SUPPLEMENTARY MATERIAL

Phytochemical Investigations And Evaluation Of Antimutagenic Activity Of The Alcoholic Extract Of *Glycosmis Pentaphylla* And *Tabernaemontana Coronaria* By Ames Test

Ashish Kumar[a], Nilanjana Banerjee[b,*], Venugopal Singamaneni[c], Sudheer K Dokuparthi[c], Tulika Chakrabarti[d], Sibabrata Mukhopadhyay[a,*]

[a]*Organic & Medicinal Chemistry Division, CSIR-Indian Institute of Chemical Biology, Kolkata-700032*

[b]*Cell Biology & Physiology Division, CSIR-Indian Institute of Chemical Biology, Kolkata-700032*

[c]*National Institute of Pharmaceutical Education and Research, CSIR-Indian Institute of Chemical Biology, Kolkata-700032*

[d]*Chemistry Division, Sir Padmapat Singhania University, Udaipur, Rajasthan , India*

** Joint correspondence to: Dr Nilanjana Banerjee and Dr Sibabrata Mukhopadhyay, at: 2006.nilanjana@gmail.com and sbm.chem@gmail.com*

Abstract

Chemical investigation of root bark of *Glycosmis pentaphylla* and stem bark of *Tabernaemontana coronaria* led to the isolation of three carbazole alkaloids glycozoline, glycozolidine, methyl carbazole 3-carboxylate, two furoquinoline alkaloids- skimmianine, dictamine an acridone alkaloid- arborinine three monomeric indole alkaloids coronaridine, 10-methoxy coronaridine, tabernaemontanine two dimeric indole alkaloids voacamine, tabernaelegantine B. Their structures were established by detailed spectral analysis. Mutagenic and antimutagenic potential of methanol extract of both plant materials were evaluated by Ames test against known positive mutagens 2-aminofluorine , 4-nitro-O-phenylenediamine and sodium azide using *Salmonella typhimurium* TA 98 and TA 100 bacterial strains both in the presence and absence of S9. Both the extracts were non-mutagenic in nature. Both the extracts of *G. pentaphylla* and *T. Coronaria* exhibited significant antimutagenic activity against NPD and sodium azide for *S. typhimurium* TA98 & TA100 strains. The results indicated that the extracts could counteract the mutagenicity induced by different genotoxic compounds.

Keywords

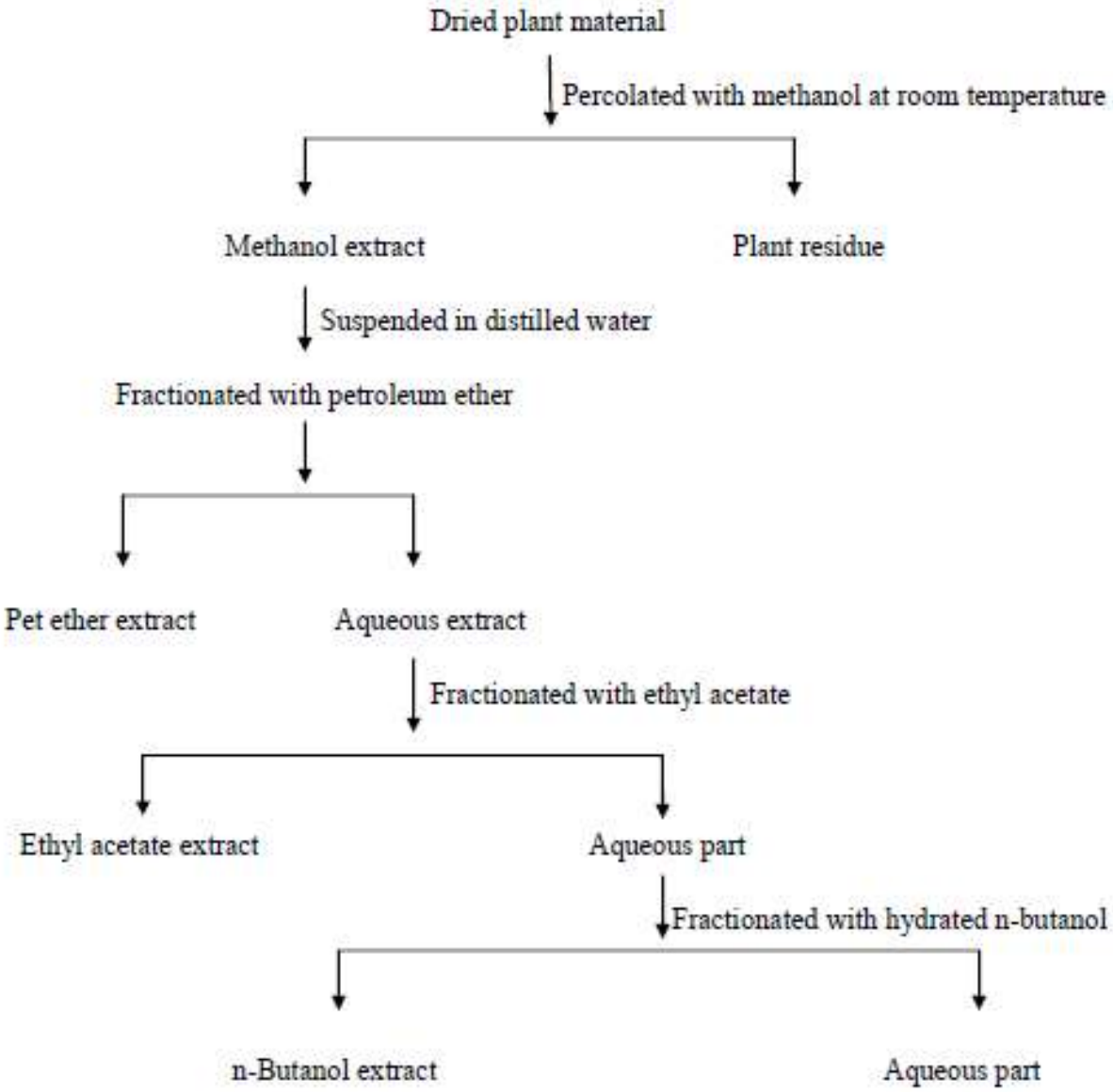
Ames Test; Antimutagenic activity; *Glycosmis pentaphylla*; *Tabernaemontana coronaria*; Alkaloids.

Experimental

Reagents. Dimethyl sulphoxide (DMSO), biotin, histidine, NADP, glucose 6-phosphate, ampicillin trihydrate, $NaNH_4HPO_4$ $4H_2O$, agar, Sodium azide (SA), 4-nitro-o-phenylenediamine (NPD), 2-aminofluorene (2-AF) and absolute ethanol were purchased from Sigma Chemical Co. (St Louis, MO). $MgSO_4$ and citric acid monohydrate were purchased from Merck, India. K_2HPO_4 and D-glucose were purchased from Qualigens Fine Chemicals (Mumbai, India).

***Plant material*.** The plant material was collected from Jhargram, India and identified at the Indian Botanical Garden, Howrah, India. A voucher specimen (Number 374 for *Glycosmis pentaphylla*, Number 375 for *Tabernaemontana coronaria*) has been deposited at the Chemistry Division of the CSIR-Indian Institute of Chemical Biology, Kolkata, India.

***Extraction and isolation*.** The air dried powdered root bark (1 kg) of *G. pentaphylla* and stem bark of *T. coronaria* were cold extracted with methanol for 48 hrs. The extracts were filtered and then evaporated under reduced pressure. Six known alkaloids from *G. pentaphylla* and 5 known alkaloids from *T. coronaria* were isolated by repeated silica gel (100-200 mesh size, Merck India) and neutral alumina column chromatography with gradient elution of benzene, chloroform and methanol. The structures of the isolated compounds were established by detailed spectral analysis and compared with the literature (Chakraborty et al., 1992). The concentration of the extract of the plant materials have been carried out under reduced pressure with the help of rotary evaporator at 40^{O} C.



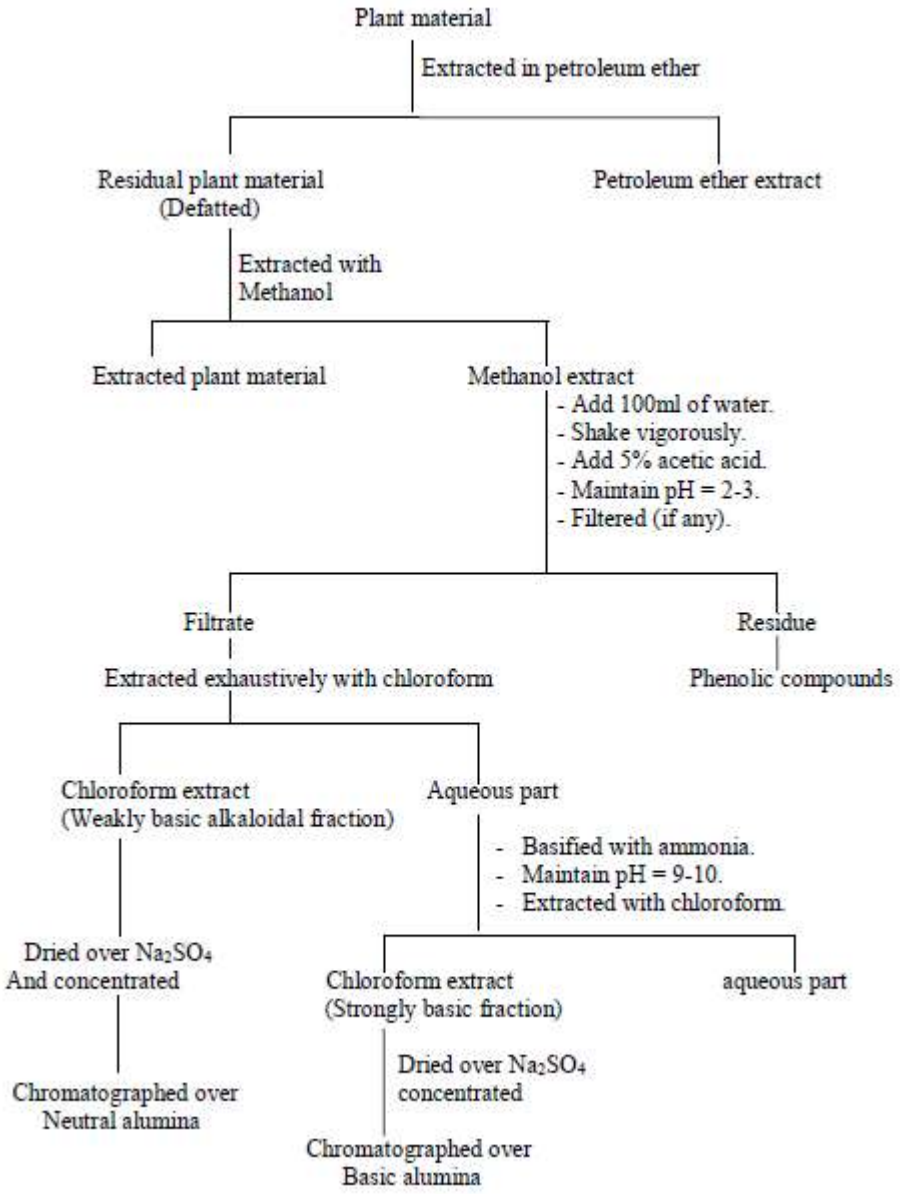
The dry powder of the root bark of *Glycosmis pentaphylla* was percolated with methanol at room temperature for 72 hours (three times) and resulting methanol extract was concentrated under reduced pressure. The methanol extract was fractionated with pet ether / water, ethyl acetate and hydrated n-butanol in consecutive way.

The plant material of *Tabernaemontana coronaria* was subjected to extraction with the various solvent systems at room temperature

1. Petroleum ether

2. Methanol

3. Chloroform



Bacterial strains. For mutagenicity and antimutagenicity assays, *Salmonella* strains TA98 and TA100 were used. These strains were kindly provided by Dr. Bruce N. Ames, Biochemistry Division, University of California, Berkeley, USA.

Preparation of S9 fraction. The procedure used for the preparation of rat liver homogenate S9 was followed as reported in literature (Ames et al., 1973). All the steps were performed at 0 °C to 4 °C with cold and sterile solutions and glassware. The S9 fractions were distributed in 2 mL aliquots in small sterile plastic tubes, quickly frozen and stored at -80 °C. The S9 mix was prepared following the reported method (Maron and Ames, 1983).

Mutagenicity and antimutagenicity assays. Standard plate incorporation tests: Plate incorporation tests were performed as previously described (Maron and Ames, 1983) using *S. typhimurium* tester strains (TA100 and TA98) with and without an exogenous metabolic system: S9 fraction in S9 mix. The methanol extracts of *G. pentaphylla and T coronaria* were dissolved in DMSO at a concentration of 200 mg/ml and different concentrations were prepared by serial dilution .Different concentrations (10, 100, 1000, 5,000 & 10,000 μg/ plate) of this extract were used for both the mutagenicity and antimutagenicity assays against known positive mutagens, with or without metabolic activation (S9). The plates were incubated for 1 hour and then inverted and placed in a dark vented incubator at 37 °C for 48 hours. Presence of background lawn on all the plates was confirmed after 48 hours of incubation. Four plates were used for each concentration. After 48 hours of incubation, the revertant colonies on the test plates were counted. A similar experiment was also carried out using liver homogenate (S9) fractions. The spontaneous reversion rates of these Salmonella strains were checked which were similar as reported earlier (Bhattacharya et al., 2011). After the specific time of incubation, the numbers of revertant colonies were calculated. Similarly, antimutagenicity assays of the fractions of extracts of *G. pentaphylla* and *T. coronaria* plant extracts were carried out against known positive compounds: NPD for TA98, SA for TA100 in the without S9 experiments and 2-AF was used for strains TA98 and TA100 in the with S9 experiments.

Statistical analysis. Graph Pad Instant software (Graph Pad software Inc., La Jolla, CA, USA) was used for statistical analyses. All data are expressed as the Mean ± SD. The differences between the control and the experimental groups were determined by one-way analysis of variance (ANOVA) and post tests were done using One way ANOVA with Tukey- Kramer Multiple comparison post test to determine the level of significance; $p<0.05$ and $p<0.001$ were considered to be significant.

The work has been approved by the ethical committee of CSIR-Indian Institute of Chemical Biology, Kolkata, India.

NMR Data of isolated compounds

Chemical structures of isolated compounds 1-11 from *Glycosmis pentaphylla* and *Tabernaemontana coronaria* are shown in Fig.1 and described below:

Glycozoline (1): White crystalline solid; m.p. 181-182^{o}C; UV λ_{max} nm: 252, 264, 304, 343; IR υ_{max} (KBr) cm^{-1}: 3398, 2999, 1492, 810; ESI-MS *m/z* 212.14 [M+H] $^{+}$; ^{1}H NMR (600 MHz, $CDCl_3$) : δ_H 7.82 (s, 1H, H-4), 7.80 (s, 1H, NH), 7.52 (d, *J* =1.8 Hz, 1H, H-5), 7.27 (m, 3H, H-1, H-2, H-8), 7.03 (dd, *J* = 1.7 and 8.8 Hz, 1H, H-7), 3.92 (s, 3H, 6-OCH_3), 2.52 (s, 3H, 3-CH_3); ^{13}C NMR (150 MHz, $CDCl_3$): δ_C 153.6 (C-6), 138.5 (C-9a), 134.7 (C-8a), 128.2

(C-3), 127.1 (C-2), 123.4 (C-4a), 123.2 (C-4b), 120.0 (C-4), 114.8 (C-7), 111.2 (C-8), 110.4 (C-1), 103.0 (C-5), 56.02 (OCH_3), 21.39 (CH_3).

Glycozolidine (2): Compound 2 is similar to compound1except at position 2 there is one more methoxyl group is present. White crystalline solid; m.p. 161-162^{o}C; UV λ_{max} nm: 234, 260, 310, 371, 375; ESI-MS *m/z* 242.14 $[M+H]^+$; ^{1}H NMR (600 MHz, $CDCl_3$): δ_H 7.73 (s, 1H, H-4), 7.73 (s, 1H, NH), 7.43 (s, 1H, H-5), 7.23 (m, 1H, H-8), 6.94 (dd, *J* = 8.8 and 2.5Hz, 1H, H-7), 6.76 (s, 1H, H-1), 3.90 (s, 3H, 6-OCH_3), 3.87 (s, 3H, 2-OCH_3), 2.35 (s, 3H, 3-CH_3); ^{13}C NMR (150 MHz, $CDCl_3$): δ_C 157.4(C-2), 153.8 (C-6), 134.2(C-8a), 133.9 (C-4b), 126.0 (C-9a), 121.4 (C-4), 118.9 (C-3), 112.9 (C-8), 110.9 (C-7), 106.2 (C-4a), 102.5 (C-5), 92.5 (C-1), 56.4 (6-OCH_3), 56.0 (2-OCH_3), 16.7 (3-CH_3).

Methyl carbazole 3-carboxylate (3): Colorless powder; m.p. 188-170^{o}C; UV λ_{max} nm: 346, 332, 320, 287, 273, 251, 241; IR υ_{max} (KBr) cm^{-1}: 3330, 1690, 1605; ESI-MS *m/z* 248.04 $[M+Na]^+$; ^{1}H NMR (600 MHz, $CDCl_3$): δ_H 8.51 (s, 1H, NH), 8.26 (s, 1H, H-4), 7.79(dd, *J*=8.5, 1.4 Hz, 1H, H-2), 7.55 (d, *J*= 8.0 Hz, 1H, H-5), 7.51 (d, *J*=8.5 Hz, 1H, H-1), 7.40 (d, *J*=8.0 Hz, 1H, H-8), 7.08 (m, 1H, H-7), 7.00 (m, 1H, H-6), 3.88 (s,3H, 3-$COOCH_3$). ^{13}C NMR (150 MHz, $CDCl_3$): δ_C 167.91 (C$\underline{O}$OCH$_3$), 142.28 (C-8a), 139.92 (9a), 127.43 (C-4), 126.55 (C-7), 123.30 (C-4b), 123.11 (C-4a), 122.88 (C-5), 121.34 (C-3), 120.61 (C-2), 120.32 (C-6), 110.92 (C-8), 110.16 (C-1), 51.96 (COO$\underline{C}$H$_3$).

Skimmianine (4): Colorless prism crystals; m.p. 178-179^{o}C; UV λ_{max} nm: 333.6, 320.8, 251.6; IR υ_{max} (KBr) cm^{-1}: 3118, 1767, 2943, 2839, 1619, 1496, 1368, 1264, 1090; ESI-MS *m/z* 260.17$[M+H]^+$; ^{1}H NMR (600 MHz, $CDCl_3$): δ_H 7.95 (d, *J* = 9.6 Hz, 1H, H-6), 7.47 (d, *J* = 2.4 Hz, 1H, H-2'), 7.17 (d, *J* = 9.6 Hz, 1H, H- 5), 6.94 (d, *J* = 2.5 Hz, 1H, H-3'), 4.43(s, 3H, 4-OCH_3), 4.12 (s, 3H, 8-OCH_3), 4.03(s, 3H, 7-OCH_3); ^{13}C NMR (150 MHz, $CDCl_3$): δ_C 164.2 (C-2), 101.8 (C-3), 157.1 (C-4), 114.6 (C-4a), 118.1 (C-5), 111.7 (C-6), 152.0 (C-7), 141.5 (C-8), 141.0 (C-8a), 142.7 (C-2'), 104.5 (C-3'), 58.8 (4-OCH_3), 56.56 (7-OCH_3), 61.45 (8-OCH_3).

Dictamine (5): Colorless powder; m.p. 126-128^{o}C; UV λ_{max} nm: 330, 308, 241, 237; IR υ_{max} (KBr) cm^{-1}: 1624, 1582, 1508, 1375, 1298, 1209, 1119, 1086, 980; ESI-MS *m/z* 200.09$[M+H]^+$; ^{1}H NMR (600 MHz, $CDCl_3$): δ_H 8.27 (dd, *J* = 8.4, 1H, 1.2 Hz, H-8), 8.02 (dd, *J* = 7.8, 1.2 Hz, 1H, H-5), 7.68 (dt, *J* = 7.8, 7.2, 1.2Hz, 1H, H-6), 7.62 (d, *J* = 2.4 Hz, 1H, H-2´), 7.44 (dt, *J* = 8.4, 7.2, 1.2 Hz, 1H, H-7), 7.07 (d, *J* = 2.7 Hz, 1H, H-3´), 4.44 (s, 3H, 4-OCH_3); ^{13}C NMR (150 MHz, $CDCl_3$): δ_C 164.0(C-2), 104.3(C-3), 157.8(C-4), 119.1(C-4a), 122.3(C-5), 123.7(C-6), 129.6 (C-7), 127.8 (C-8), 147.7 (C-8a), 143.5 (C-2'), 104.7 (C-3´), 59.0 (4-OCH_3).

Arborinine (6): Yellow needle-shaped crystals; m.p. 176-178^{o}C; UV λ_{max} nm: 272.5, 397.0, 265, 321.0; IR υ_{max} (KBr) cm^{-1}: 3436, 2920, 2850, 1642, 1592, 1558, 1462, 1322, 1190, 1108, 1060; ESI-MS *m/z* 285.91$[M+H]^+$; ^{1}H NMR (600 MHz, $CDCl_3$) : δ_H 8.47 (d, *J* = 8.0 Hz, 1H, H-8), 7.73 (dd, *J* = 8.7, 7.2 Hz, 1H, H-6), 7.51 (d, *J* = 8.7 Hz, 1H, H-5), 7.30 (dd, *J* = 8.0, 7.2 Hz, 1H, H-7), 6.30 (s, 1H, H-4), 4.02 (s, 3H, 3-OCH_3), 3.93 (s, 3H, 2-OCH_3), 3.85 (s, 3H, N-CH_3); ^{13}C NMR (150 MHz, $CDCl_3$): δ_C 180.5 (C-9),159.1 (C-3), 155.8 (C-1), 141.7 (C-14), 140.2 (C-11), 133.8 (C-6), 129.9 (C-2), 126.2 (C-8), 121.3 (C-7), 120.4 (C-13), 114.5 (C-5), 105.5 (C-12), 86.6 (C-4), 60.7 (2-OCH_3) , 55.9 (3-OCH_3), 33.9 (N-CH_3).

Coronaridine (7) ESI MS m/z 339.01 $[M+H]^+$. **^{1}H NMR:** δ 7.40 (1H, d, *J* = 7.8 Hz), 7.23 (1H, d, *J* = 7.8 Hz), 7.01 (1H, dd, *J* = 7.8, 1.2 Hz), 6.97 (1H, dd, *J* = 7.8, 1.2 Hz), 3.68 (3H, s, OCH_3), 0.90 (3H, t, *J* = 7.2 Hz, CH_3) and

between δ 1.20-4.29.(m, aliphatic protons). **^{13}C NMR:** 176.6 ($\underline{C}OOCH_3$), 138.6 (C-2), 137.7 (C-13), 129.7 (C-8), 122.3(C-11), 119.6 (C-10), 118.8 (C-9), 111.7 (C-12), 110.8 (C-7), 58.0 (C-21), 56.4(C-16) , 54.9 (C-3), 54.3(C-5), 53.0 (OCH_3), 40.1 (C-20), 37.1 (C-17), 35.0, 33.3 (C-15), 29.0 (C-14), 28.1(C-19), 22.9 (C-6), 12.2 (C-18),

10-methoxy coronaridine (8) Compound **8** was highly similar to coronaridine with the exception that the C-10 methine (CH) in coronaridine was replaced by a quaternary carbon due to the presence of a methoxy group at δ 154.0. This assignment was confirmed by ^{1}H-^{1}H COSY and HMBC correlation of δ 3.87 to C-10 (δ 154.0).

ESI-MS m/z 369.36 $[M+H]^+$.,**^{1}H NMR:** δ 7.14 (1H, d, J = 8.4 Hz, H-12) 6.92 91H, d, J = 1.8Hz, H-9), 6.81 (1H, dd, J = 8.4, 1.8 Hz, H-1) 3.87 (3H, s,$OC\underline{H}_3$), 3.71 (3H, s, $COOC\underline{H}_3$), 3.57 (1H, m, H-21), 3.22 (1H, m, H-3), 3.14, 1H, m, H-3, H-6), 2.96 (1H, m, H-6), 2.82 (2H, m, H-5), 2.57, 1H, m, H-17), 1.91 (2H, m, H-14, 17), 0.90 (3H, t, J = 7.2 Hz, H-18). , **^{13}C NMR:** 175.8 ($\underline{C}OOCH_3$), 154.1 (C-10), 137.6 (C-2), 130.7 (C-13), 129.3 (C-8), 112.0 (C-11), 111.2 (C-12), 110.2 (C-7), 100.9 (C-9), 57.6 (C-21), 56.2 (C-16), 56.2 ($O\underline{C}H_3$), 53.3 (C-5), 52.7 ($COO\underline{C}H_3$), 51.7 (C-3), 39.2 (C-20), 36.6 (C-17), 32.1 (C-15), 27.4 (C-19), 26.8 (C-14), 22.3 (C-6), 11.8 (C-18).

Tabernaemontanine (9) - ESI MS m/z 355.11 $[M+H]^+$**, ^{1}H NMR:** δ 7.70 (1H, d, J = 8.4 Hz, H-9), 7.34 (1H , s H-11), 7.32 (1H, s H-12), 7.11(1H, s., H-10), 3.94 (1H, m, H-5), 3.48, (1H, m, H-14), 2.84 (1H, m, H-14), 3.44 (1H, m, H-6), 3.30 (1H, m, H-6), 3.29 (1H, m, H- 21), 2.49 (1H, m, H- 21), 3.01 (1H, m, H-16), 2.71 (1H, m, H-15), 2.61 (3H, s, $COOC\underline{H}_3$), 2.56 (3H, s, $NC\underline{H}_3$), 1.71 (1H, m, H-19), 1.50 (1H, m, H-19), 1.53 (1H, m, H-20), 0.97 (3H, t, J = 7.2Hz, H-18)**., ^{13}C NMR:** 190.7 (C-3), 172.1 (C-17), 136.3 (C-13), 134.0 (C-2), 128.4 (C-8), 126.6 (C-11), 120.9(C-9), 120.7 (C-7), 120.3 (C-10), 111.6 (C-12), 56.8 (C-5), 50.2 ($COO\underline{C}H_3$), 46.5 (C-21), 45.6 (C-14), 43.4 (C-16), 43.0 ($N\underline{C}H_3$), 42.5 (C-20), 31.7 (C-15), 25.3 (C-19), 18.4 (C-6), 12.7 (C-18).

Voacamine (10) – ESI-MS m/z 705.10 [M+H]**, ^{1}H NMR:** δ 7.69 (brs, NH), 7.45 (brs, NH), 7.53 (1H, d, J = 4.8, H-9´), 7.04 (brs, H-10´, 11´, 12´), 6.92 (1H, s, H-9), 6.69 (1H, s, H-12), 5.30 (1H, dd, J = 13.2, 6.6 Hz, H-19´), 5.12 (1H, m, H-16´), 4.03 (1H, m H-5´), 3.99 (3H, s, 10-OCH_3), 3.64 (3H, s, 22- OCH_3), 3.49, (1H, m, H-21), 3.77, 2.97 (2H, m, H-21´), 3.51, 3.24 (2H, m, H-6´), 3.77 (1H, m, H-14´), 3.38, 3.20 (2H, m, H-5), 3.09, 2.97 (2H, m, H-6), 2.85, 2.70 (2H, m, H-3), 1.73, 1.08 (1H, m, H-15), 2.53, 1.80 (2H, m, H-17), 1.53, 1.42 (2H, m, H-19), 2.53, 1.99 (2H, m, H-17´), 1.80 (1H, m, H-14), 1.28 (1H, m, H-20), 0.87 (3H, t, J = 7.2 Hz, H-18), 1.66 (3H, d, J = 6.6 Hz, H-18´), 2.45 (3H, s, 22´- OCH_3), 2.58 (3H, s, NCH_3)**., ^{13}C NMR:** δ 175.2, (C-22), 171.6 (22´), 150.8 (C-10), 138.0 (C-2´), 137.1 (C-2, C-20´), 135.7 (C-13´), 130.2 (C-13), 129.9 (C-11), 129.7 (C-8´), 128.3 (C-8), 109.9 (C-7, C-7´), 54.9 (C-16). **CH** 121.4(C-11´), 118.8 (C-10´), 118.5 (C-19´), 117.4 (C-9´), 110.3 (C-12), 109.7 (C- 12´), 99.14 (C-9), 59.8 (C-5´), 57.1 (C-2´), 47.0 (C-3´), 38.9 (C-20), 37.2 (C-16´), 33.5 (C-14´), 27.3 (C-14). **CH_2** 53.0 (C-5), 52.4 (C-21´), 51.7 (C-3), 36.4 (C-17, C-17´), 31.9 (C-15), 26.7 (19), 22.2 (C-6), 19.4 (C-6´) **CH_3** 56.0(10´ OCH_3), 52.4 (OCH_3-22), 49.8 (OCH_3-22'), 42.2 (N-CH_3), 12.2 (C-18´), 11.6 (C-18).

Tabernaelegantine B (11) - ESI-MS m/z 707.50 $[M+H]^+$**., ^{13}C NMR:** 175.2 ($COOCH_3$), 172.3 ($COOCH_3$), 152.6 (C-11´), 137.0 (C-13, C-13´), 135.7 (C-1), 135.0 (C-2), 130.2 (C-8), 127.1 (C-10´), 122.9 (C-8') 121.3 (C-11), 120.3 (C-10), 118.7 (C-9), 117.2 (C-9´), 110.9 (C-7´), 109.8 (C-7), 109.8 (C-12), 98.9 (C-12´), 59.4 (C-5), 57.0 (C-21´), 55.9 ($ArOCH_3$), 54.8 (C-16´), 53.0 (C-5´), 52.3 ($COOCH_3$), 51.8 (C-3´), 49.7 ($COOCH_3$), 46.8 (C-21), 43.8 (C-16), 43.0 (NCH_3), 42.9 (C-20), 38.9 (C-20´), 36.9 (C-14), 36.3 (C-17´), 34.8 (C-3), 33.8 (C-15), 31.8 (C-15´), 27.2 (C-14´), 26.6 (C-19´), 25.6 (C-19), 22.1 (C-6´), 17.4 (C-6), 12.8 (C-18), 11.5 (C-18´).