) murine mammary epithelial cell line with equivalent concentrations of each purified chemical, and the crude parental essential oil extract. The two purified chemicals displayed distinct cytotoxicity profiles, with β-bisabolene rather than α-bisabolol exhibiting preferential cytotoxicity in the tumourigenic cell line compared to its non-tumourigenic counterpart (Figure 2a). Not only did β-bisabolene exhibit the most potent cytotoxicity in tumourigenic 4T1 cells, it was the least toxic in non-tumorigenic Eph4 cell lines (Figures 2a-b). Opoponax induced a marked decline in viability of the non-tumorigenic EpH4 cells, resulting in substantial loss of cells in adherent culture, yet it increased the viability of the tumourigenic cell line. The mechanism behind this apparent survival effect is unknown and likely relates to other molecular species within the crude essential oil extract. This highlights the complexity of these essential oils and further underscores the need to identify the biological properties of the constituent fractions.

These results led us to investigate further the specificity of β-bisabolene in tumour cell lines in vitro and in vivo. As α-bisabolol had previously displayed anti-tumour effects on ErbB2+ tumours in mice (Costarelli et al., 2010), we assessed the toxicity of β-bisabolene in the ErbB2-positive murine cell line, MG1361 (Figure 2c). β-bisabolene induced an equivalent loss of viability in both the undifferentiated (basal-like) 4T1 and ErbB2+ (luminal) MG1361 tumour cell lines (IC₅₀s of 48.99µg/ml and 65.49 µg/ml), with complete loss of cell viability achieved at 100µg/ml (Figure 2c). At this concentration no loss in viability was observed in non-
tumourigenic EpH4 cells (IC\textsubscript{50} of >200 µg/ml) (Figure 2c). β-bisabolene also induced time dependent decreases in cell viability for 4T1 cells (Figure 2d). A significant decrease in viability relative to vehicle control was observed only after 72 hours of culture with β-bisabolene and not at earlier time points in this particular experiment.

Cytotoxicity was also confirmed in a panel of human breast cancer cell lines, representing different ER and HER2 status. ER\textsuperscript{+} and/or HER2\textsuperscript{+} cells with luminal characteristics (SKBR3, MCF-7 and BT474) were most sensitive to β-bisabolene (IC\textsubscript{50} of 70.62µg/ml, 66.91µg/ml and 74.30µg/ml respectively), while the basal-like tumour cell line, MDA-MB-231, was significantly more resistant (IC\textsubscript{50} of 98.39µg/ml) (Figure 2e). However these effects were limited to a narrow dose range in human cell lines in culture, as the non-tumourigenic cell line, MCF-10A, were also susceptible to β-bisabolene at marginally elevated concentrations (IC\textsubscript{50} of 114.3µg/ml).

β-bisabolene induces apoptosis in mammary cancer cell lines

In order to establish whether β-bisabolene induced an active programmed cell death, we performed complementary assays for apoptosis following β-bisabolene treatment of tumourigenic cell lines. Treatment of MDA-MB-231 cells with β-bisabolene for 18 hours disrupted membrane polarity in a significant proportion of cells (Figure 3a). 22% (±5.9%) and 66% (±1.8%) of the cell population bound annexin V following treatment with 100µg/ml and 200µg/ml β-bisabolene respectively (Figure 3b), indicating a dose-responsive increase in apoptosis. No difference in propidium iodide staining was observed over the same timecourse, indicating that loss of membrane integrity, a sign of necrotic cell death, was not induced by treatment (Figure 3b). A comparative analysis of relative caspase activity in tumourigenic versus non-tumourigenic cells was also performed. Here, caspase 3/7
activity was found to be induced by β-bisabolene only in the tumourigenic cell line, but not in non-tumourigenic cells (Figure 3c). Thus β-bisabolene specifically induces apoptosis in tumourigenic mammary cell lines.

**β-bisabolene treatment reduces the rate of tumour growth in vivo**

We next investigated the effect of β-bisabolene on the growth of tumours in vivo. An earlier study had shown that tumour initiation was inhibited by α-bisabolol but it was unclear whether growth of existing tumours could be inhibited by bisabolene (Costarelli et al., 2010). Here we tested the effects of β-bisabolene on mice bearing pre-established mammary tumours.

β-bisabolene was administered by intraperitoneal injection into female Balb/C mice bearing a single orthotopic tumour of transplanted 4T1 cells after two weeks post-transplant. A tolerated dose of twice weekly injections (1.12g/Kg) resulted in a significant decrease (37.5% decrease in volume by endpoints) in the growth rate of tumours in β-bisabolene treated mice relative to vehicle controls (Figure 4a). Dosing at 100mg/Kg three-times weekly had no significant effect on tumour growth (data not shown). Histology of the tumours from the effective treatment group revealed a marked increase in cell death within the β-bisabolene treated tumours (Figure 4b). β-bisabolene treatment induced a significant increase in cleaved caspase-3 positive cells (4.6% versus 1.2% in vehicle controls) indicating that the treatment induces apoptotic cell death in 4T1 tumors. (Figure 4c). On the other hand, the number of proliferating cells as indicated by Ki-67 staining were reduced (2.6% versus 10.9% in controls) in β-bisabolene treated tumors (Figure 4d). Accompanying histology of vital organs and tissues within these animals revealed no associated increases in cytotoxicity (data not shown).
Discussion

This study describes the identification of an anti-cancer agent isolated from the essential oil opoponax that specifically induces apoptosis in breast cancer cells of mouse or human origin. Opoponax (*C. guidotti*) has previously been demonstrated to have anti-cancer properties but the active constituents of this essential oil had not been established (Mazzio & Soliman, 2009). We identified a minor constituent, the sesquiterpene, β-bisabolene, to possess tumour specific pro-apoptotic properties. Surprisingly opoponax and α-bisabolol did not share the anticancer properties attributed to them in other studies (Cavalieri et al., 2004; Costarelli et al., 2010; Mazzio & Soliman, 2009). This may be due to the cancer cell type, and in the case of α-bisabolol, the fact that we were looking at induction of apoptosis in breast cancer cell lines rather than prevention of sporadic tumour formation in vivo.

Despite this it was clear that the essential oil contains a number of fractions that have distinct cytotoxic properties. The discovery that β-bisabolene is more effective at targeting breast cancer cells compared to α-bisabolol is significant because it illustrates the importance of examining both major and minor constituents of medicinal extracts for agents with particular medicinal properties suitable for specific purposes. The other major constituents of Opoponax essential oil that we described were trans-β-ocimene, α-santalene and α-bisabolene. These agents have not been shown to possess anti-tumour properties as separate entities on their own and it would be interesting to assess their cytotoxic efficacies.

It is worth noting that β-bisabolene has recently been described to be the major constituent of *Duguetia gardneriana* essential oil which exhibits cytotoxicity against melanoma, hepatocellular carcinoma and leukemia cells (Rodrigues et al., 2015). However, in the study by Rodrigues et al., they found that β-bisabolene was
less effective in inducing cytotoxicity in vitro relative to the essential oil from 
*Duguetia gardneriana* as a whole. This would suggest that the other components of 
*Duguetia gardneriana* essential oil have a larger contribution than β-bisabolene 
towards the cytotoxic effects observed. For that reason, they only tested the anti-
tumour effects of *Duguetia gardneriana* essential oil but not β-bisabolene in vivo. 
Based on the selective cytotoxicity against breast cancer cells by β-bisabolene that we 
observed in vitro, we went on to show for the first time that it possesses anti-tumour 
properties in vivo.

A recent report describing the pro-apoptotic effects of a closely related isomer, 
γ-bisabolene, in oral squamous cell carcinoma cells suggests that bisabolene isomers 
may share certain chemical features which contribute to their anti-tumour functions 
(Jou et al., 2015). Accordingly, the selective induction of apoptosis in cancer cells that 
we observed were also demonstrated by γ-bisabolene against oral squamous 
carcinoma cells but not normal oral fibroblasts. Mechanistically, γ-bisabolene was 
shown to activate p53 mediated apoptosis through PP1/HDAC2 and ERK signaling 
(Jou et al., 2015). If indeed the bisabolene isomers share a similar mechanism of 
action, the specificity towards cancer cells that we observe may be due to differential 
HDAC2 activity in breast cancer cells. It is possible that the higher levels of HDAC2 
observed in breast cancers (Müller et al., 2013; Roy et al., 2014; Seo et al., 2014) play 
an essential role in mitigating the pro-apoptotic effects of p53 (Harms & Chen, 2007), 
making breast cancer cells more susceptible to inhibition of HDAC2 by bisabolene. It 
will be interesting to address this and compare the efficacies of all bisabolene isomers 
to identify that which is most efficient at inducing apoptosis.

We have shown that tumour cells can be successfully targeted in vivo by 
intraperitoneal injection of β-bisabolene. This is a product of the specificity of β-
bisabolene’s cytotoxicity in the mouse model. Although we only observed an effect after more than 3 weeks of treatment in vivo (Figure 4a), this is consistent with the fact that we see a time-dependent response for in vitro cytotoxicity assays (Figure 2d). The dose applied in our in vivo studies (1.12 g/kg) were about ten times less than the reported oral LD$_{50}$ in mice (>13.36g/kg) (Hoffman-LaRoche, 1967b). The oral LD$_{50}$ in rats have also been reported to be >5g/kg (Moreno, 1974), indicating that the doses we used are within the sub-toxic range. Nonetheless, it may be possible to alter the dosing regimen such that the concentration of bisabolene administered is reduced but compensated by a higher frequency of doses. Efficacious treatment was achieved with twice weekly doses of 1.12g/Kg in our studies. While a regime of three weekly doses at 100mg/Kg exhibited no effect on tumour growth (data not shown), it is conceivable that a daily dosing regimen could be both well tolerated and effective at lower doses.

Additionally, before β-bisabolene is used in a clinical setting, it would be of great benefit if its solubility properties in aqueous matrices were significantly enhanced through a formulation development exercise. These optimizations could improve the potential clinical benefits of β-bisabolene as a therapeutic in breast cancers.
Acknowledgement

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Conflict of Interest

Dr. Ahmed Ali is research director at Compton Group. The other authors declare no conflict of interest.
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Figure legends

Figure 1. GC-MS analysis of opoponax ethanolic extract and its constituent β-bisabolene. (a) Total ion current (TIC) generated gas chromatogram of the essential oil extract of opoponax highlighting its main constituents and the TIC generated gas chromatogram of β-bisabolene (inset chromatogram). The chemical components of opoponax essential oil were identified by gas chromatography-mass spectrometry (GC-MS), applied in the electron positive (EI +ve) mode. A number of terpenes (mono-and sesquiterpenes) were identified and are listed below according to the peak numbers shown in the chromatogram with % peak area data in brackets: 1) cis-α-ocimene (1.4%), 2) trans-β-ocimene (11.5%), 3) cis-α-bergamotene (2.7%), 4) α-santalene (21.9), 5) trans-α-bergamotene (9.0%), 6) epi-α-santalene (1.0%), 7) β-caryophyllene (0.6%), 8) epi-α-Santalene (0.7%), 9) cis-β-farnesene (1.0%), 10) curzerene (0.4%), 11) cis-α-bisabolene (27%), 12) β-Bisabolene (5.1%), 13) γ-bisabolene (3.9%), 14) cis-α-santalol (4.0%) and 15) unknown (0.7%). (b) EI +ve mass spectrum of β-bisabolene and its chemical structure (inset).

Figure 2. β-bisabolene treatment decreases the viability of mammary cancer cell lines. Cells plated in 96-well format and treated with respective compound for 24 hours. The resulting viability was then quantified as fluorescence and normalized to vehicle control treated cells. (a) EpH4 and 4T1 cells treated with opoponax extract, α-bisabolol or β-bisabolene at a concentration of 50μg/ml for 24 hours. (b) Representative images of EpH4 and 4T1 cell after vehicle, α-bisabolol or β-bisabolene treatment at a concentration of 50μg/ml for 24 hours. (c) Dose response curves for EpH4, 4T1 and MG1361 cells treated with varying concentrations of β-bisabolene. (d) Time course experiment with 4T1 cells treated with vehicle or β-
bisabolene for 24, 48 or 72 hours. (e) Dose response curves for MCF-10A, MCF-7, SKBR3, BT474 and MDA-MB-231 cell lines treated with varying concentrations of β-bisabolene. Data points represent triplicates from two independent experiments (n=6) ± SEM. ** indicates p<0.01, * indicates p<0.05 (two-tailed t-test).

Figure 3. β-bisabolene induces apoptosis in mammary cancer cell lines. (a) Flow cytometric analysis of MDA-MB-231 cells stained with Annexin-V and propidium iodide (PI). Representative dot-plots of MDA-MB-231 cells after treatment with increasing concentrations of β-bisabolene. (b) Graph showing the percentage of annexin-V^+ only or annexin-V^+ PI^- populations in MDA-MB-231 cells after treatment with β-bisabolene. Data represent average of triplicates (n=3) ± SEM. (c) Graph showing the levels of caspase 3/7 activity in EpH4 and 4T1 cells after treatment with β-bisabolene. Values were normalized against vehicle controls and are means of triplicated experiments (n=3) ± SEM. *, ** denotes statistical significance where p<0.05 and p<0.001 respectively (two-tailed t-test).

Figure 4. β-bisabolene treatment reduces the rate of tumour growth in vivo. (a) Growth curves of 4T1 tumours in Balb/C mice treated with β-bisabolene or vehicle control (n=4 for each cohort). Arrow indicates the starting point for treatment. * indicates statistical significance, p<0.05 (two tailed t-test). (b) Haematoxylin and eosin stained sections of 4T1 tumours from β-bisabolene or vehicle control treated mice demonstrating elevated numbers of pyknotic bodies in β-bisabolene treated tumours. (c) Immuno-histology for cleaved-caspase 3 staining in 4T1 tumours treated with vehicle control or β-bisabolene along with quantification. (d) Immuno-histology
for Ki67 staining in 4T1 tumours treated with vehicle control or β-bisabolene along with quantification.
For Peer Review

![Graph a](image1)

![Graph b](image2)

![Graph c](image3)

![Graph d](image4)

![Graph e](image5)
a) 

b) 

Caspase activity relative to vehicle control

Vehicle Control 100ug/ml Bisabolene 200ug/ml Bisabolene

Caspase activity relative to vehicle control

Eph4 4T1

Annexin-V

Annexin V only Annexin V and PI

% of total cell population

Vehicle Control 100ug/ml 200ug/ml

Vehicle Control 50 µg/ml 100 µg/ml 200 µg/ml
**Tumour volume, mm³**

- **B-Bisabolene (1.12g/kg)**
- **Vehicle Control**

**Days Post Transplant**

- 12
- 19
- 26
- 33
- 40

**% Ki-67 positive cells**

- **Vehicle Control**
- **B-bisabolene**

**% Cleaved caspase-3 cells**

- **Vehicle Control**
- **B-bisabolene**