

Online Research @ Cardiff

This is an Open Access document downloaded from ORCA, Cardiff University's institutional repository: <https://orca.cardiff.ac.uk/id/eprint/85106/>

This is the author's version of a work that was submitted to / accepted for publication.

Citation for final published version:

Yeo, Syn Kok, Ali, Ahmed Y., Hayward, Olivia A., Turnham, Daniel, Jackson, Troy, Bowen, Ifor D. and Clarkson, Richard ORCID: <https://orcid.org/0000-0001-7389-8673> 2016. Bisabolene, a sesquiterpene from the essential oil extract of opoponax (*Commiphora guidottii*), exhibits cytotoxicity in breast cancer cell lines. *Phytotherapy Research* 30 (3) , pp. 418-425.
10.1002/ptr.5543 file

Publishers page: <http://dx.doi.org/10.1002/ptr.5543>
<<http://dx.doi.org/10.1002/ptr.5543>>

Please note:

Changes made as a result of publishing processes such as copy-editing, formatting and page numbers may not be reflected in this version. For the definitive version of this publication, please refer to the published source. You are advised to consult the publisher's version if you wish to cite this paper.

This version is being made available in accordance with publisher policies.

See

<http://orca.cf.ac.uk/policies.html> for usage policies. Copyright and moral rights for publications made available in ORCA are retained by the copyright holders.





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

Journal:	<i>Phytotherapy Research</i>
Manuscript ID	PTR-15-0554.R1
Wiley - Manuscript type:	Full Paper
Date Submitted by the Author:	11-Oct-2015
Complete List of Authors:	Yeo, Syn; Cardiff University, School of Biosciences Ali, Ahmed; Cardiff University, School of Biosciences Hayward, Olivia; Cardiff University, School of Biosciences Turnham, Daniel; Cardiff University, School of Biosciences Jackson, Troy; Cardiff University, School of Biosciences Bowen, Ifor; Cardiff University, School of Biosciences Clarkson, Richard; Cardiff University, School of Biosciences
Keyword:	opoponax, bisabolene, apoptosis, breast cancer, <i>Commiphora guidotti</i> .

SCHOLARONE™
Manuscripts

1
2
3 **β -bisabolene, a sesquiterpene from the essential oil extract of Opoponax**
4
5 **(*Commiphora guidottii*) exhibits cytotoxicity in breast cancer cell lines**
6
7

8
9
10 Syn Kok Yeo¹, Ahmed Y Ali², Olivia A Hayward¹, Daniel Turnham¹, Troy Jackson²,
11
12 Ifor D Bowen² and Richard Clarkson^{1*}
13

14
15
16
17
18 *Corresponding author
19

20 Phone: +44(0)2920 870249
21

22 Fax: +44(0)2920 874116
23

24 Email: clarksonr@cardiff.ac.uk
25
26
27

28
29 ¹European Cancer Stem Cell Research Institute, School of Biosciences, Cardiff
30 University, Hadyn Ellis Building, Maindy Road, Cathays, Cardiff, CF24 4HQ
31
32

33
34
35
36 ²School of Biosciences, Cardiff University, Museum Avenue, Cardiff, CF10 3AX
37
38
39
40
41
42
43
44

45 Keywords: opoponax, scented myrrh, bisabolene, apoptosis, *Commiphora guidottii*,
46
47 breast cancer, sesquiterpene
48
49

50
51
52 Running title: Anti-tumour properties of β -bisabolene
53
54
55
56
57
58
59
60

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 μ g/ml, MG1361: 65.49 μ g/ml, 4T1: 48.99 μ g/ml) and human breast cancer cells (IC₅₀ in normal MCF10A: 114.3 μ g/ml, MCF7: 66.91 μ g/ml, MDA-MB-231: 98.39 μ g/ml, SKBR3: 70.62 μ g/ml and BT474: 74.3 μ g/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 *Commiphora guidottii* (also known as opoponax, scented myrrh, Sweet myrrh or habak haddi), some of its traditional applications involve treatment of wounds,

1
2
3 diarrhoea, stomach discomforts and removal of the placenta after childbirth (Thulin,
4 1999; Thulin & Claeson, 1991). The components of opoponax have also been
5 reported to exhibit pharmacological properties such as smooth muscle relaxing effects
6 (Andersson et al., 1997; Claeson et al., 1991) and anti-microbial properties (de
7 Rapper et al., 2012).

8
9
10
11
12
13
14 In this study, we set out to investigate the cytotoxic properties of opoponax
15 essential oil with regards to breast cancer. The constituents of opoponax essential oil
16 have been identified for decades (Craveiro et al., 1983; Ishwar & Levi, 1966) and it is
17 known to be rich in sesquiterpenes (Baser et al., 2003; Tian & Shi, 1996), naturally-
18 occurring molecules known to exert anticancer effects (Ahn et al., 2015; Han et al.,
19 2014; Martins et al., 2014; Pitchai et al., 2014). Among these is bisabolene, the main
20 constituent of opoponax of which α , β , and γ isomers constitute more than one third of
21 the essential oil. Noteworthy, the alcoholic analogue of the α -isomer of bisabolene,
22 the main component of *Matricaria chamomilla* essential oil, has selective cytotoxicity
23 against glioma cells (Cavalieri et al., 2004) and mammary tumours (Costarelli et al.,
24 2010); yet the anti-tumour properties of the other chemically related isoforms of
25 bisabolene have not been determined until recently. β -bisabolene was found to be the
26 major component of the essential oil of leaves from *Duguetia gardneriana* which
27 exhibited potent antitumor properties against melanoma, hepatocellular carcinoma
28 and leukemia cells (Rodrigues et al., 2015). However, the efficacy of β -bisabolene
29 against breast cancer cells and its potential as an anti-tumour agent *in vivo* has not
30 been investigated. Hence, following primary analysis of the constituents of opoponax,
31 the focus of this study was to assess the potential of β -bisabolene as a selective
32 therapeutic agent against breast cancers.
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

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 0.25 μ m 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

1
2
3 cells were cultured in L-15 media. All of the respective culture media were
4
5 supplemented with 10% FBS, 1% L-Glutamine and 20 units of Penicillin-
6
7 Streptomycin (Invitrogen, UK). For MG1361 cells, media was also supplemented
8
9 with non essential amino acids. MCF-10A cells were cultured in an equal mixture of
10
11 DMEM/F12 media supplemented with 10% horse serum, 1% L-Glutamine, 20 units
12
13 of Penicillin-Streptomycin, 10ug/ml insulin, 100ng/ml cholera toxin, 0.5ug/ml
14
15 hydrocortisone and 20ng/ml EGF. The cell lines used were between passages 5 to 40
16
17 and maintained at 37⁰C with 5% CO₂.
18
19

20 21 22 23 **Cell viability assay**

24
25 For viability assays, murine cells were plated at a density of 10,000 cells/well and
26
27 human cells were plated at 20,000 cells/well into a 96-well plate. Cells were treated
28
29 24 hours after seeding with respective agents prepared in media. The viability of cells
30
31 in each well was then quantified using Cell Titer Blue (Promega, UK) according to
32
33 manufacturer's instructions 24 hours after treatment.
34
35
36
37
38

39 40 **Apoptosis assays**

41
42 Cell lysates after specified treatments were analysed with Caspase Glo 3/7 assay kit
43
44 (Promega) according to manufacturer's instructions. Annexin-V/PI analysis was
45
46 performed using Dead Cell Apoptosis Kit (Invitrogen) according to manufacturer's
47
48 instructions and stained cells were analysed using a BD FACSCanto flow cytometer.
49
50

51 52 **In vivo administration of β -bisabolene**

53
54 1.12g/kg of the oily compound *β -bisabolene*, solubilised in corn oil, was administered
55
56 intra-peritoneally, two times a week, for two weeks, did not result in any signs of
57
58
59
60

1
2
3 distress in all animals. Upon necropsy, the histology of liver, kidney, spleen and lung
4
5 of these mice appeared normal (data not shown). When a dose of 2.24g/kg was
6
7 administered, mice showed signs of morbidity after single treatment. Hence, we used
8
9 a maximum non-lethal dose of 1.12g/kg β -bisabolene in our tumour growth studies.
10
11 The treatment regime consisted of intra-peritoneal (i.p.) injections twice weekly, once
12
13 palpable tumour were detected (at 2 weeks) and until mice have reached the endpoint
14
15 of the experiment or showed signs of morbidity.
16
17
18
19
20

21 **Orthotopic cell transplants and tumour measurements**

22
23 All animal experiments were conducted in accordance with the UK Animals
24
25 (Scientific Procedures) Act 1986, under Home Office licence 30/2849. 4T1 cells were
26
27 trypsinized and dissociated into single cell suspensions before transplantation into the
28
29 abdominal mammary fat pads of wild type BALB/C mice which were between 8-12
30
31 weeks old. Mice were then checked for palpable tumours and the resulting tumours
32
33 were measured using calipers three times weekly. At appropriate experimental
34
35 endpoints, tumour, mammary gland, lung and liver tissues were harvested. Tissue
36
37 samples were then fixed in 4% formaldehyde at 4⁰C overnight before processing for
38
39 histological analysis.
40
41
42
43
44

45 **Immunohistology**

46
47 Fixed tissues were embedded in paraffin, sectioned into 5 μ m slices, mounted onto
48
49 poly-L-lysine coated slides and stained with haematoxylin and eosin (H&E).
50
51 Antibodies for cleaved caspase-3 (Cell Signaling Technologies) and Ki-67
52
53 (VectorLabs) were used for immunohistology according to manufacturer's
54
55 instructions.
56
57
58
59
60

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.

For Peer Review

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-tumourigenic 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 (IC_{50} s of 48.99 μ g/ml and 65.49 μ g/ml), with complete loss of cell viability achieved at 100 μ g/ml (Figure 2c). At this concentration no loss in viability was observed in non-

1
2
3 tumourigenic EpH4 cells (IC_{50} of $>200 \mu\text{g/ml}$) (Figure 2c). β -bisabolene also induced
4
5 time dependent decreases in cell viability for 4T1 cells (Figure 2d). A significant
6
7 decrease in viability relative to vehicle control was observed only after 72 hours of
8
9 culture with β -bisabolene and not at earlier time points in this particular experiment.
10

11
12 Cytotoxicity was also confirmed in a panel of human breast cancer cell lines,
13
14 representing different ER and HER2 status. ER^+ and/or $HER2^+$ cells with luminal
15
16 characteristics (SKBR3, MCF-7 and BT474) were most sensitive to β -bisabolene
17
18 (IC_{50} s of $70.62\mu\text{g/ml}$, $66.91\mu\text{g/ml}$ and $74.30\mu\text{g/ml}$ respectively), while the basal-like
19
20 tumour cell line, MDA-MB-231, was significantly more resistant (IC_{50} of $98.39\mu\text{g/ml}$)
21
22 (Figure 2e). However these effects were limited to a narrow dose range in human cell
23
24 lines in culture, as the non-tumourigenic cell line, MCF-10A, were also susceptible to
25
26 β -bisabolene at marginally elevated concentrations (IC_{50} of $114.3\mu\text{g/ml}$).
27
28
29
30
31

32 **β -bisabolene induces apoptosis in mammary cancer cell lines**

33
34 In order to establish whether β -bisabolene induced an active programmed cell
35
36 death, we performed complementary assays for apoptosis following β -bisabolene
37
38 treatment of tumourigenic cell lines. Treatment of MDA-MB-231 cells with β -
39
40 bisabolene for 18 hours disrupted membrane polarity in a significant proportion of
41
42 cells (Figure 3a). 22% ($\pm 5.9\%$) and 66% ($\pm 1.8\%$) of the cell population bound
43
44 annexin V following treatment with $100\mu\text{g/ml}$ and $200\mu\text{g/ml}$ β -bisabolene
45
46 respectively (Figure 3b), indicating a dose-responsive increase in apoptosis. No
47
48 difference in propidium iodide staining was observed over the same timecourse,
49
50 indicating that loss of membrane integrity, a sign of necrotic cell death, was not
51
52 induced by treatment (Figure 3b). A comparative analysis of relative caspase activity
53
54 in tumourigenic versus non-tumourigenic cells was also performed. Here, caspase 3/7
55
56
57
58
59
60

1
2
3 activity was found to be induced by β -bisabolene only in the tumourigenic cell line,
4
5 but not in non-tumourigenic cells (Figure 3c). Thus β -bisabolene specifically induces
6
7 apoptosis in tumourigenic mammary cell lines.
8
9

10 11 **β -bisabolene treatment reduces the rate of tumour growth in vivo**

12
13 We next investigated the effect of β -bisabolene on the growth of tumours *in*
14
15 *vivo*. An earlier study had shown that tumour initiation was inhibited by α -bisabolol
16
17 but it was unclear whether growth of existing tumours could be inhibited by
18
19 bisabolene (Costarelli et al., 2010). Here we tested the effects of β -bisabolene on
20
21 mice bearing pre-established mammary tumours.
22
23

24
25 β -bisabolene was administered by intraperitoneal injection into female Balb/C
26
27 mice bearing a single orthotopic tumour of transplanted 4T1 cells after two weeks
28
29 post-transplant. A tolerated dose of twice weekly injections (1.12g/Kg) resulted in a
30
31 significant decrease (37.5% decrease in volume by endpoints) in the growth rate of
32
33 tumours in β -bisabolene treated mice relative to vehicle controls (Figure 4a). Dosing
34
35 at 100mg/Kg three-times weekly had no significant effect on tumour growth (data not
36
37 shown). Histology of the tumours from the effective treatment group revealed a
38
39 marked increase in cell death within the β -bisabolene treated tumours (Figure 4b). β -
40
41 bisabolene treatment induced a significant increase in cleaved caspase-3 positive cells
42
43 (4.6% versus 1.2% in vehicle controls) indicating that the treatment induces apoptotic
44
45 cell death in 4T1 tumors. (Figure 4c). On the other hand, the number of proliferating
46
47 cells as indicated by Ki-67 staining were reduced (2.6% versus 10.9% in controls) in
48
49 β -bisabolene treated tumors (Figure 4d). Accompanying histology of vital organs and
50
51 tissues within these animals revealed no associated increases in cytotoxicity (data not
52
53 shown).
54
55
56
57
58
59
60

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 sesquiterpine, 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

1
2
3 less effective in inducing cytotoxicity *in vitro* relative to the essential oil from
4
5 *Duguetia gardneriana* as a whole. This would suggest that the other components of
6
7 *Duguetia gardneriana* essential oil have a larger contribution than β -bisabolene
8
9 towards the cytotoxic effects observed. For that reason, they only tested the anti-
10
11 tumour effects of *Duguetia gardneriana* essential oil but not β -bisabolene *in vivo*.
12
13 Based on the selective cytotoxicity against breast cancer cells by β -bisabolene that we
14
15 observed *in vitro*, we went on to show for the first time that it possesses anti-tumour
16
17 properties *in vivo*.
18
19

20
21 A recent report describing the pro-apoptotic effects of a closely related isomer,
22
23 γ -bisabolene, in oral squamous cell carcinoma cells suggests that bisabolene isomers
24
25 may share certain chemical features which contribute to their anti-tumour functions
26
27 (Jou et al., 2015). Accordingly, the selective induction of apoptosis in cancer cells that
28
29 we observed were also demonstrated by γ -bisabolene against oral squamous
30
31 carcinoma cells but not normal oral fibroblasts. Mechanistically, γ -bisabolene was
32
33 shown to activate p53 mediated apoptosis through PP1/HDAC2 and ERK signaling
34
35 (Jou et al., 2015). If indeed the bisabolene isomers share a similar mechanism of
36
37 action, the specificity towards cancer cells that we observe may be due to differential
38
39 HDAC2 activity in breast cancer cells. It is possible that the higher levels of HDAC2
40
41 observed in breast cancers (Müller et al., 2013; Roy et al., 2014; Seo et al., 2014) play
42
43 an essential role in mitigating the pro-apoptotic effects of p53 (Harms & Chen, 2007),
44
45 making breast cancer cells more susceptible to inhibition of HDAC2 by bisabolene. It
46
47 will be interesting to address this and compare the efficacies of all bisabolene isomers
48
49 to identify that which is most efficient at inducing apoptosis.
50
51
52
53

54
55 We have shown that tumour cells can be successfully targeted *in vivo* by
56
57 intraperitoneal injection of β -bisabolene. This is a product of the specificity of β -
58
59
60

1
2
3 bisabolene's cytotoxicity in the mouse model. Although we only observed an effect
4
5 after more than 3 weeks of treatment *in vivo* (Figure 4a), this is consistent with the
6
7 fact that we see a time-dependent response for *in vitro* cytotoxicity assays (Figure 2d).
8
9 The dose applied in our *in vivo* studies (1.12 g/kg) were about ten times less than the
10
11 reported oral LD₅₀ in mice (>13.36g/kg) (Hoffman-LaRoche, 1967b). The oral LD₅₀
12
13 in rats have also been reported to be >5g/kg (Moreno, 1974), indicating that the doses
14
15 we used are within the sub-toxic range. Nonetheless, it may be possible to alter the
16
17 dosing regimen such that the concentration of bisabolene administered is reduced but
18
19 compensated by a higher frequency of doses. Efficacious treatment was achieved with
20
21 twice weekly doses of 1.12g/Kg in our studies. While a regime of three weekly doses
22
23 at 100mg/Kg exhibited no effect on tumour growth (data not shown), it is conceivable
24
25 that a daily dosing regimen could be both well tolerated and effective at lower doses.
26
27 Additionally, before β -bisabolene is used in a clinical setting, it would be of great
28
29 benefit if its solubility properties in aqueous matrices were significantly enhanced
30
31 through a formulation development exercise. These optimizations could improve the
32
33 potential clinical benefits of β -bisabolene as a therapeutic in breast cancers.
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Acknowledgement

We would like to thank the Cardiff University animal facility for their assistance and also Derek Scarborough for help with histology. This work was funded by a Breast Cancer Campaign PhD studentship grant (2007NovPhD06) and the Compton Group, UK.

Conflict of Interest

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

References

- 1
2
3
4
5 Ahn, J.H., Lee, T.W., Kim, K.H., Byun, H., Ryu, B., Lee, K.T., Jang, D.S., Choi, J.H. 2015.
6 6-Acetoxy Cyperene, a Patchoulane-type Sesquiterpene Isolated from
7 *Cyperus rotundus* Rhizomes Induces Caspase-dependent Apoptosis in
8 Human Ovarian Cancer Cells. *Phytother Res*.
9
10 Al-Harbi, M., Qureshi, S., Raza, M., Ahmed, M., Giangreco, A., Shah, A. 1994.
11 Anticarcinogenic effect of Commiphora molmol on solid tumors induced
12 by Ehrlich carcinoma cells in mice. *Chemotherapy*, **40**(5), 337-347.
13
14 Andersson, M., Bergendorff, O., Shan, R., Zygmunt, P., Sterner, O. 1997. Minor
15 components with smooth muscle relaxing properties from scented myrrh
16 (*Commiphora guidotti*). *Planta Med*, **63**(3), 251-4.
17
18 Baser, K., Demirci, B., Debeko, A., Dagne, E. 2003. Essential oils of some *Boswellia*
19 spp., myrrh and opoponax. *Flavour and fragrance journal*, **18**, 153-156.
20
21 Brooks, M.D., Burness, M.L., Wicha, M.S. 2015. Therapeutic Implications of
22 Cellular Heterogeneity and Plasticity in Breast Cancer. *Cell Stem Cell*,
23 **17**(3), 260-71.
24
25 Cavalieri, E., Mariotto, S., Fabrizi, C., Carcereri de Prati, A., Gottardo, R., Leone, S.,
26 Berra, V., Lauro, G., Ciampa, A., Suzuki, H. 2004. α -Bisabolol, a nontoxic
27 natural compound, strongly induces apoptosis in glioma cells. *Biochemical*
28 *and Biophysical Research Communications*, **315**, 589-594.
29
30 Claeson, P., Andersson, R., Samuelsson, G. 1991. T-cadinol: a pharmacologically
31 active constituent of scented myrrh: introductory pharmacological
32 characterization and high field ^1H - and ^{13}C -NMR data. *Planta Med*, **57**(4),
33 352-6.
34
35 Costarelli, L., Malavolta, M., Giacconi, R., Cipriano, C., Gasparini, C., Tesei, S.,
36 Pierpaoli, S., Orlando, F., Suzuki, H., Perbellini, L., Piacenza, F., Emanuelli,
37 M., Mocchegiani, E. 2010. In vivo effect of alpha-bisabolol, a nontoxic
38 sesquiterpene alcohol, on the induction of spontaneous mammary tumors
39 in HER-2/neu transgenic mice. *Oncol Res.*, **18**(9), 409-418.
40
41 Craveiro, A., Corsano, S., Proietti, G., Strappaghetti, G. 1983. Constituents of
42 essential oil of *Commiphora guidotti*. *Planta Med*, **48**(2), 97-8.
43
44 Da Rocha, A., Lopes, R., Schwartzmann, G. 2001. Natural products in anticancer
45 therapy. *Current Opinion in Pharmacology*, **1**, 364-369.
46
47 de Rapper, S., Van Vuuren, S.F., Kamatou, G.P., Viljoen, A.M., Dagne, E. 2012. The
48 additive and synergistic antimicrobial effects of select frankincense and
49 myrrh oils--a combination from the pharaonic pharmacopoeia. *Lett Appl*
50 *Microbiol*, **54**(4), 352-8.
51
52 Han, J., Bae, S.Y., Oh, S.J., Lee, J., Lee, J.H., Lee, H.C., Lee, S.K., Kil, W.H., Kim, S.W.,
53 Nam, S.J., Kim, S., Lee, J.E. 2014. Zerumbone suppresses IL-1 β -induced cell
54 migration and invasion by inhibiting IL-8 and MMP-3 expression in
55 human triple-negative breast cancer cells. *Phytother Res*, **28**(11), 1654-
56 60.
57
58 Harms, K.L., Chen, X. 2007. Histone deacetylase 2 modulates p53 transcriptional
59 activities through regulation of p53-DNA binding activity. *Cancer Res*,
60 **67**(7), 3145-52.
61
62 Hoffman-LaRoche, I. 1967b. Acute toxicity, eye and skin irritation tests on
63 aromatic compounds.

- Ishwar, C., Levi, L. 1966. Essential oils and their constituents : XXXII. Gas chromatography of sesquiterpene hydrocarbons. *Journal of Chromatography A*, **23**, 217-226.
- Jou, Y.J., Chen, C.J., Liu, Y.C., Der Way, T., Lai, C.H., Hua, C.H., Wang, C.Y., Huang, S.H., Kao, J.Y., Lin, C.W. 2015. Quantitative phosphoproteomic analysis reveals γ -bisabolene inducing p53-mediated apoptosis of human oral squamous cell carcinoma via HDAC2 inhibition and ERK1/2 activation. *Proteomics*.
- Liu, W., Furuta, E., Shindo, K., Watabe, M., Xing, F., Pandey, P.R., Okuda, H., Pai, S.K., Murphy, L.L., Cao, D., Mo, Y.Y., Kobayashi, A., Iizumi, M., Fukuda, K., Xia, B., Watabe, K. 2011. Cacalol, a natural sesquiterpene, induces apoptosis in breast cancer cells by modulating Akt-SREBP-FAS signaling pathway. *Breast Cancer Res Treat*, **128**(1), 57-68.
- Martins, A., Mignon, R., Bastos, M., Batista, D., Neng, N.R., Nogueira, J.M., Vizetto-Duarte, C., Custódio, L., Varela, J., Rauter, A.P. 2014. In vitro antitumoral activity of compounds isolated from *Artemisia gorgonum* Webb. *Phytother Res*, **28**(9), 1329-34.
- Mazzio, E., Soliman, K. 2009. In vitro screening for the tumoricidal properties of international medicinal herbs. *Phytother Res*, **23**(3), 385-98.
- Moreno, O. 1974. Report to RIFM.
- Müller, B.M., Jana, L., Kasajima, A., Lehmann, A., Prinzler, J., Budczies, J., Winzer, K.J., Dietel, M., Weichert, W., Denkert, C. 2013. Differential expression of histone deacetylases HDAC1, 2 and 3 in human breast cancer--overexpression of HDAC2 and HDAC3 is associated with clinicopathological indicators of disease progression. *BMC Cancer*, **13**, 215.
- Pitchai, D., Roy, A., Banu, S. 2014. In vitro and in silico evaluation of NF- κ B targeted costunolide action on estrogen receptor-negative breast cancer cells--a comparison with normal breast cells. *Phytother Res*, **28**(10), 1499-505.
- Rodrigues, A.C., Bomfim, L.M., Neves, S.P., Menezes, L.R., Dias, R.B., Soares, M.B., Prata, A.P., Rocha, C.A., Costa, E.V., Bezerra, D.P. 2015. Antitumor Properties of the Essential Oil From the Leaves of *Duguetia gardneriana*. *Planta Med*, **81**(10), 798-803.
- Roy, S.S., Gonugunta, V.K., Bandyopadhyay, A., Rao, M.K., Goodall, G.J., Sun, L.Z., Tekmal, R.R., Vadlamudi, R.K. 2014. Significance of PELP1/HDAC2/miR-200 regulatory network in EMT and metastasis of breast cancer. *Oncogene*, **33**(28), 3707-16.
- Seo, J., Min, S.K., Park, H.R., Kim, D.H., Kwon, M.J., Kim, L.S., Ju, Y.S. 2014. Expression of Histone Deacetylases HDAC1, HDAC2, HDAC3, and HDAC6 in Invasive Ductal Carcinomas of the Breast. *J Breast Cancer*, **17**(4), 323-31.
- Tabassum, D.P., Polyak, K. 2015. Tumorigenesis: it takes a village. *Nat Rev Cancer*, **15**(8), 473-83.
- Thulin, M. 1999. *Flora of Somalia* Royal botanic gardens, Kent.
- Thulin, M., Claeson, P. 1991. The botanical origin of scented myrrh (bissabol or habak hadi). *Economic Botany*, **45**, 487-494.
- Tian, J., Shi, S. 1996. Studies on the constituents of essential oil of imported Myrrh and gum opoponax. *Zhongguo Zhongyao Zazhi*, **21**, 235-237.

- 1
2
3 Wang, G., Li, X., Huang, F., Zhao, J., Ding, H., Cunningham, C., Coad, J., Flynn, D.,
4 Reed, E., Li, Q. 2005. Antitumor effect of beta-elemene in non-small-cell
5 lung cancer cells is mediated via induction of cell cycle arrest and
6 apoptotic cell death. *Cell Mol Life Sci*, **62**(7-8), 881-893.
7
8 Wani, M., Taylor, H., Wall, M., Coggon, P., McPhail, A. 1971. Plant antitumor
9 agents. VI. The isolation and structure of taxol, a novel antileukemic and
10 antitumor agent from *Taxus brevifolia*. *J Am Chem Soc*, **93**, 2325-2327.
11 Zhang, M., Tsimelzon, A., Chang, C.H., Fan, C., Wolff, A., Perou, C.M., Hilsenbeck,
12 S.G., Rosen, J.M. 2015. Intratumoral heterogeneity in a Trp53-null mouse
13 model of human breast cancer. *Cancer Discov*, **5**(5), 520-33.
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

For Peer Review

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 50ug/ml for 24 hours. (b) Representative images of EpH4 and 4T1 cell after vehicle, α -bisabolol or β -bisabolene treatment at a concentration of 50ug/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 -

1
2
3 bisabolene for 24, 48 or 72 hours. (e) Dose response curves for MCF-10A, MCF-7,
4
5 SKBR3, BT474 and MDA-MB-231 cell lines treated with varying concentrations of
6
7 β -bisabolene. Data points represent triplicates from two independent experiments
8
9 (n=6) \pm SEM. **indicates $p < 0.01$, * indicates $p < 0.05$ (two-tailed t-test).
10
11

12
13
14
15 **Figure 3. β -bisabolene induces apoptosis in mammary cancer cell lines.** (a) Flow
16
17 cytometric analysis of MDA-MB-231 cells stained with Annexin-V and propidium
18
19 iodide (PI). Representative dot-plots of MDA-MB-231 cells after treatment with
20
21 increasing concentrations of β -bisabolene. (b) Graph showing the percentage of
22
23 annexin-V⁺ only or annexin-V⁺ PI⁺ populations in MDA-MB-231 cells after treatment
24
25 with β -bisabolene. Data represent average of triplicates (n=3) \pm SEM. (c) Graph
26
27 showing the levels of caspase 3/7 activity in EpH4 and 4T1 cells after treatment with
28
29 β -bisabolene. Values were normalized against vehicle controls and are means of
30
31 triplicated experiments (n=3) \pm SEM. *, *** denotes statistical significance where
32
33 $p < 0.05$ and $p < 0.001$ respectively (two-tailed t-test).
34
35
36
37
38
39
40
41

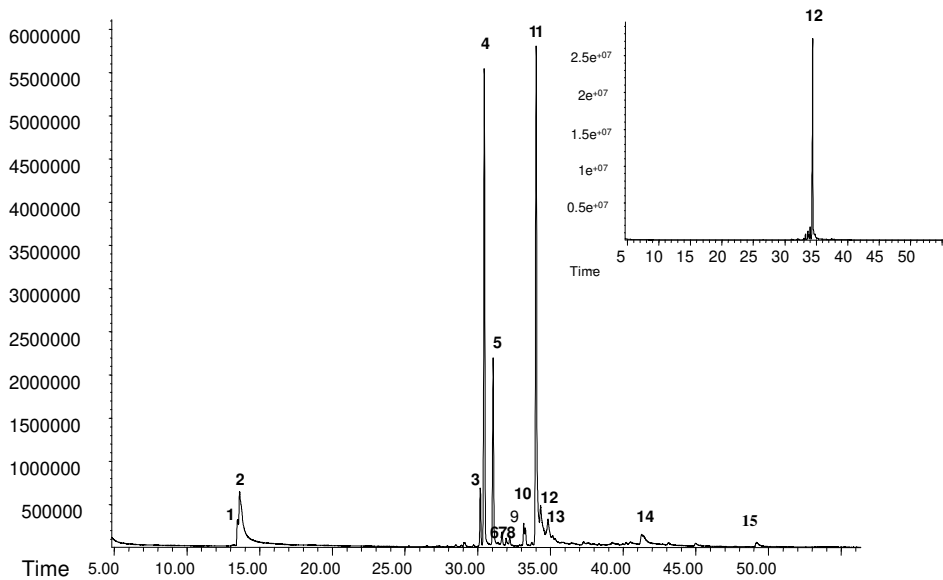
42 **Figure 4. β -bisabolene treatment reduces the rate of tumour growth *in vivo*.** (a)
43
44 Growth curves of 4T1 tumours in Balb/C mice treated with β -bisabolene or vehicle
45
46 control (n=4 for each cohort). Arrow indicates the starting point for treatment.
47
48 *indicates statistical significance, $p < 0.05$ (two tailed t-test). (b) Haematoxylin and
49
50 eosin stained sections of 4T1 tumours from β -bisabolene or vehicle control treated
51
52 mice demonstrating elevated numbers of pyknotic bodies in β -bisabolene treated
53
54 tumours. (c) Immuno-histology for cleaved-caspase 3 staining in 4T1 tumours treated
55
56 with vehicle control or β -bisabolene along with quantification. (d) Immuno-histology
57
58
59
60

1
2
3 for Ki67 staining in 4T1 tumours treated with vehicle control or β -bisabolene along
4
5 with quantification.
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

For Peer Review

a

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20



b

21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58

