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Antifouling Alkaloids from *Crinum augustum* (Amaryllidaceae)

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ABSTRACT

Fractionation and purification of the ethanolic extract of the bulbs of *Crinum augustum* Rox. (Amaryllidaceae) cultivated in Egypt yielded five alkaloids 6-methoxy-crinamine (**1**), crinamine (**2**), buphanisine (**3**), ungeremine (**4**), and hippadine (**5**); two fatty acid derivatives: myristic acid ethyl ester (**6**) and palmitic acid ethyl ester (**7**); four terpenoidal and steroidal compounds: ursolic acid (**8**), ß-sitosterol-O- ß glucopyranoside (**9**) and mixture of ß-sitosterol (**10**) and stigmasterol (**11**). The structures of all compounds were determined by interpretation of their spectroscopic data; 1D (^{1}H and ^{13}C), 2D (HSQC, COSY, DQF, NOE and HMBC) NMR; MS and UV analyses. The compounds (**1**-**4**) and (**6**-8) were tested towards biofouling activity using larvae of barnacle *Balance amphitrie*. Significant activities of **1**, **2** and **3** with EC_{50} 1.8, 1.2 and 0.75 µg/mL respectively, were observed.

KEYWORDS: *Crinum augustum*, Amaryllidaceous, anti-fouling, natural products

INTRODUCTION

Amaryllidaceae is a big family and contains about 90 genera and 1310 species (1). They occur naturally throughout the tropics, subtropics and warm temperate regions of the world in Asia, Australia, Africa and America (2). Members of Amaryllidaceae attract considerable attention due to various medicinal properties such as anti-tumor, immunostimulant, analgesic, antiviral, anti-malarial, antibacterial and antifungal activities. They are subjected to chemical, cytological and pharmacological investigations due to their richness in pharmacologically active alkaloids (3) such as lycorine, a principal alkaloid possessing a strong antiviral activity (4, 5) and galanthamine which has anti-cholinesterase activity (5) that accounts for its use in Alzheimer's disease (6). The genus *Crinum* is productive and well known with its diversity of chemical and biological activities (7, 8). Settlement of higher organisms such as macroalgae and invertebrates may threaten the survival of individuals of benthic invertebrates. Therefore, benthic invertebrates have developed various defense systems against biofouling, such as biofilm (9). Biofouling on ships hulls, offshore structures or aquaculture equipment is a major global economic and technical problem. The organotin compounds have until recently been widely used for biofouling control, but after 2008 their use will be prohibited worldwide, Therefore, the development of environmentally safe antifouling substances is urgently needed (10). Finally, the main task of the current paper is the discovering of new natural products with biological activity and/or novel chemical structures from *Crinum augustum*, particularly those having eco-environmental activity such as anti-fouling. Thus, successive fractionation of the ethanolic extract of *Crinum augustum* by vacuum liquid chromatography (VLC) over normal silica, yielded **1**-**11** (Figure 1). The effect of compounds **1**, **2** and **3** on settlement and variability of cyprids (Table 1 and figure 2), with EC_{50}; 1.8, 1.2 and 0.75 µg/mL respectively. $CuSO_4$ (EC_{50} = 0.23 µg/mL) was used as a positive control.

MATERIALS and METHDS

General procedures- Melting points were measured using Stuart Scientific (SMPI) melting point apparatus and were uncorrected. UV spectra were determined using Spectronic® Genesys™ 2PC UV spectrophotometer. ^1H and ^{13}C-NMR spectra were recorded on a JEOL-JNM-EX-400 spectrometer (400 MHz for ^1H and 100 MHz for ^{13}C, respectively). ESIMS data were obtained with a JEOL JMS–700T mass spectrometer. Pre-coated silica gel 60 GF_{254} plates (E. Merck) were used for TLC. The compounds were detected by UV absorption at λ_{max} 254 and 366 nm followed by spraying with anisaldehyde/H_2SO_4 reagent and heating at 110 ^0C for 1-2 min. All solvents were distilled prior to use.

Plant material

The bulbs of *Crinum augustum* Rox. were collected from the farm of Faculty of Pharmacy, Assuit University, Egypt. The plant material was kindly identified by Prof. Dr. A. Fayed, Professor of plant taxonomy at Assuit University. A voucher specimen (C-1) was deposited in herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Minia University, Minia, Egypt.

Extraction and isolation

The air-dried powdered bulbs (4 Kg) were extracted by 95% EtOH (3 X 5 L), and evaporated under reduced pressure into viscous extract (795 g). The solvent-free residue (795 g) was partitioned between 5% aqueous HCl and $CHCl_3$ (1:1). The $CHCl_3$ fraction (F001, 487 g). The mother liquor was rendered alkaline by 5% NH_4OH and successively extracted with $CHCl_3$ and EtOAc, yielded F002 (10.3 g) and F003 (11.4 g), respectively. The solution (mother liquor) was saturated with Na_2CO_3 and then re-extracted with $CHCl_3$, resulted in F004 (1.32 g). Finally, the aqueous mother liquor was concentrated to give fraction F005 (201.5 g). F001 (20 g) was fractionated on NP-silica (100 x 5 cm, 500 g, 60-120 mesh, E-Merck) employing gradient elution using pet. ether– EtOAc (98:2; 95:5; 90:10; 70:30; 50:50, 0:100), (1.35, 4.0, 0.7, 2.55, 5 and 3.53 g) and washed with MeOH (1.2 g), 250 mL each of fraction. The column was monitored by TLC, the similar fractions were pooled into 14 pools. F012 was fractionated on NP-silica employing gradient technique using pet. ether-$CHCl_3$ (99:1, 98:2, 97:3) and then purified on NP-silica, using pet. ether-$CHCl_3$ (99:01) to yield **6** (12.7 mg) and pet. ether-$CHCl_3$ (99:01) to yield **7** (199 mg). F00I5 was purified on NP-silica, using gradient pet. ether-EtOAc (97:3, 95:5 and 93:7), led to isolation of **5** (20 mg), re-crystallized by MeOH. F016 fractionated on NP-silica, employing pet. ether-EtOAc (95:5, 93:7, 90:10, 88:12) resulted in mixture of (**10** and **11**) (105 mg) and **8** (26.5 mg). F0111 was purified by NP-silica, using $CHCl_3$-MeOH (99:1, 98:2, 97:3, 95:5, 93:7) to yield **9** (26 mg). F0113 was purified by using alkaline silica NP-silica, and employing pet. ether-EtOAc (30:70, 25:75, 20:80, 10:90) and further purification on NP-silica using $CHCl_3$-MeOH (98:2), yielded **1** (39 mg). F0114 was purified by using NP-silica, and employing pet. ether-EtOAc (30:70, 20:80, 10:90) and further purification on NP-silica using $CHCl_3$-MeOH (98:2) yielded **3** (34.5 mg). F02 fractionated on NP-silica (100 x 5 cm, 310 g, 60-120 mesh, E-Merck), employing pet. ether-EtOAc (90:10; 70:30 ; 50:50; 25:75; 0:100) (0.1, 0.5, 0.5, 1.6, 2.5g, respectively) and washed with EtOAc-MeOH (50:50 and 0:100) (2.3 and 1.0 g), led to F021-27. F024 was fractionated on alkaline NP-silica using pet. ether-$CHCl_3$ (40:60), yielded **2** (60 mg). F004 was fractionated on alkaline NP-silica (30 x 2 cm 30 g, 60-120 mesh, E-Merck), elutied with pet. ether-EtOAc (75:25, 50:50, 0:100) (20, 30, 80 mg, respectively), and washed with EtOAc-MeOH (80:20 and 0:100) (100, 340 mg) yielded **4** (42 mg). F045 was fractionated on NP-silica (100 x 5, 340 g, 60-120 mesh, E-Merck) eluted with pet. ether-EtOAc (70:30, 10:90, 0:100) yielded **4** (42 mg).

BIOLOGICAL ACTIVITY

Antifouling Assay

The culture of Cyprid larvae has been reported previously (11). The samples were dissolved in MeOH and aliquots were pipetted into 24-well polystyrene tissue culture plates and air-dried. Two mL of filtered seawater diluted to 80% by deionized water (80% filtered seawater) and 4-8 cyprids (2-3 days fold) were added to each well. Each level of the experiments was carried out with four wells (10-18 cyprids). The plates were kept in the dark at 25 ˚C and the number of larvae that attached, metamorphosed, dried or did not settle, was counted after 48 H (9, 12), cyprids that did not move had extended appendages and did not respond after a light touch by metal were regarded as dead. The assay of seven compounds and controls was repeated 5 times with different batches of larvae. Each concentration was replicated 3-5 times. Normality of the distribution was verified with Shapiro-Wilk´s test. When necessary, the percentages of settled and dead larval were analyzed after arcsine transformation. ANOVA followed by Dunnet´s comparison test was used for multiple comparisons of treatment means with control. The antifouling activity of the compounds was expressed as ED_{50} values. The ED_{50} values were calculated by prohibt analyses. A non toxic solution was defined as one in which cyprids did not settle but remained alive after 48 h.

RESULTS AND DISCUSSION

This class of compounds has been tested for the first time for their anti-fouling activity. There is no previous publication of antifouling activity based on a computer survey. This paper represents the first report of alkaloids with anti-fouling activity which opens the gate to the other researcher to find new antifouling activity from terrestrial natural product research.

Positive HR-ESI-MS of **1** revealed a molecular ion peak at *m/z* 332.14 $[M+H]^+$ to have molecular formula $C_{18}H_{21}NO_5$. Decoupled ^{13}C and DEPT NMR spectra of **1** showed eighteen resonances (Table 3). Eight of eighteen elements of unsaturation, as indicated by the molecular formula of **1**, could be attributed to four carbon-carbon double bonds and the molecule thus, has five rings. The NMR spectroscopic data further enabled all but one hydrogen atom of **1** to be attached to carbons; hence it was evident that the remaining one is present in the molecule as hydroxyl function. UV spectral data of **1**, exhibited three maxima absorptions (λ_{max}292, 241 and 205 nm), indicating the presence of a methylenedioxy-substituted benzene ring (13) revealing the presence of crinine nucleus. Interpretation of the 2D NOESY correlations is simplified assignment of the two pararomatic proton to be H-10 and H-7, respectively. From this spectral data, it was clear that H-10 (δ_H 6.9) has correlation with H-1 and H-7 (δ_H 6.71) is correlated with H-6. From ^1H NMR spectral data of **1**, four characteristic signals, two of them indicates olefinic protons δ_H 6.47 (d, *J* = 10.5 Hz, H-1) and δ_H 5.89 (dd, *J* = 10, 5.1 Hz, H-2); and the remaining two are doublets at δ_H 5.83 and 5.80 attributed to methylenedioxy group with a geminal coupling. The typical AB pattern of the benzylic H-6 protons at δ_H 4-4.5 that is characteristic for all C-6 unsubstituted alkaloids of the crinine series was absent in the ^1H NMR spectrum of **1**, and instead it showed only one singlet at δ_H 5.05 for H-6α. The absence of the typical AB pattern together with the pronounced deshielding effect on H-6α as well as C-6 in ^{13}C-NMR spectrum suggested oxylation of C-6. Further investigation of ^1H NMR spectrum of **1**, indicated that the large coupling constant measured from the splitting of H-4a signal δ_H 3.79 (dd, *J* = 13.6 Hz) and H-4α, led to *trans*-diaxial configuration (14). A multiplet at δ_H 3.74 was assigned to H-3, whereas the two (ddd) at δ_H 1.8 and 1.5 were assigned to H-4α and H-4ß, respectively. The large coupling constant between H-3 and H-4α (13.6 Hz) as well as the NOE correlation between H-3 and H-4α were indicative to the *cis* relationship between the C-3 pseudoequatorial substituent and the 5,10b-ethano bridge. It was clear from ^{13}C spectral chemical shift of the two methoxyl groups δ_C 56.9 (6-OCH_3), 56.7(3-OCH_3), that they are aliphatic, this deduction supported by ^1H chemical shifts δ_H 3.32 (s, 6-OCH_3) and 3.25 (s, 3-OCH_3). The former was further deshielded due to the nitrogen atom of ring B. The observed NOESY and HMBC correlations between the 6-OMe group and H-6α, and C-6ß, respectively, proved the presence of the methoxyl group at C-6. The coupling pattern of protons of the ethano-bridge that was represented by only three (dd) at δ_H 3.65, 2.74 and 2.02, together with the observed deshielding of H-11*endo* as well as C-11 in ^{13}C-NMR in comparison with the related C-11 unsubstituted alkaloids indicated the oxylation at this position. The NOE effect between H-10 and H-11*endo* was consistent with a hydroxyl substituent at the *exo*-position. The ^{13}C-NMR spectrum of compound **1** showed a similarity to its related alkaloid crinamine but with a pronounced deshielding of C-6 to be at δ_C 87.3 and the appearance of an additional aliphatic signal at δ_C 56.9 for C_6-OMe group. All these findings together with the coincidence of its physical and spectral data (15, 16) indicated that compound **1** is 6-methoxy crinamine, which was previously isolated from *C. zeylanicum* (15) and this is the first time to be isolated from *Crinum augustum* Rox.

Negative and positive modes HR-ESI-MS of **2** revealed molecular ion peak at *m/z* 300.110 $[M-H]^-$ and *m/z* 302.13 $[M+ H]^+$ respectively, showed it to have the molecular formula $C_{17}H_{19}NO_4$. UV spectral data of **2**, indicated to the presence of three maxima absorption (λ_{max}292, 241 and 208), which indicated the presence of a methylenedioxy-substituted benzene ring (13), indicated the presence of crinine nucleus. ^{13}C NMR spectra (^1H decoupled) of **2**, showed seventeen resonances (Table 3). ^1H NMR spectral data of **2** showed two singlets for the two para aromatic protons of ring A at δ_H 6.71 and 6.39 that were assigned to H-10 and H-7, respectively. H-10 could be distinguished from H-7 through the observed NOESY correlations of H-10 with H-1 and H-7 with 2H-6. Another broad singlet was observed at δ_H 6.16 for two olefinic protons, their multiplicities were in agreement with the *cis*-relationship between the C-3 substituent and the 5,10b-ethano-bridge. This olefinic part of the spectrum showed a close resemblance with that of alkaloids of the (+)-crinane-skeleton (14). The methylenedioxy group was assigned by the presence of the characteristic doublets δ_H 5.81 and 5.80 (*J* =1.4 Hz, H_2-

15). Two doublets characteristic for the typical AB pattern of the benzylic H-6 protons were observed at δ_H 4.2 and 3.58 assigned to H-6ß and H-6α, respectively, with a large coupling constant (J = 17 Hz). 2D NOE correlations between H-4a and the low field signal at δ_H 4.2 and between H-12 *endo* and the up field signal δ_H 3.58 confirmed the previous assignment. The spectrum showed a doublet of doublet δ_H 3.88 was assigned for H-3. The large coupling between H-3 and H-4α (J = 9 Hz) as well as the NOE correlation between H-3 and H-4α were also indicative of the *cis*-relationship between the C-3 pseudoequatorial substituent and the 5,10b-ethano-bridge (16). Another double doublet δ_H 3.14 was assigned to H-4a and two (ddd) at δ_H 2.04 and 1.98 were assigned to H-4α and H-4ß, respectively. A singlet δ_H 3.32 was indicative for the C-3 methoxy group. The coupling pattern of protons of the 5,10b-ethano bridge that were represented by the three doublets at δ_H 3.92, 3.30 and 3.25 together with the pronounced deshielding of H-11*endo* as well as C-11 in ^{13}C NMR in relation to alkaloids with no bridge substituents were indicative to substitution at C-11. In addition, the NOE effect between H-10 and H-11*endo* was consistent with a hydroxyl substituent at the *exo*-position. The carbon signal of C-9 was assigned at lower field than C-8 because of its three bond connectivities with H-7, in addition to, the quaternary carbons C-6a and C-10a were ascribed by means of their HMBC correlations with H-10 and H-7, respectively. The aliphatic region of the spectrum was characterized by one singlet for the quaternary carbon C-10b, three singlets for the methine carbons C-3, C-4a and C-11, three singlets for the methylene carbons C-4, C-6 and C-12, and one singlet for the methoxyl group at C-3. From the previous data, the compound **2** is crinamine, which was previously isolated from *Crinum augustum* Rox. (17).

Positive and negative modes HR-ESI-MS of **3** revealed the molecular ion peak at *m/z* 286.154 $[M+H]^+$ and 284.0 $[M-H]^-$ respectively and showed it has the molecular formula $C_{17}H_{19}NO_3$. UV spectrum of **3**, exhibited three maxima absorption (λ_{max}292, 241 and 208 nm) which indicated the presence of a methylenedioxy-substituted benzene ring and is coincided with the crinine nucleus (13). The ^{13}C NMR spectrum of **3** showed 17 signals. The aliphatic region of the spectrum was characterized by one singlet for the quaternary carbon, two singlets for the methine carbons, four singlets for the methylene carbons, and one singlet for the methoxy group. ^{1}H NMR spectrum of **3** showed two singlets for the two-para aromatic protons H-10 and H-7 of ring A at δ_H 6.77 and 6.47, respectively. The observed NOE correlations is between H-10 and H-1; H-7 and H-6. The olefinic part of the spectrum showed a close resemblance with that of alkaloids of the (-)-crinine-skeleton (18). The two olefinic protons appeared at δ_H 6.38 (d, J = 10 Hz, H-1) and δ_H 6.04 (ddd, J = 10, 5.1, 1.1 Hz, H-2) as an AB pattern of an ABX system partially overlapped by the methylenedioxy signals that appeared as doublet at δ_H 5.85 and 5.87 (J = 1.4 Hz) due to geminal coupling. The olefinic proton H-2 was further splitted by H-4ß. Two doublets characteristic for the typical AB pattern of the benzylic H-6 protons were observed at δ_H 4.63 ppm and 4.06 ppm assigned to H-6α and H-6ß, respectively. H-6α was shifted downfield due to the nitrogen lone pair (19). The 2D NOE correlations between H-4a and the low field signal at δ_H 4.63 ppm and between H-12 *endo* and the up field signal at δ_H 4.06 distinguished the two protons and confirmed the previous assignment. Their large coupling (J = 16.3 Hz) is due to their *trans*-diaxial configuration (18). The multiplet at δ_H 3.77 was assigned to H-3, while the singlet at δ_H 3.27 was assigned to the methoxy group at C-3. Its stereochemistry could be determined at the pseudoaxial configuration due to the large coupling (J = 5.1 Hz) between H-3 and H-2, the small coupling (J = 3.9 Hz) between H-3 and H-4ß, the small coupling (J = 1.9 Hz) between H-3 and H-4α and the absence of allylic coupling between the vinylic H-1 and the allylic H-3. A doublet of doublet at δ_H 3.69 was assigned to H-4a which coupled with H-4α (J = 3.9 Hz) and H-4ß (J = 13.4 Hz). Both signals of H-4α and H-4ß were further splitted into (ddd) at δ_H 2.4 and 1.62, respectively, due to their mutual coupling (J = 13.4 Hz). The protons of the ethano-bridge were represented by the (ddd) at δ_H 2.2, 2.07, 3.12 and 3.84 excluding substitution at both C-11 and C-12. The lower field signal at δ_H 3.84 was attributed to H-12 *exo* not to H-12 *endo* because of its co-planarity with the nitrogen lone pair and the NOE correlation with H-4ß (22). The assignment of 2H-4, H-4a, 2H-11 and 2H-12 were confirmed by ^{1}H-^{1}H COSY and NOESY experiments. The previous assignments pointed at the alkaloid buphanisine, which was isolated from *Crinum augustum* (20).

Negative mode HR-ESI-MS of **4** revealed molecular ion peak at *m/z* 264.00 $[M-H]^-$ and indicates a molecular mass of 265 which corresponds to the molecular formula $C_{16}H_{11}NO_3$. UV spectrum of **4** exhibited three maxima absorption (λ_{max}259, 226 and 208 nm) characteristic for alkaloids of the lycorine type (15). ^{1}H

NMR spectrum showed six singlets in addition to two other triplet-like signals. The absence of the AB system of the benzylic H-7 protons that was replaced by the most downfield singlet at δ_H 9.26 was characteristic to the olefinic proton H-7 which is more deshielded by the nitrogen in these quaternary alkaloids of the lycorine type (21). Four aromatic singlets at δ_H 7.93, 7.59, 7.53 and 7.25 ppm, evidently indicated the presence of two sets of tetra-substituted benzene ring system that were assigned to H-11 and H-8 of ring A as well as H-1 and H-3 of ring C, respectively. The other singlet at δ_H 6.28 assigned to methylenedioxy group attached to ring A as a common feature among Amaryllidaceae alkaloids. Protons of ring D wre represented by the two triplet-like signals at δ_H 5.13 and 3.66 with a vicinal coupling constant of 6.6 assigned to 2H-5 and H_2-4, respectively; the former suffering more deshielded due to the neighboring nitrogen atom. The physical, chromatographic properties and spectral data of compound **4** are consistent with the data reported in literature (21), so that it was identified as the alkaloid ungeremine, which was previously isolated from *Crinum augustum* Rox. (22).

ESI-MS spectrum m/z 255.00 $[M-H]^-$ (negative mode) of **6**, showed to have the molecular formula $C_{16}H_{32}O_2$. Investigation of ^1H and spectral data led to classification of compound **6** as an aliphatic compound with a single double bond. ^1H NMR spectrum of **6** showed a triplet at δ_H 0.78 (J = 7 Hz) that was assigned to CH_3-14 representing a vicinal coupling with an adjacent methylene group. The crowded multiplets at δ_H 1.11-1.20 were suggestive of a long carbon chain of methylene groups. A triplet at δ_H 2.18 (J = 7.5) that was assigned to CH_2-2 indicated the attachment of this methylene group to a carbonyl function while the magnitude of the coupling constant were indicative to a vicinal coupling with an adjacent methylene group that was assigned to CH_2-3 and resonated as a multiplet at δ_H 1.51. The quartet at δ_H 4 (J = 7), that was assigned to CH_2-$1^\backslash$, was characteristic for a direct attachment to an oxygen atom and a vicinal coupling with an adjacent methyl group CH_3-$2^\backslash$, the later was overlapped with the crowded region at δ_H 1.11-1.20. The previously suggested coupling patterns told that the ^1H NMR spectrum of **6** is typical for a saturated fatty acid ethyl ester, that together with the obtained molecular formula, it could be identified as myristic acid ethyl ester. Myristic acid was previously isolated from *C. americanum* L. and *C. augustum* Rox. (23), while this is the first time for isolation of myristic acid ethyl ester from the genus *Crinum*.

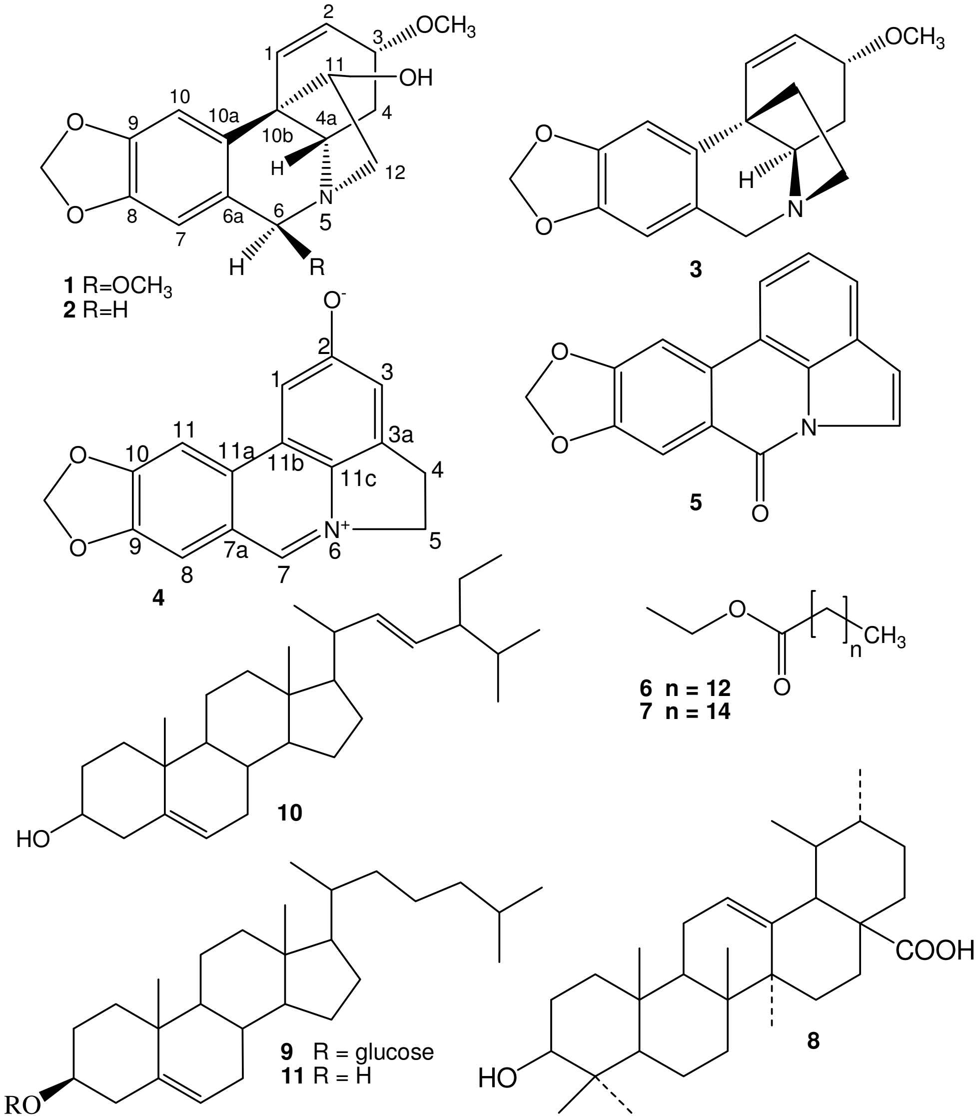
The HR-ESI-MS (negative mode) spectrum of compound **7** showed it to have the molecular formula $C_{18}H_{36}O_2$. ^1H NMR spectrum of **7** showed a large similarity to that of **6** indicating that they belong to the same homologous series. The triplet at δ_H 0.78 was assigned to CH_3-16, while the triplet at δ_H 2.2 (J = 7.3 Hz) that was assigned to CH_2-2 indicated the attachment of this methylene group to the carbonyl group of the carboxyl function and the magnitude of the coupling constant was typical to a vicinal coupling with the adjacent CH_2-3 group that resonated as a multiplet at δ_H 1.9-2.0. The ethyl group of the fatty acid ethyl ester could be simply detected through the quartet at δ_H 4 (J = 7.3) that was assigned to CH_2-$1^\backslash$ adjacent to the carboxyl function and was coupled with CH_3-$2^\backslash$ that resonated as a triplet in the crowded region of the other methylene groups of the fatty acid chain at δ_H 1.16-1.25. The crucial step of determining the chain length of the fatty acid could be attained through the HR-ESI-MS indicating that compound **7** is palmitic acid ethyl ester. Palmitic acid as well as its methyl ester were previously isolated from *C. bulbispermum* Milne. and *C. augustum* Rox. (26), while this is the first time for isolation of palmitic acid ethyl ester from the genus *Crinum*.

Finally, compounds **1**, **6**, **7** and **8** are first report in *Crinum augustum*. Compound **1** is isolated from *C. zeylanicum* (15) and this is the first time to be isolated from *Crinum augustum* Rox. Myristic acid was previously isolated from *C. americanum* L. and *C. augustum* Rox. (23), while this is the first time for isolation of myristic acid ethyl ester from the genus *Crinum*

The effect of compounds **1**, **2** and **3** on settlement and variability of cyprids (Table 1 and figure 2), with EC_{50}; 1.8, 1.2 and 0.75 µg/mL respectively. $CuSO_4$ (EC_{50} = 0.23 µg/mL) was used as a positive control. Compounds **1** and **2** significantly affected settlement at 1 µg/mL and completely inhibited at 3 µg/mL. The other compounds (**4**, **6**, **7**, **8**) showed no activity up to10 µg/mL. Compounds **1**-**3** have the same nucleus, thus, their mechanism of action are probably similar. This suggestion explains the similarity of their safety which coincided with $CuSO_4$ safety.

Many of the amaryllidaceous alkaloids are biosynthetically created by a phenol activated oxidative coupling reaction. In the laboratory a large number of oxidising agents have been successful in emulating nature (25).

***Figure 1**: Compounds isolated from Crinum augustum*



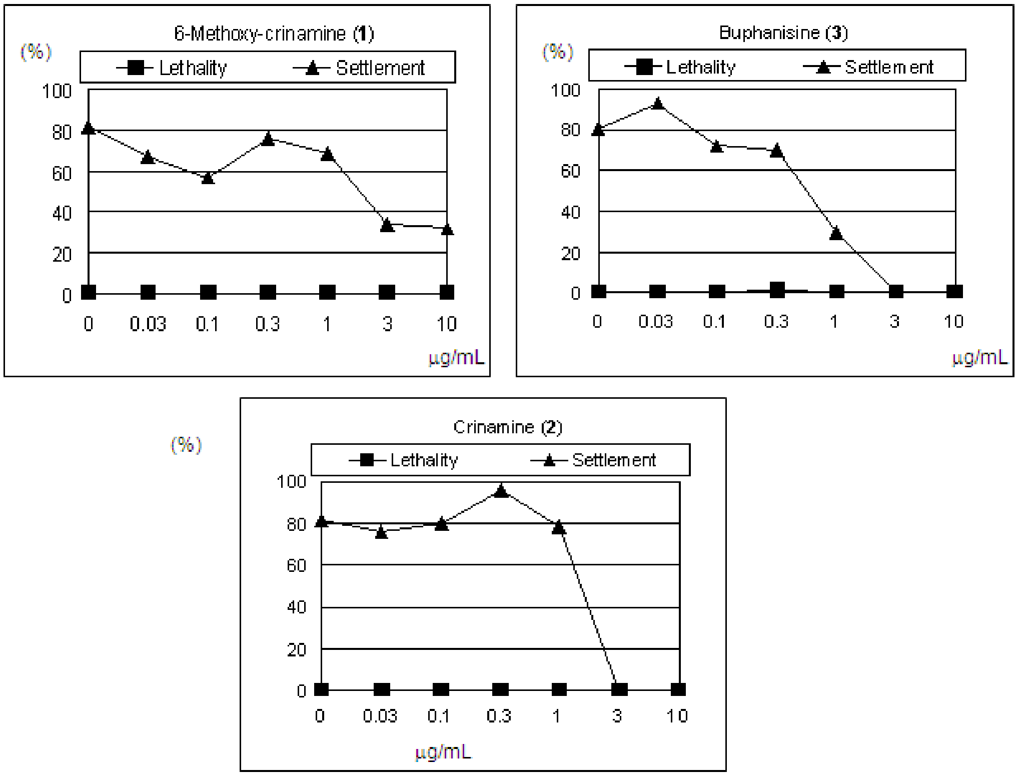


Figure 2: Antifouling activity and toxicity of 1-3 and $CuSO_4$ against Cyprid larvae after 48 h. The rate of settlement of Cyprid (▲) and mortality (■) in different concentrations were plotted.

Table 1: Antifouling activity and toxicity of 1-3 and $CuSO_4$ against Cyprid larvae after 48 h

Comp. No.	Effect (%)	Concentration (µg/mL)							*EC_{50} µg/mL*
		0.0	0.03	0.10	0.30	1.00	3.00	10.0	
1	Lethality	0	0	0	0	0	0	0	*1.80*
	Settlement	82	67	57	76	69	34	32	
2	Lethality	0	0	0	0	0	0	0	*1.20*
	Settlement	81	76	80	96	78	0	0	
3	Lethality	0	0	0	1	0	0	0	*0.75*
	Settlement	81	93	72	70	29	0	0	
$CuSO_4$	Settlement	85	87	79	35	05	0	0	0.23

Table 2: ^{1}H [CDCl$_3$, 400 MHz] Spectral Data for Compounds 1-3

Position	**δ ^{1}H** (Hz)		
	1	**2**	**3**
1	6.47 d (10.5)	6.16 br.s	6.38 d (10)
2	5.89 ddd (10, 5.1)	6.16 br.s	6.04 ddd (10, 5.1, 1.1)
3	3.74 m	3.88 dd (9, 6)	3.77 m
4α	1.80 ddd (13.6, 13.6, 4)	2.04 ddd (13, 11.7, 9)	2.40 ddd (13.4, 3.9, 1.9)
4β	1.50 ddd (13.6, 4.4, 4.1)	1.98 ddd (11.7, 6, 4.6)	1.62 ddd (13.4, 13.4, 3.9)
4a	3.79 dd (13.6, 4.4)	3.14 dd (13, 4.6)	3.69 dd (13.4, 3.9)
6α	5.05 s	3.58 d (17)	4.63 d (16.3)
6β	-------	4.20 d (17)	4.06 d (16.3)
6a	-------	-------	-------
7	6.71 s	6.39 s	6.47 s
8	-------	-------	-------
9	-------	-------	-------
10	6.90 s	6.71 s	6.77 s
10a	-------	-------	-------
10b	-------	-------	-------
11endo	3.65 dd (9, 4.6)	3.92 dd (6, 4)	2.20 ddd (12.7, 9, 4)
11 exo	-------	-------	2.07 ddd (12.7, 11, 6.6)
12endo	2.74 dd (13.2, 9)	3.30 dd (13.9, 6)	3.12 ddd (13.4, 9, 6.6)
12 exo	2.02 dd (13.2, 4.6)	3.25 dd (13.9, 4)	3.84 ddd (13.4, 11, 4)
13	3.25 s	3.32 s	3.27 s
14	3.32 s	-------	-------
15	5.83 d – 5.80 d (1.2)	5.81 d – 5.80 d (1.3)	5.85 d – 5.87 d (1.4)

Table 3: ^{13}C NMR [CDC$_3$l, 75.5 MHz] spectral data for compounds 1-3

Position	**δ_C**		
	1	**2**	**3**
1	139.0	135.9	130.1
2	126.3	123.7	127
3	72.6	76.1	71.6
4	28.3	30.2	26.7
4a	62.4	66.2	64.3
6	87.3	63.6	60.3
6a	127.6	126.7	121.0
7	109.9	106.8	107.5
8	146.4	146.2	147.3
9	147.7	146.5	147.8
10	102.9	103.2	103.7
10a	137.6	135.5	135.9
10b	45.3	50.3	45.3
11	77.6	80.0	42.1
12	57.1	61.2	52.8
13	56.7	55.7	57.2
14	56.9	------	------
15	102.7	100.8	101.7

6-OMe-Crinamine (1): (39 mg, 99% purity); yellowish oil; R$_f$ 0.48, silica gel 60 F$_{254}$ (Pet. ether-EtOAc 1:1); failed to respond to many trials of crystallization; $[\alpha]_D^{20}$= + 20.2° (MeOH; *c*. 0.0025 g/ml). UV λ_{max} nm: 292 (ε = 96652), 241 (ε = 79771), 205 (ε = 67855); ^{1}H-NMR (400 MHz, CDCl$_3$): δ 6.9 (1H, s, H-10), 6.71 (1H, s, H-7), 6.47 (1H, d, J = 10.5 Hz, H-1), 5.89 (1H, ddd, J = 10, 5.1 Hz, H-2), 5.83–5.80 (2H, 2d, J = 1.2 Hz, OCH$_2$O), 5.05 (1H, s, H-6α), 3.79 (1H, dd, J = 13.6, 4.4 Hz, H-4a), 3.74 (1H, m, H-3), 3.65 (1H, dd, J = 9, 4.6 Hz, H-11 endo), 3.32 (3H, s, 6-OCH$_3$), 3.25 (3H, s, 3-OCH$_3$), 2.74 (1H, dd, J = 13.2, 9 Hz, H-12 endo), 2.02 (1H, dd, J = 13.2, 4.6 Hz, H-12 exo), 1.8 (1H, ddd, J = 13.6, 13.6, 4 Hz, H-4α), 1.5 (1H, ddd, J = 13.6, 4.4, 4.1 Hz, H-4β), ^{13}C-NMR (75 MHz, CDCl$_3$): 147.7 (s, C-9), 146.4 (s, C-8), 139 (d, C-1), 137.6 (s, C-10a), 127.6 (s, C-6a), 126.3 (d, C-2), 109.9 (d, C-7), 102.9 (d, C-10), 102.7 (t, OCH$_2$O), 87.3 (t, C-6), 77.6 (d, C-11), 72.6 (d, C-3), 62.4 (d, C-4a), 57.1 (t, C-12), 56.9 (q, 6-OCH$_3$), 56.7 (q, 3-OCH$_3$), 45.3 (s, C-10b), δ 28.3 (t, C-4); HR-ESI-MS [positive mode] *m/z* 332.14 [M+H]$^+$ (calculated for C$_{18}$H$_{21}$NO$_5$, 331.14203).

Crinamine (2); (60 mg, 100% purity); colorless needles; R$_f$ 0.41, silica gel 60 F$_{254}$ (Pet. ether-EtOAc 1:1); mp.200 °C ;$[\alpha]_D^{20}$ = + 356.8° (MeOH; *c*. 0.0025 g/ml). UV λ_{max} nm: 292 (ε = 87892), 241 (ε = 72541), 208 (ε = 62608). ^{1}H-NMR (400 MHz, CDCl$_3$): *δ* 6.71 (1H, *s*, H-10), 6.39 (1H, *s*, H-7), 6.16 (2H, *s*, H-1 and H-2), 5.81–5.80 (2H, 2*d*, *J* = 1.3 Hz, OCH$_2$O), 4.20 (1H, *d*, *J* = 17 Hz, H-6β), 3.92 (1H, *dd*, *J* = 6, 4 Hz, H-11 *endo*), 3.58 (1H, *d*, *J* = 17 Hz, H-6α), 3.32 (3H, *s*, 3-OCH$_3$), 3.30 (1H, *dd*, *J* = 13.9, 6 Hz, H-12 *endo*), 3.25 (1H, *dd*, *J* = 13.9, 4 Hz, H-12 *exo*), 3.14 (1H, *dd*, *J* = 13, 4.6 Hz, H-4a), 2.04 (1H, ddd, *J* = 13, 11.7, 9 Hz, H-4α), 1.98 (1H, ddd, J = 11.7, 6, 4.6 Hz, H-4β). ^{13}C-NMR (75 MHz, CDCl$_3$): *δ* 146.5 (*s*, C-9), 146.2 (*s*, C-8), 135.9 (*d*, C-1), 135.5 (*s*, C-10a), 126.7 (*s*, C-6a), 123.7 (*d*, C-2), 106.8 (*d*, C-7), 103.2 (*d*, C-10), 100.8 (*t*, OCH$_2$O), 80 (*d*, C-11), 76.1 (*d*, C-3), 66.2 (*d*, C-4a), 63.6 (*t*, C-6), 61.2 (*t*, C-12), 55.7 (*q*, 3-OCH$_3$), 50.3 (*s*, C-10b), 30.2 (*t*, C-4). HR-ESI-MS [positive mode] *m/z* 302.13 [M+H]$^+$ and [negative mode] *m/z* 300.11 [M-H]$^-$ (calculated for C$_{17}$H$_{19}$NO$_4$, 301.13147).

Buphanisine (3); (34.5 mg, 100% purity); yellowish oil; R$_f$ 0.49, silica gel 60 F$_{254}$ (Pet. ether-EtOAc 1:1); failed to respond to many trials of crystallization; $[\alpha]_D^{21}$ = -39.2° (MeOH; *c* 0.0025 g/ml). UV λ_{max} nm: 292 (ε = 83320), 241 (ε = 68685), 208 (ε = 59280). ^{1}H-NMR (400 MHz, CDCl$_3$): *δ* 6.77 (1H, *s*, H-10), 6.47 (1H, *s*, H-7), 6.38 (1H, *d*, *J* = 10 Hz, H-1), 6.04 (1H, *ddd*, *J* = 10, 5.1, 1.1 Hz, H-2), 5.85–5.87 (2H, 2*d*, *J* = 1.4 Hz, OCH$_2$O), 4.63 (1H, *d*, *J* = 16.3 Hz, H-6α), 4.06 (1H, *d*, *J* = 16.3 Hz, H-6β), 3.84 (1H, *ddd*, *J* = 13.4, 11, 4 Hz, H-12 *exo*), 3.77 (1H, *m*, H-3), 3.69 (1H, *dd*, *J* = 13.4, 3.9 Hz, H-4a), 3.27 (3H, *s*, 3-OCH$_3$), 3.12 (1H, *ddd*, *J* = 13.4, 9, 6.6 Hz, H-12 *endo*), 2.4 (1H, *ddd*, *J* = 13.4, 3.9, 1.9 Hz, H-4α), 2.2 (1H, *ddd*, *J* = 12.7, 9, 4 Hz, H-11 *endo*),), 2.07 (1H, *ddd*, *J* = 12.7, 11, 6.6 Hz, H-11 *exo*), 1.62 (1H, *ddd*, *J* = 13.4, 13.4, 3.9 Hz, H-4β). ^{13}C-NMR (75 MHz, CDCl$_3$): *δ* 147.8 (*s*, C-9), 147.3 (*s*, C-8), 135.9 (*s*, C-10a), 130.1 (*d*, C-1), 127 (*d*, C-2), 121 (*s*, C-6a), 107.5 (*d*, C-7), 103.7 (*d*, C-10), 101.7 (*t*, OCH$_2$O), 71.6 (*d*, C-3), 64.3 (*d*, C-4a), 60.3 (*t*, C-6), 57.2 (*q*, 3-OCH$_3$), 52.8 (*t*, C-12), 45.3 (*s*, C-10b), 42.1 (*t*, C-11), 26.7 (*t*, C-4). HR-ESI-MS [positive mode] m/z 286.154 [M+H]$^+$ and [negative mode] *m/z* 284 [M-H]$^-$ (calculated for C$_{17}$H$_{19}$NO$_3$, 285.13657).

Ungeremine (4); (42 mg, 99% purity); yellow crystals; R$_f$ 0.39, silica gel 60 F$_{254}$ (CHCl$_3$-MeOH 8:2); mp. 256-257 °C ;UV λ_{max} nm: 259 (ε = 68635), 226 (ε = 59890), 208 (ε = 55120). ^{1}H-NMR (400 MHz, CD$_3$OD): *δ* 9.26 (1H, *s*, H-7), 7.93 (1H, *s*, H-11), 7.59 (1H, *s*, H-8), 7.53 (1H, *s*, H-1), 7.25 (1H, *s*, H-3), 6.28 (2H, *s*, OCH$_2$O), 5.13 (2H, *t-like*, *J* = 6.6 Hz, 2H-5), 3.66 (2H, *t-like*, *J* = 6.6 Hz, 2H-4), HR-ESI-MS [negative mode] *m/z* 264 [M-H]$^-$ (calculated for C$_{16}$H$_{11}$NO$_3$, 265.07393).

Myristic acid ethyl ester (6); (199 mg, 100% purity); yellowish oil; R$_f$ 0.46, silica gel 60 F$_{254}$ (pet. ether-CHCl$_3$ 8:2); ^{1}H-NMR (400 MHz, CDCl$_3$): *δ* 4.00 (2H, *q*, *J* = 7 Hz, 1'-CH$_2$), 2.18 (2H, *t*, *J* = 7.5 Hz, 2-CH$_2$), 1.51 (2H, *p*, *J* = 7.3 Hz, 3-CH$_2$), 1.11-1.20 (*m*, 2′-CH$_3$ and other CH$_2$ protons), 0.78 (3H, *t*, *J* = 7 Hz, 14-CH$_3$). HR-ESI-MS [negative mode] *m/z* 255 [M-H]$^-$ (calculated for C$_{16}$H$_{32}$O$_2$, 256.24036).

Palmitic acid ethyl ester (7); (12.7 mg, 99% purity); yellowish oil; R$_f$ 0.69, silica gel 60 F$_{254}$ (pet. ether-CHCl$_3$ 8:2); ^{1}H-NMR (400 MHz, CDCl$_3$): *δ* 4.00 (2H, *q*, *J* = 7.3 Hz, 1'-CH$_2$), 2.2 (2H, *t*, J = 7.3 Hz, 2-CH$_2$), 1.9-2 (2H, *m*, 3-CH$_2$), 1.16-1.25 (*m*, 2′-CH$_3$ and other CH$_2$ protons), 0.78 (3H, *t*, *J* = 7.3Hz, 16-CH$_3$). HR-ESI-MS [negative mode] *m/z* 283.26 [M-H]$^-$ (calculated for C$_{18}$H$_{36}$O$_2$, 284.27168).

Ursolic acid (8); (26.5 mg, 99% purity); yellowish white powder; R$_f$ 0.49, silica gel 60 F$_{254}$ (pet. ether-EtOAc 9:1); mp.260-262 °C; ^{1}H-NMR (400 MHz, CDCl$_3$): *δ* 5.12 (1H, *br.s*, H-12), 4.4 (1H, d, J = 7, H-3α), 2.18 (1H, *d*, *J* = 11.5, H-18β), 0.9-1.9 (*m*, CH$_2$ groups), 0.76-0.82 (*m*, CH$_3$ groups). HR-ESI-MS [negative mode] *m/z* 455 [M-H]$^-$ (calculated for C$_{30}$H$_{48}$O$_3$, 456.36054).

β-sitosterol-O-glucoside (9); (26 mg, 100% purity); white amorphous powder; R$_f$ 0.23, silica gel 60 F$_{254}$ (CHCl$_3$-MeOH 9:1); mp.276-277 °C.

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