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First Reporting on the Chemistry and Biological Activity of a Novel Boswellia chemotype: The Methoxy Alkane Frankincense

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Keywords: *decyl methyl ether, frankincense, cytotoxicity, antimicrobial, enantiomeric distribution, methoxy alkane.*

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FIRSTREPORTINGONTHECHEMISTRYANDBIOLOGICALACTIVITYOFANOVELBOSWELLIACHEMOTYPETHEMETHOXYALKANEFRANKINCENSE

Strictly as per the compliance and regulations of :

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Prabodh Satyal [α] & Robert S. Pappas [σ]

Abstract- Two oleogum resin essential oils (from two different seasons: fall and summer), of *Boswellia spp.* (collected from Somalia), were obtained by hydrodistillation and analyzed by GC-MS. Out of 147 peaks, components were identified among the two essential oils accounting for 100%, and 99.7% of the oils, respectively. The two essential oils were dominated by the 1-methoxy alkane [octyl methyl ether (5.5-11.7%), and decyl methyl ether (30.6-54.9%)], α-pinene (0.3-11.5%), sabinene (2.1-7.2%), and α-bourbonene (1.7-5.7%). This is the first report of a methoxy alkane chemotyped frankincense essential oils as well as the first reporting of the natural occurance of decyl methyl ether (methyoxy decane) and octyl methyl ether (methyoxy octane). Monoterpenes chiral distributions were also measured and it was found that both of the oils have same enantiomeric ratio. Large chemical variation was attributed to seasonal variation. The essential oil harvested on fall season had also exhibited notable antimicrobial activities [*Aspergillus niger* (MIC = 39 µg/mL), *Candida albicans* (MIC = 78 µg/mL), *Bacillus cereus* (MIC = 78 µg/mL), *Staphylococus aureus* (MIC = 78 µg/mL), and *Escherichia coli* (MIC = 78 µg/mL)], the essential oil also showed pronounced cytotoxic activities (100% kill on MCF-7 cells at 100 µg/mL).

Keywords: decyl methyl ether, frankincense, cytotoxicity, antimicrobial, enantiomeric distribution, methoxy alkane.

I. Introduction

Boswellia spp (also known as frankincense or Olibanum) is one of the most popular essential oils in aromatherapy from the Bursearaceae family. word frankincense is derived from an ancient French word which means "Franc = pure + enens=incense" and is mentioned frequently in many sacred text including the Bible. Frankincense essential oil is obtained from a variety different species of the genus *Boswellia*. The most prevalent species in today's world market are *B. carterii (Somaliland), B. serrata (India), B. sacra (Oman), B. frereana (Somaliland)* and *B. papyrifera (Ethiopia).* The bark of the *Boswellia* tree is filled with oleo-gum-resin reservoirs. When the reservoirs are penetrated, a milky juice is excreted onto the bark. When the milky juice becomes exposed to air it begins to harden producing the oleogum resin. [Tucker, 1986]. The resin typically contains between 5 and 15% essential oil [Mertens, et al., 2009]. Frankincense oil is used medicinally in many cultures still today. For example, frankincense essential oil has been claimed to contain chemical components present that aid in the removal of scars and stretch marks, in addition to having antibacterial and antifungal properties [Mikhaeil, et al.,2003]. The oil has also been studied in reference to pharmaceutical properties as well as clinical trials [Vuuren, et al., 2010].

Frankincense resin is traditionally used in treatment of inflammation, wound healing, skin diseases, urinary tract infections, etc. Its application in medicines and cosmetics product formulation are increasing daily.

For a number of years there has been considerable controversy [Paul, et al., 2011, Woolley, et al., 2012] in identifying the correct botanical name of certain frankincense species. Paul et al have proposed a simple TLC method for identification of three olibanum resins on the basis of biomarker compounds. According to their findings, the presence of incensole and incensyl acetate confirms the presence of *Boswellia papyrifera*, caryophyllene oxide confirms *Boswellia carterii* and/or *Boswellia sacra*, *Boswellia serrata* has neither incensole acetate nor caryophyllene oxide, but has a remarkable amount of serratol present in addition to trace amount of incensole. It has been claimed for some time that *B. sacra* is actually the same as *B. carterii* [Thulin and Warfa, 1987], but the enantiomeric studies by Woolley et al [Wooley, et al., 2012] as well as the finding of this laboratory give strong evidence that they are indeed different species.

The current study was conducted to report the unique chemical composition (first time presence of alkyl methyl ether as the natural volatile component in an essential oil as well as first reporting of its presence in nature in general), enantiomeric distribution, and biological activities of the oleogum resin essential oil of *Boswellia spp* from Somalia.

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Picture 1 : Methoxy alkane chemotyped *Boswellia* resin in collected in Northern Somaliland

II. Essential Oils Composition

The Frankincense oil was obtained in 1-1.5% yields, a notably lower oil yield than the typical resin from Somaliland. A total of 147 compounds were identified, accounting for about 100% of compound identification (see Table 1). The essential oils contained α-pinene (0.3-11.6%), sabinene (2.1-7.2%), octyl methyl ether (5.5-11.7%), decyl methyl ether (30.6-54.9%), and α-bourbonene (1-5.7%). These results are qualitatively different than any result previously published in a significant review paper [Mertens, et al., 2009; Niebler & Buttner, 2016; Niebler et al., 2016]. On analyzing twenty commercial essential oil samples (mostly carterii, neglecta, sacra, thurifera, frereana) from South Africa, revealed those oils were composed of α-pinene (2.0-64.7%), α-thujene (0.3-52.4%), β-pinene (0.3-13.1%), myrcene (1.1-22.4%), sabinene (0.5-7.0%), limonene (1.3-20.4%), p-cymene (2.7-16.9%), and β-caryophyllene (0.1-10.5%) [Vuuren et al. 2010]. None of the samples exhibited the presence of methoxy alkane (octyl methyl ether, decyl methyl ether) even in trace quantities. Frankincense the North east region of Somalia has also been studied and according to this report α-pinene (10.3-37.7%), α-phellandrene (12.2-41.8%), limonene (6.4-19.6%) were the major components [Vuuren, et al., 2010]. Presence of 9.84% decyl methyl ether (Table-4) in dichloromethane extract indicated natural occurrence of decyl methyl ether in *Boswellia* spp.

It has been shown that enantiomeric distribution is important in properly identifying frankincense species [Woolley et al., 2012], so on our enantiomeric studies of two resin samples harvested at different times show similar enantiomeric distribution from each other as shown in table 3.

In addition to genetic variation, other factors such as age, vegetative cycle stage, climate, season, soil composition, etc. are among several things responsible for the considerable variation in essential oil compositions [Satyal, et al., 2012]. Based on the observed chemical composition, this variety of Somaliland frankincense may be treated as a distinct and novel chemotype.

III. Cytotoxicity, Antimicrobial Activity

Frankincense oil showed notable biological activity on all of the tested microorganisms (Table 3): *Bacillus cereus* (MIC = 78 μg/mL), *Staphylococcus aureus* (MIC = 78 μg/mL), *Escherichia coli* (MIC = 78 μg/mL) and *Aspergillus niger* (MIC = 39 μg/mL). Several previous studies have reported antibacterial and antifungal [Vuuren et al 2010] activities for *Frankincense* essential oils, consistent with our results. α–pinene and

decyl methyl ether, especially in synergy with other essential oil components, are likely responsible for the antimicrobial activities as shown in the table 3.

Frankincense essential oil demonstrated notable *in-vitro* cytotoxic activity against MCF-7 breast tumor cells (100% kill at 100 µg/mL). Note that decyl methyl ether, α-pinene, and β-pinene, are not appreciably cytotoxic, either alone (Table 3).

Table 1 : Chemical Composition of methoxy alkane chemotyped Frankincense EO from Somaliland

RI[1]	Compounds	%[2a] Fall	%[2b] Summer
821	Hexyl methyl ether	TR	ND
863	n-Hexanol	TR	ND
881	2-Butyl furan	TR	ND
900	Nonane	TR	ND
919	Hasheshene	0.10	ND
921	Tricyclene	0.03	ND
924	α-Thujene	2.01	0.38
932	α-Pinene	11.55	0.34
941	Thujadiene	0.05	ND
948	Camphene	0.37	ND
951	Thuja-2,4(10)-diene	0.21	ND
953	α-Fenchene	0.02	ND
971	Sabinene	7.20	2.1
976	β-Pinene	0.56	0.04
982	Hept-5-en-2-one <6-methyl->	0.02	ND
988	Myrcene	0.26	0.04
1003	Octanal	ND	0.02
1004	Pseudolimonene	0.10	ND
1006	α-Phellandrene	0.87	0.06
1008	3-Carene	0.19	ND
1011	Hexyl acetate	ND	0.01
1014	1,4-Cineole	0.02	0.01
1016	α-Terpinene	0.49	0.17
1023	*p*-Cymene	1.58	0.61
1025	Octyl methyl ether	5.53	11.72
1028	Limonene	1.45	0.13
1030	β-Phellandrene	1.16	ND
1031	1,8-cineole	0.11	ND
1034	(*Z*)-β-Ocimene	0.13	0.04
1036	2,5-dimethylcyclohexanol A	0.05	0.03
1042	2,5-Dimethylcyclohexanol B	ND	0.06
1045	(*E*)-β-Ocimene	0.13	ND
1057	γ-Terpinene	0.88	0.31
1066	*o*-Cymenene	TR	0.04
1069	Octanol	0.09	0.39
1075	2-Decanol methyl ether	0.21	0.41
1080	Unidentified	ND	0.07
1084	Terpinolene	0.19	0.05
1089	*p*-Cymenene	0.10	0.03

RI[1]	Compounds	%[2a] Fall	%[2b] Summer
1099	Linalool	0.19	0.09
1101	2-Nonanol	TR	0.04
1112	4,8 Dimetyl-nona-1,3,7-triene	0.13	0.46
1117	β-Thujone	TR	ND
1118	3-Octyl acetate	TR	0.01
1121	*cis*-Non-3-en-1-ol, methyl ether	TR	ND
1123	*cis*-*p*-Menth-2-en-1-ol	0.06	ND
1126	Methyl nonyl ether	1.37	2.21
1128	Hexyl propanoate	0.11	0.07
1138	*trans*-Sabinol	TR	ND
1139	*trans*-Pinocarveol	0.22	ND
1140	*cis*-Verbenol	0.12	ND
1142	Camphene hydrate	ND	0.02
1144	*trans*-Verbenol	0.27	ND
1149	α-Felandren-8-ol	0.13	ND
1156	Sabina ketone	TR	ND
1158	β-Pinene oxide	TR	ND
1159	*trans*-Pinocamphone	TR	ND
1168	α-Phellandrene epoxide	TR	ND
1170	*p*-Mentha-1,5-dien-8-ol	0.32	ND
1171	n-Nonanol	ND	0.05
1179	Terpinen-4-ol	1.27	0.47
1185	*p*-Cymen-8-ol	0.07	0.02
1191	Hexyl butanoate	0.17	0.14
1194	α-Terpineol	0.24	0.02
1206	*cis*-Decen-9-ol methyl ether	0.34	0.34
1207	*trans*-Piperitol	TR	ND
1208	(2*E*,4*E*)-Methyl dodeca-2,4-dienoate	ND	0.03
1209	Octyl acetate	0.15	0.58
1214	*trans*-Decen-9-ol, methyl ether	0.26	0.4
1218	*cis*-Decen-1-ol, methyl ether	0.37	0.74
1222	trans-Decen-1-ol, methyl ether	ND	0.06
1227	Decyl methyl ether	30.65	54.91
1242	*cis*-Dec-2-en-1-ol, methyl ether	0.72	1.17
1248	Linalyl acetate	TR	ND
1252	Piperitone	ND	0.02
1262	*trans*-Dec-2-enal	ND	0.02
1271	Decanol	0.63	2.1
1283	Bornyl acetate	0.27	ND
1319	*cis*-Undecen-1-ol, methyl ether	0.11	0.09
1322	Methyl decanoate	0.00	0.03
1326	Undecyl methyl ether	0.20	0.29
1345	α-Cubebene	0.59	0.32
1367	α-Ylangene	0.37	0.28
1374	α-Copaene	1.68	1.74

RI[1]	Compounds	%[2a] Fall	%[2b] Summer
1382	α-Bourbonene	5.72	1.69
1385	Hexyl hexanoate	0.89	0.21
1387	β-Elemene	1.12	0.24
1390	Sativene	0.07	0.05
1393	1,5-di-epi-α-Bourbonene	0.13	ND
1402	*cis*-Caryophyllene	0.07	0.02
1405	α-Gurjunene	0.20	0.11
1408	Decyl acetate	0.09	1.18
1416	β-Ylangene	0.43	0.12
1417	*trans*-Caryophyllene	0.83	0.72
1428	β-Copaene	0.75	0.22
1442	α-Aromadendrene	0.29	0.07
1447	*cis-Muurola-3,5-diene*	0.13	0.13
1453	α-Humulene	0.32	0.18
1458	Alloaromadendrene	0.20	0.17
1466	β-Aromadendrene	0.15	0.08
1470	*cis*-Cadina-1(6),4-diene	0.11	0.07
1473	*trans*-Cadina-1(6),4-diene	0.73	0.64
1476	*cis*-4,10-epoxy-Amorphane	TR	0.03
1479	Germacrene D	1.52	1.23
1483	Heptylhexanoate	ND	0.02
1487	β-Selinene	0.48	0.07
1489	*trans*-Muurola-4(14),5-diene	0.24	0.19
1493	α-Selinene	0.41	0.23
1496	α-Muurolene	0.31	0.21
1500	γ-Amorphene	0.06	0.03
1503	β-Dihydroagarofuran	0.04	0.03
1511	δ-Amorphene	0.20	0.16
1513	Cubebol	0.30	0.45
1516	δ-Cadinene	1.09	0.82
1520	Tridecyl methyl ether	0.38	0.5
1522	Methyl dodecanoate	TR	ND
1527	Kessane	0.06	0.07
1530	*trans*-Cadine-1,4-diene	0.06	0.05
1534	Isokessane	0.07	0.07
1539	Italicene ether	ND	0.02
1539	α-Calcorene	0.02	0.02
1546	α-Elemol	0.20	0.22
1557	Dodecanoic acid	ND	0.06
1559	*cis*-Muurol-5-en-4-α-ol+*trans*-Nerolidol	0.69	0.88
1565	Zierone	ND	0.02
1568	Caryophyllenol	0.05	0.11
1570	*trans*-Tridec-2-en-1-al	0.06	0.11
1576	Germacrene-D-4-ol	ND	0.02
1579	Caryophyllene oxide	TR	ND

RI[1]	Compounds	%[2a] Fall	%[2b] Summer
1592	Viridiflorol	0.11	ND
1602	Ledol	TR	0.03
1606	1,10 di-*epi*-Cubenol	0.24	0.16
1611	8-*epi*-γ-Eudesmol	1.10	1.38
1625	1-*epi*-Cubenol	TR	0.03
1641	Cubenol	0.11	0.03
1641	epi-α-Cadinol	ND	0.01
1644	δ-Cadinol	TR	0.03
1652	Unidentified Sesquiterpineol	ND	0.26
1653	α-Eudesmol	0.20	0.15
1673	Bulnesol	TR	0.06
1696	Tridec-4-en-1-yl acetate	ND	0.02
1791	α-Phellandrene dimer	0.05	ND
1950	*3E*-Cembrene A	0.49	0.31
1995	α-Pinacene	0.09	0.06
2007	Verticilla-4(20),7,11-triene	0.33	0.14
2131	Neocembrene A	0.14	0.12
2142	Incensole	0.79	0.43
2144	Serratol	0.41	0.55
	Total identified	100%	99.74%

Note: Where RI[1] Retention Index determined to a series of n-Alkanes on DB-5 column; compounds are listed in order of elution (Increasing RI), %[2a] and %[2b] refers to Percent of total oil collected in fall season and percent of total oil collected in summer season respectively. "TR" indicates trace components (<0.01%), and "ND" indicates non detected compounds on the provided conditions.

Table 2 : Enantiomeric Distribution of Frankincense EO collected in fall and summer season from Somalia

Monoterepene Components	Fall	Summer
(1R)+-α-thujene: (1S)-(-)-α-thujene	43 to 57	36 to 64
(1R,5R)-(+)-α-pinene: (1S,5S)-(–)-α-pinene	83 to 17	76 to 34
(4R)-(+)-limonene :(4S)-(-)-limonene	73 to 27	65 to 35
(4S-(+)-terpinen-4-ol : (4R)-(-)-terpinen-4-ol	81 to 19	71 to 29
(4S)-(+)-sabinene :(4R)-(-)-sabinene	93 to 7	98 to 2

Table 3 : Biological activities of *Frankincense* essential oils and major essential oil components

Bioassay	EO	Methoxydecane	α-Pinene	β-Pinene
MCF-7 cytotoxicity (% kill at 100 µg/mL)	100±1.6	80±10.6	16.8±2.6	30.4±7.7
Antimicrobial (MIC, µg/mL)				
Bacillus cereus	78	78	1250	1250
Staphylococcus aureus	78	313	1250	1250
Escherichia coli	78	78	1250	1250
Aspergillus niger	39	313	156	156
Candida albicans	78	313	625	313

Table 4 : Chemical composition of dichlromethane extract of methoxy alkane chemotyped frankincense resin from Somalia

RI[a]	Compounds	%[b]
924	α-Thujene	0.26
932	α-Pinene	0.26
971	Sabinene	1.08
1024	*p*-Cymene	0.13
1025	Octyl methyl ether	3.34
1057	γ-Terpinene	0.06
1075	2-Decanol, methyl ether	0.09
1112	2-Methyl-6-methylen-octa-1,7-dien-3-one	0.11
1126	Methyl nonyl ether	0.39
1209	Octyl acetate	0.06
1214	*cis*-Decen-1-ol, methyl ether	0.07
1218	*trans*-Decen-1-ol, methyl ether	0.11
1227	Decyl methyl ether	9.84
1242	*cis*-Dec-2-en-1-ol, methyl ether	0.21
1271	Decanol	0.47
1375	α-Copaene	0.36
1383	α-Bourbonene	0.35
1408	Decyl acetate	0.24
1419	*trans*-Caryophyllene	0.11
1474	*trans*-Cadina-1(6),4-diene	0.18
1480	Germacrene D	0.37
1494	*epi*-Cubebol	0.07
1514	Cubebol	0.47
1517	δ-Cadinene	0.2
1520	Tridecyl methyl ether	0.17
1547	Elemol	0.32
1558	Dodecanoic acid	0.41
1561	Prenopsan-8-ol	0.75
1570	*2E*-Tridecen-1-al	0.16
1608	1,10-di-*epi*-Cubenol	0.18
1613	5-*epi*-7-*epi*-α-Eudesmol	2.48
1652	8-epi-γ-Eudesmol	0.67
1654	α-Eudesmol	0.36
1674	iso-Bulensol	0.1
1952	*3E*-Cembrene A	1.36
1996	α-Pinacene	0.43
2009	Verticilliol	0.72
2133	Neocembrene A	7.1
2145	Incensole	43.39
2147	Serratol	20.23
2253	Incensole oxide A	0.2
2263	Incensole oxide B	1.04
2269	Isoincensole	0.46
2293	Isoincensole oxide	0.35
2350	Cembra-2,7,11-trien-4,5-diol isomer	0.29
	Total Identified	100%

IV. Experimental

a) Plant Material

Oleo-gum-resin were collected from the city Ufeyn (10.6500° N, 49.7500° E, 470 m above sea level) in the Puntland region of Somalia for two times of year [2014 April 20th and 2015 September 9th].

The voucher specimen of resin collected plant was stored in, Somalia. Local botanist has identified this resin as *Boswellia carterii,* but on the basis of previously published articles, it did not match chemical composition, so throughout of this article, we mention it as Frankincense or Boswellia spp to avoid controversy in taxonomy. The air-dried sample (250 g) were hydrodistilled using a Clevenger Apparatus for 4 hours to yield a translucent, yellow essential and colorless oil. The essential oil was stored at room temperature until analysis was carried out.

In addition to steam distillation, this resin was also extracted with methylene chloride and analyzed by GC/MS (see Table-4) in order to confirm the presence of the methoxy alkane components in the resin itself. This experiment was done to avoid any possible arguments against these components occurring naturally, ruling out the possibility that the methoxy alkanes are arising from a secondary reaction occurring during the distillation process.

b) Gas Chromatographic-Mass Spectral Analysis

The essential oil of Boswellia was analyzed by GC-MS using a Shimadzu GCMS-QP2010 Ultra operated in the EI mode [(electron energy = 70eV), scan range = 3.0 scans/sec], and GCMS Solution software. The GC column was Zebron ZB-5MS fused silica capillary column with a (5% phenyl)-polymethyl siloxane stationary phase a film thickness of 0.25 mm. The career gas was helium with a column head pressure 80 psi and flow rate of 1.37 ml/min. Injector temperature was 250°C and the ion source temperature was 200°C, increase in temperature rate 2°C/min to 260°C. The GC oven temperature program was programmed for 50°C initial temperature, increase in rate 2°C/min to 260°C. A 5% w/v solution of the sample in CH_2Cl_2 was prepared and 0.1 μL was injected in splitting mode (30:1). Identification of the oil components was based on their retention indices determined by reference to a homologous series of n-alkanes, and by comparison of their mass spectral fragmentation patterns with those reported in the literature [Adams], and stored in the MS library.

c) Chiral Gas Chromatographic-Mass Spectral Analysis

Chiral analysis of the essential oils was performed on a Shimadzu GCMS-QP2010S operated in the EI mode [(electron energy=70eV), scan range = 3.0 scans/sec]. GC equipped with a RestekB-Dex 325 capillary column (30 m×0.25 mm ID×0.25 _m film). Oven temperature was started at 50◦C, and then gradually raised to 120◦C at 1.5 ◦C/min. The oven was then raised to 200◦C at 2◦C/min and held for 5 min. Helium was the carrier gas and flow rate was maintained at 1.8 ml/min. Samples were diluted 3% w/v with CH_2Cl_2 and then a 0.1 μL sample was injected in a split mode with a split ratio of 1:45.

d) Antimicrobial Screening

The essential oil was screened for antimicrobial activity against Gram-positive bacteria, *Bacillus cereus* (ATCC No. 14579) and *Staphylococcus aureus* (ATCC No. 29213); Gram-negative bacteria, *Pseudomonas aeruginosa* (ATCC No. 27853) and *Escherichia coli* (ATCC No. 10798). Minimum inhibitory concentrations (MICs) were determined using the microbroth dilution technique [Satyal et al., 2013]. Dilutions of the crude extracts were prepared in cation-adjusted Mueller Hinton broth (CAMHB) beginning with 50 μL of 1% w/w solutions of crude extracts in DMSO plus 50 μL CAMHB. The extract solutions were serially diluted (1:1) in CAMHB in 96-well plates. Organisms at a concentration of approximately 1.5×10^8 colony-forming units (CFU)/mL were added to each well. Plates were incubated at 37°C for 24 hours; the final minimum inhibitory concentration (MIC) was determined as the lowest concentration without turbidity. Geneticin® was used as a positive antibiotic control; DMSO was used as a negative control. Antifungal activity against *Aspergillus niger* (ATCC No. 16888) was determined as above using YM broth inoculated with *A. niger* hyphal culture diluted to a McFarland turbidity of 1.0. Amphotericin B was the positive control.

e) Cytotoxic Activity

Human MCF-7 breast adenocar-cinoma cells (ATCC No. HTB-22) [Satyal et al, 2014] were grown in a 3% CO_2 environment at 37°C in RPMI-1640 medium, supplemented with 10% fetal bovine serum, 100,000 units penicillin and 10.0 mg streptomycin per liter of medium, 15mM of Hepes, and buffered with 26.7 mM $NaHCO_3$, pH 7.35. Cells were plated into 96-well cell culture plates at 2.5×10^4 cells per well. The volume in each well was 100 µL. After 48 h, supernatant fluid was removed by suction and replaced with 100 µL growth medium containing 1.0 µL of DMSO solution of the essential oil (1% w/w in DMSO), giving a final concentration of 100 µg/mL for each well. Solutions were added to wells in four replicates. Medium controls and DMSO controls (10 µL DMSO/mL) were used. Tingenone [Satyal et al, 2015] was used as a positive control. After the addition of compounds, plates were incubated for 48 h at 37°C in 5% CO_2; medium was then removed by suction, and 100 µL of fresh medium was added to each well. In order to establish percent kill rates, the MTT assay for cell viability was carried out [Satyal et al., 2012]. After colorimetric readings were recorded (using a Molecular Devices SpectraMAX Plus

microplate reader, 570 nm), average absorbances, standard deviations, and percent kill ratios ($\%kill_{cmpd}/\%kill_{DMSO}$) were calculated.

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