**Multi-layered antimicrobial synergism of (*E*)-caryophyllene with minor compounds, tecleanatalensine B and normelicopine, from the leaves of *Vepris gossweileri* (I. Verd.) Mziray**

Moses K. Langat[a,*], Yisau Mayowa[a], Nicholas Sadgrove[a], Mohammed Danyaal[a,b], Thomas A. K. Prescott[a], Teva Kami[c], Sianne Schwikkard[b], James Barker[b], Martin Cheek[d]

[a]*Jodrell Laboratory, Natural Capital and Plant Health Department, Royal Botanic Gardens, Kew, Richmond, Surrey, TW9 3DS, United Kingdom*

[b]*School of Life Sciences, Pharmacy and Chemistry, Kingston University, London, Kingston, KT1 2EE, United Kingdom*

[c]*Herbier National, Institut National en Sciences Exactes et Naturelles, (IRSEN), Cité Scientifique de Brazzaville, République du Congo*

[d]*Herbarium, Royal Botanic Gardens, Kew, Richmond, Surrey, TW9 3AE, United Kingdom*

**Abstract**

An aromatic alkaloid-rich 'absolute' extract from *Vepris gossweileri* inhibited *Saccharomyces cerevisiae* at 62.5 μg.mL$^{-1}$ and *Bacillus subtilis* at 500 μg.mL$^{-1}$. A loss of activity upon fractionation indicated possible synergistic effects. Three new acridones, gossweicridone A (**3**), B (**4**) and C (**5**) and known compounds from the extract were inactive. Combinations of compounds showed that a sub-fraction containing mixtures of minor compounds with (*E*)-caryophyllene augmented activity by 50-folds, with MIC values of 19.6 μg.mL$^{-1}$ for *S. cerevisiae* and 375.0 μg.mL$^{-1}$ for *B. subtilis*, demonstrating potent ΣFIC values of 0.02 and 0.375 respectively. From the active sub-fraction, three compounds were assigned as tecleanatalensine B, 13*S*-hydroxy-9*Z*,11*E*,15*E*-octadecatrienoic acid and normelicopine. In combination with (*E*)-caryophyllene they separately demonstrated MIC values of 18 μg.mL$^{-1}$, 34 μg.mL$^{-1}$ and 16 μg.mL$^{-1}$, respectively against *S. cerevisiae*. The synergistic combinations were more potent with addition of pheophytin A, suggesting that the synergistic antifungal effect of the extract is multi-layered.

*Keywords:*

*Vepris gossweileri* (I. Verd.) Mziray

Acridone

Furoquinoline

*Saccharomyces cerevisiae* strain BY4743

Synergism and potentiation

Antimicrobial effects

## 1. Introduction

In the current study we comprehensively analysed the antifungal activity of an aromatic alkaloid-rich 'absolute' extract from *Vepris gossweileri* (I. Verd.) Mziray, cultivated at the Royal Botanic Gardens Kew to understand the cessation of inhibition following fractionation and reveal the net combinations involved in synergism. The previously unstudied *V. gossweileri*, is a tecleoid species restricted to coastal thicket habitat of northern coastal Angola and southern coastal Republic of Congo. This species is easily distinguished from all other species of *Vepris* that are found in west of the Congo basin, because it has articulated, unifoliate leaves. *Vepris* Comm. ex A. Juss. (Rutaceae-Toddalieae) is a genus with 88 accepted species with 22 in Madagascar and 67 in continental Africa, with one species extending to Arabia and another endemic to India (Cheek et al. 2019, Cheek et al. 2009, Cheek et al. 2018, Kew 2020, Onana and Chevillotte 2015, Onana et al. 2019).

Due to the essential oils distributed in their leaves, and the alkaloids distributed in their roots, several species of *Vepris* have traditional medicinal value (Burkill 1997). Burkill describes the uses, essential oils and alkaloids known from five species in west Africa: *Vepris hiernii* Gereau (as *Diphasia klaineana* Pierre), *Vepris suaveolens* (Engl.) Mziray (as *Teclea suaveolens* Engl.), *Vepris afzelii* (Engl.) Mziray (as *Teclea afzelii* Engl.), *Vepris heterophylla* (Engl.) Letouzey (as *Teclea sudanica* A. Chev.) and *Vepris verdoorniana* (Exell & Mendonça) Mziray (as *Teclea verdoorniana* Exell & Mendonça) (Burkill 1997). Some of the alkaloids from the genus have shown antibacterial, antifungal and anti-oncogenic activity (Imbenzi et al. 2014). While the chemistry of *Vepris* is well represented in the published literature, sometimes this information is published under generic names no longer in current use (Wansi et al. 2008, Wansi et al. 2006). Nevertheless, it is clear that members of the genus *Vepris* predominantly afford acridones, furoquinoline alkaloids and limonoids (Atangana et al. 2017, Kouam et al. 2018). Extractives from *Vepris* are also used as synergists for insecticides (Langat 2011). In this regard, Cheplogoi et al. (2008) and Imbenzi et al. (2014) respectively list 14 and 15 species of *Vepris* that have been studied for such effects. A study of the furoquinoline alkaloids of *V. lecomteana* (Pierre) Cheek & T.Heller demonstrated pronounced non-discriminate antimicrobial activity against a panel of Gram-positive and Gram-negative bacteria. The activity only manifested using furoquinolines with secondary amines, where the *N*-methylated derivatives demonstrated no activity at the concentrations tested. Kouam et al. (2018) also noted

that the isolated alkaloids were not as active as the crude extract and concluded that a synergistic effect must be occurring. Hence, it is necessary to elucidate the pathway to antimicrobial synergism in these species to understand their mechanisms as natural antiinfective medicines in ethnobotanical traditions.

In this paper we report three previously undescribed acridone alkaloids gossweicridone A, gossweicridone B, gossweicridone C together with the known acridone alkaloids, 1,2,3,5-tetramethoxy-*N*-methylacridone (Atangana et al, 2017) and 1,3,5-trimethoxy-*N*-methylacridone (Vaquette et al. 1974), normelicopine (Wang et al. 2014), the known furoquinolines, tecleanatalensine B (Tarus et al. 2005) and delbine (Bhattacharyya and Serur 1981), (Fig. 1) pheophytin A (Smith et al. 1984), vitamin E, β-amyrin, (*E*)-caryophyllene, caryophyllene oxide, humulene, methyl-palmitate, methyl-linolenate, 13*S*-hydroxy-9*Z*,11*E*,15*E*-octadecatrienoic acid (Bang et al. 2002) and the common sterols, sitosterol and stigmasterol were isolated from the leaves of *V. gossweileri* growing at the Royal Botanic Gardens Kew (UK). The current study also reports the synergistic antimicrobial effects of the minor compounds, tecleanatalensine B and normelicopine.

## 2. Results and discussion

### *2.1 Chemistry of Vepris gossweileri*

The leaves of *V. gossweileri* were extracted using $CH_2Cl_2$ and subjected to chromatographic analysis to afford two known acridones, 1,2,3,5-tetramethoxy-*N*-methylacridone and 1,3,5-trimethoxy-*N*-methylacridone, normelicopine, three undescribed acridone alkaloids gossweicridone A (**3**), gossweicridone B (**4**) and gossweicridone C (**5**), the furoquinolines tecleanatalensine B and delbine (Fig. 1), vitamin E, β-amyrin, (*E*)-caryophyllene, caryophyllene oxide, humulene, methyl-palmitate, methyl-linolenate, and the common sterols, sitosterol and stigmasterol. The structures of the new acridone alkaloids were determined based on comprehensive spectroscopic and spectrometric analysis (Table S1) whereas the spectra of the known compounds were determined by comparing spectra with to those reported previously.

Compound **3** was isolated as an orange amorphous powder, and its molecular formula determined to be $C_{19}H_{21}NO_6$ due to a molecular ion $[M+H]^+$ at *m/z* 360.1440 (calcd. 359.1447) from HRESIMS. Ten degrees of unsaturation were calculated. The IR spectrum for **3** showed an absorption band for a conjugated ketone carbonyl group at 1719 $cm^{-1}$. The $^1H$ NMR spectrum for **3** (Table S1) exhibited five methoxy group

resonances at $\delta_H$ 4.00 (s, $OCH_3$-1), 3.94 (s, $OCH_3$-2), 4.09 (s, $OCH_3$-3), 3.88 (s, $OCH_3$-4) and 3.98 (s, $OCH_3$-5), and a *N*-methyl group at $\delta_H$ 3.65 (s). The aromatic region of the $^1$H NMR spectrum showed presence of an ABC coupled system in **3** due to $\delta_H$ 7.89 (dd, $J$ = 7.8, 1.3 Hz), 7.21 (dd, $J$ = 7.8, 7.8 Hz) and 7.13 (dd, $J$ = 1.3, 7.8 Hz) for H-8, H-7 and H-6 respectively. The H-8 resonance was assigned as the lower-fielded due to the adjacent carbonyl group of the acridone moiety (Atangana et al, 2017, Wansi et al, 2006), whereas the H-7 was assigned due to the double *ortho*-coupled $J$ values of 7.8 and 7.8 Hz, and H-6 resonance was inferred from the COSY spectrum. The $^{13}$C NMR resonances for **3** were assigned by using HSQC and HMBC experiments. The H-8 proton resonance showed a correlation in the HMBC spectrum with a carbonyl resonance at $\delta_C$ 178.8, assignable to C-9, whereas H-7 showed correlation in the HMBC spectrum with a carbon resonance at $\delta_C$ 128.3 for C-8a, and $\delta_C$ 150.8 for C-5. The C-5 carbon resonance, in turn, showed correlations with the H-6 and the $OCH_3$-5 proton resonances. The H-6 and the *N*-methyl proton resonances showed correlations in the HMBC spectrum with a carbon resonance at $\delta_C$ 138.2 that was assigned to C-10a. The *N*-methyl proton resonance showed a correlation in the NOESY spectrum with a methoxy proton resonance at $\delta_H$ 3.88 (s) that was assigned to $OCH_3$-4. This proton resonance for $OCH_3$-4 showed a correlation in the NOESY spectrum with a methoxy proton resonance at $\delta_H$ 4.09 (s) for $OCH_3$-3. The methoxy resonances at $\delta_H$ 4.00 (s) and 3.94 (s) were assigned to $OCH_3$-1 and $OCH_3$-2 respectively. The carbonyl resonances for C-1, C-2, C-3 and C-4 were assigned as $\delta_C$ 150.2,142.6, 152.4 and 140.2 respectively. Compound **3** was determined to be 1,2,3,4,5-pentamethoxy-*N*-methylacridone trivially named gossweicridone A.

Compound **4** was determined to be a *N*-demethylated derivative of compound **3**. Compound **4** was isolated as a white amorphous powder, and its molecular formula determined to be $C_{18}H_{19}NO_6$ due to a molecular ion $[M]^+$ at *m/z* 346.1281 (calcd. 346.1291) from HRESIMS. Ten degrees of unsaturation were calculated. The IR spectrum for **4** showed absorption band at 1733 cm$^{-1}$ for a conjugated ketone carbonyl group and 3422 cm$^{-1}$ for a N-H absorption band. The $^1$H NMR spectrum of compound **4** showed a proton resonance at $\delta_H$ 8.83 (br s $W_{1/2}$ 6.2 Hz) that was not correlated with a carbon resonance in the HSQCDEPT spectrum (Table S1). The *N*-methyl proton resonance in the $^1$H NMR spectrum and its corresponding up-field carbon resonance expected at *ca* $\delta_C$ 42.0 were absent, hence **4** was *N*-demethylated. The ABC system

observed in **4** was also present in **3** due to proton resonances at $\delta_H$ 8.00 (dd, $J$ = 8.0, 1.3 Hz), 7.14 (dd, $J$ = 8.0, 8.0 Hz) and 7.14 (dd, $J$ = 1.3, 8.0 Hz) for H-8, H-7 and H-6 respectively. The $^1$H NMR spectrum for **4** (Table S1) also exhibited five methoxy group resonances at $\delta_H$ 4.00 (s, $OCH_3$-1), 3.95 (s, $OCH_3$-2), 4.09 (s, $OCH_3$-3), 4.06 (s, $OCH_3$-4) and 4.04 (s, $OCH_3$-5). Compound **4** was determined to be 1,2,3,4,5-pentamethoxy-*NH*-acridone trivially named gossweicridone B.

Compound **5** was determined to be a 6-methoxy derivative of the known 1,3,5-trimethoxy-*N*-methylacridone, herein named 1,3,5,6-tetramethoxy-*N*-methylacridone, and trivially *gossweicridone C*. Compound **5** was isolated as a white amorphous powder, and its molecular formula determined to be $C_{18}H_{19}NO_5$ due to a molecular ion $[M+1]^+$ at *m/z* 330.1332 (calcd. 330.1342) from HRESIMS. Ten degrees of unsaturation were calculated. The IR spectrum for **5** showed absorption band at 1720 $cm^{-1}$ for a conjugated ketone carbonyl group. The $^1$H NMR spectrum for compound **5** showed two meta coupled aromatic proton resonances at $\delta_H$ 6.28 (d, $J$ = 2.0 Hz) for H-2 and $\delta_H$ 6.40 (d, $J$ = 2.0 Hz) for H-4 and two ortho coupled aromatic proton resonances at $\delta_H$ 6.91 (d, $J$ = 8.9 Hz) for H-7 and $\delta_H$ 8.20 (dd, $J$ = 8.9 Hz) for H-8, as were observed the known ,3,5-trimethoxy-*N*-methylacridone, but with an extra methoxy proton resonance at $\delta_H$ 3.94 (s, $OCH_3$-6). The $^1$H NMR spectrum for **5** (Table S1) exhibited three methoxy group resonances at $\delta_H$ 3.98 (s, $OCH_3$-1), 3.97 (s, $OCH_3$-4), 3.92 (s, $OCH_3$-5) and 3.78 (s, $NCH_3$). Compound **5** was tentatively determined to be 1,3,5,6-tetramethoxy-*N*-methylacridone trivially named gossweicridone C based on its $^1$H NMR and 2D spectra.

### *2.2 Antimicrobial effects of compounds from Vepris gossweileri*

The aromatic alkaloid-rich 'absolute' extract was prepared in a similar way to the standard approach used in industry for aromatic extracts (see methods). Absolutes are generally manufactured in industry to enrich the volatile organic compound content. They are marketed as perfumes or for use in aromatherapy (Sadgrove and Jones 2015). Chemical analysis of the crude absolute using NMR and GCMS experiments indicated that it is primarily comprised of 1,2,3,5-tetramethoxy-*N*-methylacridone with trace amounts of the other acridone alkaloids (**3**-**5**), 1,3,5-trimethoxy-*N*-methylacridone, (*E*)-caryophyllene, caryophyllene oxide, pheophytin A, normelicopine, tecleanatalensine

B, delbine, 13*S*-hydroxy-9*Z*,11*E*,15*E*-octadecatrienoic acid and naturally occurring methyl esters of palmitic and linolenic acid.

The absolute was screened for antimicrobial activity against *S. cerevisiae*, *E. coli* and *B. subtilis* at a starting concentration of 1 mg.mL$^{-1}$. No activity against *E. coli* was observed at the starting concentration, but against *B. subtilis* and *S. cerevisiae* MIC values of 62.5 µg.mL$^{-1}$ and 500 µg.mL$^{-1}$ were observed, respectively. The inhibitory activity was lost from sub-fractions from chromatographic analysis of the absolute extract when screened against *S. cerevisiae*. The sub-fractions were then methodically recombined to identify combinations responsible for synergistic antifungal activity. Inhibitory activity returned with a combination of the alkaloid-rich sub-fraction and *(E)*-caryophyllene. This combination was then screened against three organisms in a chequerboard assay. Synergistic activity was observed against *S. cerevisiae* and *B. subtilis*, but not *E. coli*. The MIC value was most potent against *S. cerevisiae*, which was 19.6 µg.mL$^{-1}$. This combination was comprised of 15.6 µg.mL$^{-1}$ of the alkaloids subfraction and 3.9 µg.mL$^{-1}$ of (*E*)-caryophyllene (Table S2). Activity was much weaker against *B. subtilis,* which was 375 µg.mL$^{-1}$ (125 µg.mL$^{-1}$ alkaloids subfraction and 250 µg.mL$^{-1}$ (*E*)-caryophyllene) (Table S3). The ΣFIC of the alkaloid/(*E*)-caryophyllene combination against *S. cerevisiae* was significant, at 0.02 (Table S1), in contrast with that for *B. subtilis*, at 0.375 (Table S3). According to van Vuuren and Viljoen (2011) these ΣFIC concentrations are well within the margin of synergism or potentiation.

From the subfraction that was rich with alkaloids, the major acridones were not identified as active, rather, it was the minor compounds tecleanatalensine B, normelicopine, 13*S*-hydroxy-9*Z*,11*E*,15*E*-octadecatrienoic acid and pheophytin A that were participating in the synergistic outcome. In pure form, all four compounds were inactive against *S. cerevisiae* and *E. coli* at starting concentrations of 285, 550, 160 and 275 µg.mL$^{-1}$ respectively. Against *B. subtilis* tecleanatalensine B and normelicopine were found to be active at a concentration of 72 µg.mL$^{-1}$.

In combination with *(E)*-caryophyllene only three out of the four compounds were synergistic; tecleanatalensine B, normelicopine and 13*S*-hydroxy-9*Z*,11*E*,15*E*-octadecatrienoic acid (see supplementary tables: S2-S5). When tecleanatalensine B

was combined with *(E)*-caryophyllene the combination gave an MIC of 33.4 μg.mL$^{-1}$ made up of 18 μg.mL$^{-1}$ caryophyllene and 16 μg.mL$^{-1}$ tecleanatalensine B. The combination with normelicopine gave an MIC of 35.6 μg.mL$^{-1}$, which was made up of 20 μg.mL$^{-1}$ caryophyllene and 16 μg.mL$^{-1}$ normelicopine. Lastly, the combination with 13*S*-hydroxy-9*Z*,11*E*,15*E*-octadecatrienoic acid gave an MIC of 96.9 μg.mL$^{-1}$ , which was made up of 34 μg.mL$^{-1}$ caryophyllene and 63 μg.mL$^{-1}$ 13*S*-hydroxy-9*Z*,11*E*,15*E*-octadecatrienoic acid.

The concentration of *(E)*-caryophyllene required to enact synergistic effects by the three isolated compounds was many orders of magnitude higher as compared to the pre-fractionated alkaloids-rich extract (Table S2). Hence the possibility of a potentiator was investigated. Although pheophytin A was not synergising with *(E)*-caryophyllene it was combined with each of the three actives and screened in the chequerboard assay. In this regard, only a trace amount of pheophytin A (1 – 3% mg.mg$^{-1}$) was required to augment the inhibitory effects of the *(E)*-caryophyllene and 13*S*-hydroxy-9*Z*,11*E*,15*E*-octadecatrienoic acid in combination. The MIC of the mixture against *S. cerevisiae* was reduced from 96.9 to 24.2 μg.mL$^{-1}$ (made up of 9 μg.mL$^{-1}$ *(E)*-caryophyllene, 16 μg.mL$^{-1}$ 13*S*-hydroxy-9*Z*,11*E*,15*E*-octadecatrienoic acid + pheophytin A). Interestingly, pheophytin A had no antimicrobial effects on its own at a starting concentration of 1 mg.mL$^{-1}$, nor did it have a synergistic effect with *(E)*-caryophyllene.

It was surprising to observe that the major acridone alkaloid, 1,2,3,5-tetramethoxy-*N*-methylacridone, was not involved in the synergism. Instead, it is the furoquinolines that are known for antimicrobial effects against species of bacteria and yeast (Kuete et al. 2008) and as previously mentioned synergism has already been suggested as a possible mechanism in crude extracts (Kouam et al. 2018). In this regard, it is often the case that antimicrobial extracts lose potency when they are fractionated into less complex mixtures, which evidently occurred in the current study. While this is common, it is rare for researchers to identify and explain the combinations that are responsible for the antimicrobial potency (Sadgrove and Jones 2019). However, synergism in natural products is becoming an area of interest in the wider scientific community (Caesar and Cech 2019), particularly in the context of combinations of volatile organic compounds with fixed components (Sadgrove 2020). To date, most research that is focused on antimicrobial synergism uses combinations between

conventional antibiotics (Li et al. 2008) or combinations of natural products with conventional antibiotics, to restore efficacy or antagonise resistance mechanisms (Sadgrove and Jones 2019). It is extremely rare, if not unheard of, to identify synergistic combinations between alkaloids and small lipophilic terpenes. However, this may be a consequence of a lack of emphasis in research goals, rather than rarity. Thus, the outcome of the current study leads by way of example the paradigmatic shift in antimicrobial research on natural products. Furthermore, since *S. cerevisiae* represents a eukaryotic model, the combination of (*E*)-caryophyllene and tecleanatalensine B (or 13*S*-hydroxy-9*Z*,11*E*,15*E*-octadecatrienoic acid, normelicopine and the other furoquinolines) should be tested against mammalian cells for toxic or anti-cancerous activity. Finally, these unusual potentiating effects of pheophytin A and synergistic effects of (*E*)-caryophyllene add to the plethora of biological effects that these compounds demonstrate against eukaryotic cells, including immunomodulatory and psychotropic effects (Francomano et al. 2019). While (*E*)-caryophyllene is generally regarded as a therapeutic compound, it is necessary to know if it is safe in combinations with the furoquinolines of the current study (Tables S4-S7).

## 3. Experimental

### *3.1 General experimental procedure*

Details of the general experimental procedures are provided as part of the supplementary information.

### *3.2 Plant material, extraction, and preparation of the absolute*

The leaves of *V. gossweileri* were collected from a live plant growing at the Royal Botanic Gardens, Kew raised from seed collected from Tchimpounga, Republic of Congo. The import and safe use of this wild collected plant material was enabled using Defra Plant Health Licence 2149/194627/5. The plant was identified against authenticated reference specimens at the Kew Herbarium by the last author. Voucher specimens are *T.Kami* 1427 (IEC, K, MO) and *A.L. Mpandzou* 1645 (B, BR, IEC, K, P, WAG). Herbarium codes follow Thiers (continuously updated). The freeze-dried leaves (36.9 g) from *V. gossweileri* were extracted through soaking successively with $CH_2Cl_2$ (0.5 L) and $CH_3OH$ (0.5 L) for 24 hours and then filtered. The extracts were evaporated to dryness using a rotary evaporator at 50 $^oC$ to give 3.8 g of $CH_2Cl_2$ extract and 6.7 g of $CH_3OH$ extract. The crude extracts were chromatographed over silica to isolate compounds. The crude $CH_2Cl_2$ extract was used to produce the aromatic

absolute following a modified protocol that is normally used to make absolutes in industry (Sadgrove and Jones 2015). The crude $CH_2Cl_2$ extract (2.5g) was reextracted with hexane under sonication, then centrifuged to remove particulates (2.2g removed). Approximately 12% of the extract dissolved into the hexane (0.3g). The organic phase was removed, and the aromatic resin extracted again with methanol, then centrifuged to remove particulates (0.19g: identified as fatty acids). The organic phase was removed, and a dark green amorphous aromatic resin was obtained (0.11g), representing approximately 4 – 5% by mass of the original $CH_2Cl_2$ extract.

***3.3 Isolation***

Step by step procedures of isolation of compounds are given in detail as part of the supplementary information. The pure fractions were analysed using spectroscopic and spectrometric techniques and details are as follows: -

*Gossweicridone A (**3**)*: orange solid; (16.7 mg); IR (NaCl) $\nu_{max}$ ($cm^{-1}$): 2927, 2850, 1719, 1615; $^{1}H$ and $^{13}C$ NMR are given in Table S1; HRESIMS *m/z* 360.1440 $[M+1]^+$ ($C_{19}H_{22}NO_6$ requires 360.1447).

*Gossweicridone B (**4**)*: white solid; (23.4 mg); IR (NaCl) $\nu_{max}$ ($cm^{-1}$): 3422, 2939, 2851, 1733, 1618; $^{1}H$ and $^{13}C$ NMR are given in Table S1; HRESIMS *m/z* 346.1281 $[M+1]^+$ ($C_{18}H_{20}NO_6$ requires 346.1291).

*Gossweicridone C (**5**)*: white solid; (0.9 mg); IR (NaCl) $\nu_{max}$ ($cm^{-1}$): 2928, 2849, 1720, 1616; $^{1}H$ NMR are given in Table S1; HRESIMS *m/z* 330.1332 $[M+1]^+$ ($C_{18}H_{20}NO_5$ requires 330.1342).

***3.4 GCMS and NMR analysis of the alkaloid-rich aromatic absolute***

Detailed explanation of the GCMS and NMR analysis of the aromatic alkaloid-rich absolute is given as part of the supplementary information. Three sesquiterpenes were identified, (*E*)-caryophyllene, caryophyllene oxide and humulene. Other known and common components were identified by comparison to NMR spectra from our spectral library, which included vitamin E, β-amyrin, methyl-palmitate, methyl-linolenate, and linolenic acid. NMR spectra of the less commonly known compounds were matched to published values. These included the acridone alkaloids, 1,2,3,5-tetramethoxy-*N*-methylacridone (Atangana et al. 2017) and 1,3,5-trimethoxy-*N*-methylacridone (Vaquette et al. 1974), normelicopine (Wang et al. 2014), the known furoquinolines, tecleanatalensine B (Tarus et al. 2005) and delbine (Bhattacharyya and Serur 1981), a chlorophyll derivative pheophytin A (Smith et al. 1984), and 13*S*-hydroxy-9*Z*,11*E*,15*E*-

octadecatrienoic acid (Bang et al. 2002). The specific rotation of 13*S*-hydroxy-9*Z*,11*E*,15*E*-octadecatrienoic acid was determined by optical rotations, which gave $[\alpha]_D^{25} = +5.7°$, corresponding to the *S* enantiomer (Waridel et al. 2004).

### *3.5 Antimicrobial testing*

Three model organisms were used to characterise activity against Gram-positive (*Bacillus subtilis* BKK24010), Gram-negative (*Escherichia coli* BW25113) and fungal/eukaryotic (*Saccharomyces cerevisiae* strain BY4743) organisms. Protocols used for antimicrobial testing is given as part of the supplementary information, and they followed the protocol described by the Clinical Laboratory Standards Institute (CLSI 2009) using tryptone soya broth (*B. subtilis* & *E. coli*) or SC broth (*S. cerevisiae*) and the chequerboard assay was conducted according to the protocol described by van Vuuren and Viljoen (Van Vuuren and Viljoen 2011), using an 8 x 8 square.

## 4. Conclusion

We demonstrate a synergistic/potentiating effect of antimicrobial alkaloids by (*E*)-caryophyllene against *S. cerevisiae* with an MIC value of 19.6 μg.mL$^{-1}$ and *B. subtilis* with an MIC 375.0 μg.mL$^{-1}$, which represent strong ΣFIC values of 0.02 and 0.375 respectively. The antifungal synergism augmented activity by as much as 50-folds and appears to be a consequence of 'potentiation' of the synergistic combination (alkaloid and (*E*)-caryophyllene) by pheophytin A. This synergistic/potentiating effect was observed from an aromatic alkaloid-rich absolute extract that was prepared from the leaves of *V. gossweileri* cultivated at the Royal Botanic Gardens Kew (UK). This novel finding of synergism/potentiation of compounds within a mixture was observed in a study of the furoquinoline alkaloids of *V. lecompteana* where non-discriminate antimicrobial activity against a panel of Gram-positive and Gram-negative bacteria was demonstrated (Kouam et al. 2018) but below the activity demonstrated by the crude extract. The authors tentatively suggested a synergistic effect within the extract. Surprisingly, the major alkaloids were not involved in the synergy. They were isolated from the $CH_2Cl_2$ extract of the leaves of *V. gossweileri*, three of which are the previously undescribed acridones gossweicridone A, gossweicridone B, and gossweicridone C. Several known compounds were also assigned, including the known acridones, 1,2,3,5-tetramethoxy-*N*-methylacridone and 1,3,5-trimethoxy-*N*-methylacridone, normelicopine, delbine, tecleanatalensine, vitamin E, β-amyrin, (*E*)-

caryophyllene, caryophyllene oxide, palmitic acid, linolenic acid, methyl-palmitate, methyl linolenoate, sitosterol and stigmasterol.

## ASSOCIATED CONTENT

### Supporting Information

The supporting information is available free of charge on the website at DOI: xxxxxxx

### Author information

Corresponding Author.

*Phone: +442083325365. Email: m.langat@kew.org

**ORCID**

Moses K Langat: 0000-0001-8079-7774

### Conflict of interest

The authors declare no competing financial interest.

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