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(−)-Amarbellisine, a lycorine-type alkaloid from *Amaryllis belladonna* L. growing in Egypt

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Abstract

A new lycorine-type alkaloid, named (−)-amarbellisine, was isolated from the bulbs of Egyptian *Amaryllis belladonna* L. together with the well known alkaloids (−)-lycorine, (−)-pancracine, (+)-vittatine, (+)-11-hydroxyvittatine, and (+)-hippeastrine. The new alkaloid, containing the pyrrolo[*de*]phenanthridine ring system, was essentially characterised by spectroscopic and optical methods, and proved to be the 2-methoxy-3a,4,5,7,11b,11c-hexahydro-1*H*-[1,3]dioxolo[4,5-*j*]pyrrolo[3,2,1-*de*]phenanthridinol.

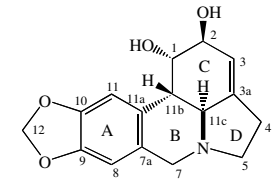
By using HPTLC technique we also carried out a comparative study of the relative and total alkaloidal content at two different stages of plant growth. Finally, the antimicrobial activity of the isolated alkaloids was assayed.

Keywords: *Amaryllis belladonna* L.; Amaryllidaceae; (−)-Amarbellisine; Lycorine-type alkaloid; Antimicrobial activity; HPTLC technique

1. Introduction

Amaryllis belladonna L. (also named *Hippeastrum equestre*) is cultivated in Egypt as an ornamental plant. Amaryllidaceae species are an exclusive source of Amaryllidaceae alkaloids that possess wide range of interesting biological activities being cytotoxic (Pettit et al., 1984) and antimicrobial compounds (Elgorashi and Staden, 2004).

Although some species of the genus *Amaryllis*, including *A. belladonna* L., have been employed in folk medicine (Pettit et al., 1984), no recent reports were noted on the alkaloids of Egyptian *A. belladonna* L. Based on our interest in some Egyptian Amaryllidaceae plants, we carried out the present study on the title plant. This investigation resulted in the isolation of six crystalline alkaloids including the well-known (−)-lycorine (**1**, obtained in very large amount), (−)-pancracine (**4**), (+)-vittatine **(5)**, and (+)-11-hydroxyvittatine (**6**). The remaining two alkaloids appeared to be (+)-hippeastrine (**3**) and a new alkaloid (**2**).



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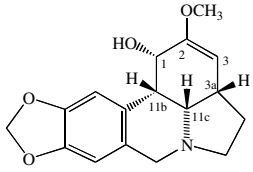

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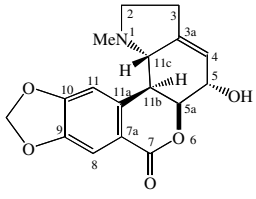

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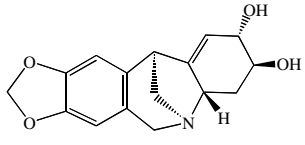

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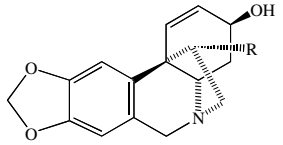

5 R=H; **6** R=OH

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This paper describes the isolation and chemical and biological characterisation of the isolated alkaloids. The structure of the new lycorine-type alkaloid, (−)-amarbellisine (**2**), was determined by extensive use of spectroscopic (IR, NMR, MS) and optical (CD) methods; the complete NMR study of hippeastrine (**3**) is also presented for the first time. In addition, a comparative study of the relative and total alkaloidal content of the plant bulbs in the preflowering and flowering stages of growth was carried out using HPTLC technique. Finally, the antimicrobial activity of isolated alkaloids was tested for the first time.

2. Results and discussion

Six crystalline alkaloids were isolated from the bulbs of *A. belladonna* L. cultivated in Egypt. Four of them were identified as (−)-lycorine (**1**), (−)-pancracine (**4**), (+)-vittatine (**5**), and (+)-11-hydroxyvittatine (**6**) on the basis of published spectral data, and by comparison with reference alkaloid samples (Co-TLC and m.mp). These alkaloids were also described as metabolites of *H. equestre* (Wagner et al., 1996; Rhee et al., 2001), and other Amaryllidaceae plants (Wildman, 1968; Ghosal et al., 1985; Labrana et al., 2002). The remaining two alkaloids were characterised by extensive use of spectroscopic (NMR and MS) and optical (CD) techniques. One of them was identified as (+)-hippeastrine (**3**), previously isolated from *A. belladonna* (Wagner et al., 1996) and from other Amaryllidaceae plants (Wildman, 1968; Ghosal et al., 1985), while the other proved to be a new alkaloid. By extensive use of NMR spectroscopy we assigned the whole proton spectrum of (+)-hippeastrine for first time. Furthermore, the ^{13}C chemical shifts of the aromatic quaternary carbons (C-7a, C-9, C-10, and C-11a) were revised with respect to the values previously reported (Jeff et al., 1985) and also reflying on NMR studies of other alkaloids belonging to lycorine- (Evidente et al., 1983) and lycorenine- (Evidente et al., 1999) type. The structure assigned to alkaloid **3** was also confirmed by mass spectra. In fact, the EI mass spectrum, beside the molecular ion at m/z 315, showed peaks m/z 190, 125, 124 and 96 generated through fragmentation mechanisms characteristic for lycorenine-type alkaloids (Ibuka et al., 1966; Jeff et al., 1985). The ESI spectrum showed clustered potassium and sodium ions at m/z 354 and 338, while the pseudomolecular ion $[M + H]^+$ was observed at m/z 316. Its absolute stereochemistry was corroborated by the CD spectrum, which is in agreement with those reported (Jeff et al., 1985; Wagner et al., 1996).

Preliminary spectroscopic data showed that **2** is correlated with (−)-lycorine (**1**). Its ^1H NMR spectrum (Table 1) differed from that of **1** (Evidente et al., 1983) only for the absence of the diol system signal present between C-1 and C-2, with the broad singlet, resonating at δ 3.48 and due to the proton of a secondary hydroxylated carbon, assigned to H-1. In the COSY spectrum (Braun et al., 1998) it correlated with the

Table 1
^1H and ^{13}C NMR data of (−)-amarbellisine (**2**). The chemical shift are in δ values (ppm) from TMS[a]

C	δ[b]	δH	J (Hz)	HMBC
1	79.8 *d*	3.48 *br s*	2.5, 2.3, 1.8	3.43, 2.14
2	154.3 *s*			3.48, 3.41, 3.28, 2.14, 1.56
3	112.9 *d*	5.56 *br s*	2.3, 2.3	3.48, 3.28
3a	58.6 *d*	3.41 *br ddd*	11.8, 5.4, 2.3	5.56, 3.48, 3.28, 2.14, 1.56
4	32.7 *t*	2.14 *ddd*	12.9, 5.4, 3.4	3.41
		1.56 *ddd*	12.9, 11.8, 3.7	
5	55.4 *t*	3.07 *dd*	11.2, 2.2	3.79
		3.02 *d*	11.2	
7	60.9 *t*	4.33 *d*	16.7	6.45, 3.07, 3.02
		3.79 *d*	16.7	
7a[c]	132.5 *s*			6.45, 4.33, 3.79, 3.28
8	107.3 *d*	6.45 *s*		6.54, 4.33, 3.79
9[c]	146.0 *s*			6.45, 5.88, 5.86, 4.33, 3.79
10[c]	146.7 *s*			6.54, 5.88, 5.86
11	106.8 *d*	6.54 *s*		6.45, 3.28
11a[c]	124.6 *s*			6.54, 4.33, 3.79, 3.28
11b	45.6 *d*	3.28 *br s*	1.8	6.54, 5.56, 3.79
11c	69.1 *d*	4.08 *br s*	3.7, 3.4, 2.5	5.56, 3.48, 2.14, 1.56
12	100.7 *t*	5.88 *d*	1.1	
		5.86 *d*	1.1	
OMe	57.6 *q*	3.43 *s*		

[a] 2D ^1H, ^1H (COSY) and 2D ^{13}C, ^1H (HSQC) NMR experiments delineated the correlations of all protons and the corresponding carbons.
[b] Multiplicities determined by DEPT spectrum.
[c] Assigned also in agreement with the value reported for the same carbons in structurally close alkaloids (Evidente et al., 1983).

broad singlet at δ 5.56, typical of an olefinic proton (H-3) (Pretsch et al., 1989), which in turn, coupled with a broad doublet of double doublet ($J = 11.8$, 5.4 and 2.3 Hz) assigned to the proton linked to C-3a. The latter represents one of the bridgehead carbon of the C/D ring-junction. If the C ring adopts a chair-like conformation H-3a should be axial according to its coupling with H-11c ($J \sim 1$–2 Hz) and with the two protons of the adjacent pyrrole methylene group (H_2C-4) at δ 2.14 and 1.56, which appeared as two doublets of double doublets ($J = 12.9$, 5.4 and 3.4, and $J = 12.9$, 11.8 and 3.7, respectively) (Sternhell, 1969; Pretsch et al., 1989). H-11c, resonating as a broad singlet at δ 4.08, is linked to the bridgehead carbon (C-11c) of both B/C and C/D rings-junction, and should be equatorial being also coupled ($J \sim 1$–2 Hz) with H-11b, also appearing as a broad singlet at δ 4.08. The latter, linked to the other bridgehead carbon (C-11b) of B/C ring-junction, should be axial also for the typical axial-equatorial coupling ($J = 1.8$ Hz) with H-1 (Sternhell, 1969; Pretsch et al., 1989), which, consequently, should be equatorial. Therefore, its geminal hydroxy group is axial, and both B/C and C/D ring fusion have a *cis*-stereoschemistry. The partial structures of the C and D rings are consistent with the typical hydroxy and olefinic bands observed in the IR spectrum of **2** (Nakanishi and Solomon, 1977), and were further supported by the correlations observed in the HSQC spectrum (Braun et al., 1998), which allowed the assignment of the chemical shift of δ 79.8, 112.9, 58.6, 32.7, 45.6 and 69.1 to C-1, C-3, C-3a, C-4, C-11b and C-11c. Other significant differences between **1** and **2** were the location of the double bond in the C ring and its substituents, and the presence of a methoxy group resonating at the typical chemical shift values of δ 3.43 (^{1}H) and 57.6 (^{13}C), respectively (Breitmaier and Voelter, 1987; Pretsch et al., 1989). The olefinic group of the C ring in **2** is always trisubstituted as in **1**, but is located between C-2 and C-3 instead of C-3 and C-3a as in **1**. This is safely deduced from the ^{1}H chemical shift and coupling constants above described for H-3, and from the typical chemical shift values of δ 112.9 and 154.3 recorded for C-3 and C-2 in the ^{13}C NMR spectrum, with C-2 linked to the methoxy group (Breitmaier and Voelter, 1987). On the basis of the correlations observed in the COSY and HSQC and the data already reported for lycorine (Evidente et al., 1983) the chemical shifts of all protons and carbons could be assigned (Table 1).

Therefore, the structure of a $\Delta^{2,3}$-2-dehydroxy-2-methoxy-3a-hydrolycorine was assigned to (−)-amarbellisine, which can be formulated as the 2-methoxy-3a,4,5,7,11b,11c-hexahydro-1*H*-[1,3]dioxolo[4,5-*j*]pyrrolo-[3,2,1-*de*]phenanthridinol. This structure was consistent with the ^{1}H, ^{13}C NMR long-range correlations and NOEs observed in HMBC (Braun et al., 1998) (Table 1) and NOESY (Braun et al., 1998) (Table 2) spectra, respectively.

Finally, the structure assigned to **2** was supported by the mass spectra data. The HR EI mass spectrum showed the molecular ion at m/z 301.1302, and significant peaks at m/z 286 and 270 (due to the expected losses of Me and MeO from the parent ion) and those at m/z 252 and 226, which are generated through characteristic and diagnostic fragmentation already described for other lycorine-type alkaloids (Ibuka et al., 1966). The ESI spectrum (positive mode) showed the potassium and sodium $[M + K]^+$ and $[M + Na]^+$, and the pseudomolecular $[M + H]^+$ ions at m/z 340, 324 and 302, respectively, while the pseudomolecular and the molecular ions by the loss of H_2O and MeO residues generated the ions observed at m/z 284 and 270, respectively.

The relative stereochemistry of (−)-amarbellisine depicted in **2** was assigned on the basis of the coupling constants described above and the CD data. **2**, which shows a *cis* B/C-ring junction, exhibited a CD spectrum different from that of lycorine and other phenanthridine-type alkaloids having a *trans* B/C ring fusion. It resembled instead those of Amaryllidaceae alkaloids belonging to other subgroups but having a *cis* B/C ring fusion (Wagner et al., 1996). This relative streochemistry is consistent with the inspection of a Dreiding model of **2** and is in agreement with the NOEs reported in Table 2.

The results of antibacterial and antifungal screening (Table 3) showed that (−)-amarbellisine, (−)-pancracine, (+)-vittatine and (+)-11-hydroxyvittatine have antibacterial activity against the Gram-positive *Staphylococcus aureus*. Both (−)-amarbellisine and (+)-vittatine exhibited activity against the Gram-negative *Escherichia coli* whereas (−)-pancracine showed activity against

Table 2
2D ^{1}H-NOE (NOESY) data obtained for (−)-amarbellisine (**2**)

Considered	Effects	Considered	Effects
6.54 (H-11)	3.28 (H-11b)	3.41 (H-3a)	5.56 (H-3), 4.08 (H-11c), 2.14 (H-4), 1.56 (H-4′)
6.45 (H-8)	4.33 (H-7), 3.79 (H-7′)	3.28 (H-11b)	6.54 (H-11), 5.56 (H-3)
5.56 (H-3)	3.43 (OMe), 3.41 (H-3a), 3.28 (H-11b)	3.07 H-5)	4.33 (H-7)
4.33 (H-7)	6.45 (H-8), 3.79 (H-7′), 3.07 (H-5), 3.02 (H-5′)	3.02 (H-5′)	4.33 (H-7), 1.56 (H-4′)
4.08 (H-11c)	3.41 (H-3a), 2.14 (H-4),	2.14 (H-4)	4.08 (H-11c), 3.41 (H-3a), 1.56 (H-4′)
3.79 (H-7′)	6.45 (H-8), 4.33 (H-7)	1.56 (H-4′)	3.41 (H-3a), 3.02 (H-5′), 2.14 (H-4)
3.43 (MeO)	5.56 (H-3), 1.56 (H-4′)		

Table 3
Results of the antibacterial and antifungal screening of different alkaloids (**1**–**6**) isolated from bulbs of *A. belladonna* L.

Alkaloid	Inhibition zone in mm				MIC (μg/ml)	
	Bacteria			Fungi		
	Gram positive	Gram-negative				
	Staphylococcus aureus	*Escherichia coli*	*Pseudomonas aeroginosa*	*Candida albicans*	*Staphylococcus aureus*	*Candida albicans*
(−)-Lycorine	–	–	–	40	–	39
(−)-Amarbellisine	22	22	–	24	125	63
(−)-Hippeastrine	–	–	–	25	125	125
(−)-Pancracine	22	–	16	15	188	188
(+)-Vittatine	19	22	–	17	63	31
(+)-11-Hydroxyvittatine	17	–	–	20	219	156

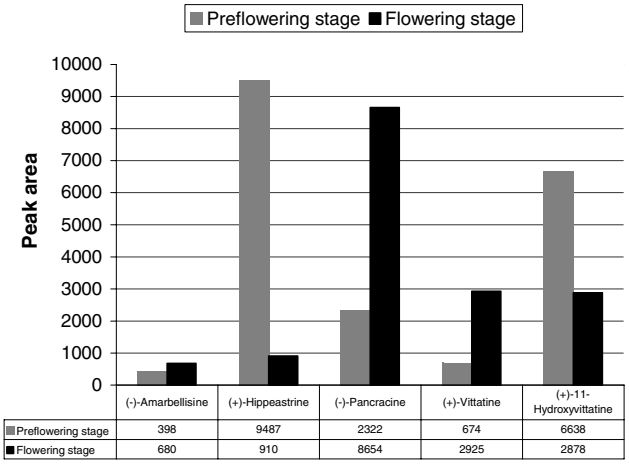

Fig. 1. Relative alkaloid content in the bulbs of *A. belladonna* L. in the preflowering and flowering stages determined using HPTLC.

Pseudomonas aeroginosea. Furthermore, all isolated alkaloids especially (−)-lycorine, (−)-amarbellisine and (+)-hippeastrine showed antifungal activity against *Candida albicans*.

The HPTLC study aimed at comparing the relative amounts of the alkaloids present in the bulbs of *A. belladonna* L. in the flowering stage (on April), and in the preflowering stage (on November). As illustrated in Fig. 1, (+)-hippeastrine is the major alkaloid in the preflowering stage, while (−)-pancracine dominates in the flowering stage. (−)-Amarbellisine and (+)-vittatine are present in higher amount in the flowering stage, while (+)-11-hydroxyvittatine is present in greater concentration in the preflowering stage. Furthermore, the total alkaloidal content is slightly higher in the preflowering stage (total peak areas = 19,519) than the flowering one (total peak areas = 16,047) (Stacey and Sherma, 2001; Jamshidi et al., 2000).

Investigation of the Amaryllidaceae alkaloids began in 1877 obtaining lycorine from *Narcissus pseudonarcissus* (Cook and Loudon, 1952), and interest in these compounds has increased ever since because of their antitumoral and antiviral activities. From 150 species belonging to 36 genera hundreds of new alkaloids have been isolated from different parts in different vegetation periods, and can be grouped in 12 ring-type alkaloids (Ghosal et al., 1985). The advances on the isolation and chemical and biological characterisation have been extensively reviewed (Bastida et al., 1998; Evidente, 2000; Evidente and Motta, 2002). Here we report the first isolation of (−)-amarbellisine (**2**), as natural occurring compound and as a metabolite of an Amaryllidaceae plant, which at best of our knowledge represents the first case of a lycorine-type alkaloid with a *cis* B/C ring junction.

3. Experimental

3.1. General

Uncorr. mps. were determined on a Sturat SMP heating stage microscope; the optical rotations were measured in $CHCl_3$ solution, unless otherwise noted, on JASCO P-1010 digital polarimeter, and the CD spectra were recorded in MeOH solution on a JASCO J-715 spectropolarimeter; IR and UV spectra were determined in KBr and MeOH, respectively, on Beckman 4210 infrared Perkin–Elmer Lambda 3B UV/VIS spectrophotometers. ^{1}H- and ^{13}C-NMR spectra were recorded at 500, 400 and 300 and at 125 and 75 MHz, respectively, in $CDCl_3$, on Varian and Bruker spectrometers. The solvent peak was used as internal standard. Carbon multiplicities were determined by DEPT spectra. DEPT and COSY-45, HSQC and HMBC experiments (Braun et al., 1998) were performed using Bruker and Varian microprograms. EI MS and high resolution EIMS were taken at 70 eV on a Fison Trio-2000 and a Fison ProSpec spectrometer, respectively. Electrospray MS were recorded on a Perkin–Elmer API 100 LC-MS; a probed voltage of 5300 V and a declustering potential of 50 V were used. Analytical and preparative TLC were performed on silica gel (Merck, Kieselgel, 60 F_{254}, 0.25 and 0.50 mm, respectively) plates; the spots were visualized by exposure to I_2, UV radiation

or Dragendorff's spray reagent. CC: silica gel (Merck, Kieselgel 60, 0.063–0.20 mm). Solvent systems: (A) $CHCl_3$–MeOH (9:1); (B) $CHCl_3$–EtOAc–MeOH (2:2:1). HPTLC comparative analysis was performed on Merck 20 cm × 10 cm silica gel 60 F_{254} (0.25 mm) plates. Sample solutions were applied by means of a Camag (Wilmington, NC) Linomat IV automated spray-on band applicator. Zones were quantified by linear scanning at 254 nm with a Camag TLC Scanner II with a deuterium source in the reflection mode, slit dimension settings of length 6 and width 0.1, monochromator bandwidth 20 nm, a scanning rate of 10 mm/s. The peak areas of chromatograms were determined using CATS TLC software (version 4.X).

3.2. Plant materials

A. belladonna L. was collected in November 2002 (preflowering stage) and in April 2002 (flowering), cultivated in Alexandria, Egypt. The plant was kindly identified by Prof. Alam El-Din Negm (Head of Ornamental Plants Department, Faculty of Agriculture, Alexandria University, Egypt). A voucher sample is deposited in the Department of Pharmacognosy, Faculty of Pharmacy, Alexandria.

3.3. Extraction and purification of alkaloids

Freshly chopped bulbs in the flowering stage (4 kg) were exhaustively extracted with EtOH by percolation. The combined extracts were concentrated under reduced pressure then defatted with petroleum ether, acidified with 5% tartaric acid to pH 2, filtered and then washed with Et_2O. The acidic aqueous phase was rendered alkaline with NH_4OH solution to pH 10, and then extracted successively with $CHCl_3$, EtOAc and *n*-BuOH. The $CHCl_3$ extracts were combined, concentrated to a small volume, at this stage a white residue (0.9 g) was precipitated and identified as (−)-lycorine **(1)** by comparison against a reference sample and filtered out. The filtrate was evaporated under reduced pressure to give a residue (3 g), which was fractionated over a silica gel column. Elution was started by $CHCl_3$, increasing the polarity with MeOH. Fractions (100 ml each) were collected monitored by TLC (solvent systems A and B). Chromatographic separation resulted in the isolation of the previously reported (−)-pancracine, (+)-vittatine and (+)-11-hydroxyvittatine (**4**, **5** and **6**) (47, 36, 40 mg, respectively) identified by using the available spectral data together with comparison with reference alkaloidal samples (Co-TLC and m.mp). Fractions eluted with 6% MeOH in $CHCl_3$ was further purified by successive prep. TLC (eluent A) to give a colourless crystalline alkaloid (15 mg, R_f 0.68), which as below described proved to be (+)-hippeastrine (**3**). The two successive fractions eluted with 8% and 10% MeOH in $CHCl_3$ were identical and proved to be a mixture of two alkaloids (R_f 0.48 and 0.49 respectively, eluent A), the most polar of which is (−)-lycorine. The two fractions were combined and the residue (0.2 g) were further purified by CC on silica gel eluted with 5% MeOH in $CHCl_3$ and then by prep. TLC using solvent system A to give a further crop of (−)-lycorine (25 mg) and a homogenous compound (12 mg, R_f 0.48 and 0.17, eluent A and B, respectively) which crystallized from methanol and being a new alkaloid as below described it was named (−)-amarbellisine (**2**).

3.4. (−)-Amarbellisine (*2*)

Compound **2**: white needles, m.p. <300 °C; $[\alpha]_D^{25}$ −39.2° (*c* 0.7): CD (*c* 1.3×10^{-4} M) $[\theta]_\lambda$: $[\theta]_{219}$ −65,332, $[\theta]_{244}$ −42,219, $[\theta]_{294}$ −3450; IR υ_{max} cm^{-1} 3439, 1645; UV $\lambda_{max}(\log \varepsilon)$ nm: 293 (2.9), 244 (2.9); ^1H and ^{13}C NMR: Table 1; HR EIMS (rel. int.) *m/z*: 301.1302 ($C_{17}H_{19}NO_4$, Calc. 301.1314, 100) $[M]^+$, 286 $[M - Me]^+$ (6), 270 $[M - OMe]^+$ (84), 252 (22), 226 (17); ESI MS (+) *m/z*: 340 $[M + K]^+$, 324 $[M + Na]^+$, 302 $[M + H]^+$, 284 $[M + H - H_2O]^+$, 270 $[M - OMe]^+$.

3.5. (+)-Hippeastrine (*3*)

Compound **3**: colourless crystals, m.p. 215 °C; $[\alpha]_D^{25}$ +152 (*c* 0.3) (see Mügge et al., 1994); CD (*c* 1.7×10^{-4} M, $[\theta]_\lambda$): $[\theta]_{234}$ −46,146, $[\theta]_{255}$ +6518, $[\theta]_{275}$ −19,649 (see Wagner et al., 1996; Jeff et al., 1985); IR υ_{max} cm^{-1} 3440, 1786, 1644; UV $\lambda_{max}(\log \varepsilon)$ nm: 308 (2.6), 268 (2.7), 236 (3.4); ^1H NMR, δ: 7.48 (1H, *s*, H-8), 6.98 (1H, *s*, H-11), 6.08 (1H, *br s*, H-12), 6.07 (1H, *br s*, H-12′), 5.70 (1H, *br s*, H-4), 4.61 (1H, *br s*, H-5a), 4.38 (1H, *br s*, H-5), 3.25 (1H, *m*, H-2), 3.04 (1H, *br d*, $J = 9.4$ Hz, H-11b), 2.73 (1H, *d*, $J = 9.4$ Hz, H-11c), 2.54 (2H, *m*, H_2-3), 2.31 (1H, *q*, $J = 9.4$ Hz, H-2′), 2.10 (3H, *s*, Me – N); ^{13}C NMR, δ: 151.9 (*s*, C-9), 148.0 (*s*, C-10), 139.1 (*s*, C-7a), 118.5 (*s*, C-11a); HR EIMS (rel. int.) *m/z*: 316 $[MH]^+$ (4), 315 $[M]^+$ (2), 297 $[M - H_2O]^+$ (10), 279 $[M - 2xH_2O]^+$ (9), 190 $[M - C_7H_{11}NO]^+$ (28), 126 $[C_7H_{12}NO]^+$ (84), 125 $[C_7H_{11}NO]^+$ (100), 124 $[C_7H_{11}NO - H]^+$ (84), 96 $[C_7H_{11}NO - HCO]^+$ (99); ESI MS (+) *m/z*: 354 $[M + K]^+$, 338 $[M + Na]^+$, 316 $[M + H]^+$.

3.6. Antibacterial and antifungal activity of isolated alkaloids from the bulbs of Amaryllis belladonna L.

Antibacterial and antifungal assays were carried out using the agar diffusion technique (Jian and Kar, 1971) against a Gram-positive bacterium *S. aureus*, two Gram-negative bacteria, *E. coli* and *P. aeroginosea*, and the fungus *C. albicans*. The used organisms are local isolates provided by the Department of Microbiology, Faculty of Pharmacy, University of Alexandria. One ml of 24-h broth culture of each of the tested organisms was separately inoculated into 100 ml of sterile molten nutrient

agar maintained at 45 °C. The inoculated medium was mixed well and poured into sterile 10 cm diameter Petri-dishes, receiving 15 ml. After setting, 10 cups, each 8 mm in diameter, were cut in the agar medium (Oxoid). Ampicillin was used as an antibacterial control (10 μg/disc) and chlorotrimazole was used as an antifungal control (10 mg/ml). Three milligrams of each alkaloid, accurately weighed, were dissolved in 1 ml DMF. The solutions were inserted in the cups and incubated at 37 °C for 24 h.

3.7. Comparative study of the alkaloidal content of Amaryllis belladonna L. bulbs at different stages of growth using HPTLC technique

Fresh chopped bulbs of *A. belladonna* L. in the pre-flowering (sample 1) and flowering stages (sample 2) (250 g each) were exhaustively extracted with 2 l EtOH. Both extracts were concentrated under reduced pressure, (−)-lycorine was precipitated and filtered out. Each extract was transferred to a 100 ml volumetric flask and completed to volume with ethyl alcohol. The band applicator was operated with the following settings: band length 6 mm, application rate 15 s/μl. The volumes applied for comparative analyses were duplicate 6 μl aliquots of each sample solution in addition to five standard alkaloids; (−)-pancracine, (+)-11-hydroxyvittatine, (+)-vittatine, (−)-amarbellisine, and (+)-hippeastrine for comparison and identification of alkaloid present in the samples. The developing system was $CHCl_3$–MeOH (9:1 + 1 drop ammonia). After development, the plate was air-dried and sample zones were quantified by linear scanning at 254 nm and the peak areas of chromatograms were determined.

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