# Isolation of stigmasterol, α-amyrin acetate and lupeol acetate from *Tabernaemontana Stapfiana* Britten

**Kipkemei Abraham Serem**

**Abstract**- Plant sterol, compound (**1**) and two pentacyclic triterpenoids, compound (**2**) and compound (**3**) were isolated from *Tabernaemontana stapfiana* Britten. The stem extracts of the plant were subjected to isolation and characterization of the compounds using several chromatographic and spectroscopic techniques.

**Index Terms**— Pentacyclic triterpenoids, sterol, isolation, structure elucidation, chromatography, NMR spectroscopy

## 1. Introduction

Kenya has four species of *Tabernaemontana; T. elegans* Stapf, *T. pachysichon* Stapf, *T. stapfiana* Britten and *T. ventricosa* A.DC. These plants are found in the central, western and coastal parts of Kenya (Beentje, Adamson and Bhanderi, 1994). The main constituents the *Tabernaemontana* species are indole alkaloids, a class of compounds with wide range of pharmacological activities (Pelletier, 1999). Besides the alkaloids, pentacyclic triterpenoids and steroids have also been isolated from the species (Nielsen, Hazell, Hazell, Ghia and Torssell, 1994, Pereira, França, Oliveira, Breves, Pereira, Sampaio, Nomizo and Dias, 2008).

## 2. Experimental

### 2.1. Plant material

Stems and root barks of *Tabernaemontana stapfiana* were collected from Kaptagat forest in Elgeiyo Marakwet County in Kenya highlands. A taxonomist authenticated these species and a voucher specimen was deposited in the Kenya National Museum Herbarium. The plant materials (stem and root barks) was dried at room temperature and then separately ground to fine powder using a Christy and Morris laboratory-grinding mill. The powdered plant materials were each weighed using a weighing balance.1.8 kgs of powdered root barks and 2.5 kgs stem barks of *Tabernaemontana stapfiana* was sequentially soaked in hexane, dichloromethane (DCM) ethyl acetate (EtOH) and finally methanol (MeOH) for 48 hours.

### 2.2. Visualizing reagent

*p*-Anisaldehyde was used as spray reagents. *p*-Anisaldehyde used was prepared by mixing 0.5ml *p*-anisaldehyde, 10 ml acetic acid, 85 ml chilled methanol and 5ml concentrated Sulphuric acid.

### 2.3. Extraction and isolation

Separation and purification of the secondary metabolite constituents from *Tabernaemontana stapfiana* crude extracts was carried out using standard chromatographic techniques which includes vacuum liquid chromatography (VLC) on Kieselgel silica gel 60G (0.040-0063 MM, Merck, Germany), Preparative TLC, column chromatography (CC) using silica gel 60G (0.63-0.2 MM, Merck Germany) and sephadex LH20 and thin layer chromatography (TLC).

The crude extracts of the stem were separately fractioned by VLC and CC using solvent system with gradual increasing polarity starting from n-hexane to 10% MeOH in DCM and eluted with a slow gradient of solvent system. The packing of both types of columns was done using slurry method with silica gel suspended in the least polar solvent in the solvent system. The sample was dissolved in the minimum possible solvent that dissolved it, mixed with an equal amount of silica gel used in the CC and ground into a fine powdery form to remove the solvent. This powder was then applied at the top of the column and finally covered by small amount of CC silica gel and cotton wool to minimize the disturbance of the sample when eluting solvent is applied.

Analytical pre-coated plastic (Polygram R sil G/UV 254) and aluminium sheets (Alugram R sil G/UV, Machery-Nagel GmbH and Co., Germany) TLC plates were used throughout the purification process. These were mainly for establishment of optimum solvent systems for separations, complexity of extracts and purity of isolated compounds. Spots on the chromatograms were detected under UV light at a $\lambda$ 254 and 366 nm for UV active compounds and visualized upon development by separately spraying Dragendorff and *p*-Anisaldehyde and heating for 10 minutes at 110$^0$C in an oven. Fractions that showed homogeneity were combined and concentrated together to give pure compounds or impure compounds for further purification. Sephadex columns were used during the purifications. The Sephadex columns were run using 1:1 ratio of DCM to methanol or in pure methanol.

Compound of hexane extract of *Tabernaemontana stapfiana* (15 g) were separated using column chromatography and the fractions obtained at 7:3 (Hex:DCM). The fraction was not UV ac-

tive but gave a blue color with *p*-anisaldehyde spray reagent. The fraction was purified further using column chromatography and at a solvent system of 4:1 (Hex:DCM), 35 mg was obtained. A prep-TLC was done on this fraction using 4:1 (Hex:DCM) solvent system and 31 mg of compound (**1**) obtained.

Vacuum liquid chromatography was used to fractionate 40g of *Tabernaemontana stapfiana* stem bark DCM extract. The fractions obtained at 3:2 (Hex: DCM) were 2-16 (9.55g) and 21-37 (0.70 g). The fractions 2-16 were pooled since they gave simillar spots on TLC using *p*-anisaldehyde. The fraction obtained after pooling was further fractionated using CC. The fractions 11-26 obtained from CC showed similar spots on TLC using *p*-anisaldehyde. They were pooled and the resulting fraction was separated using a sephadex column. The fraction 23-46 showed two spots on TLC using *p*-anisaldehyde and was separated using prep-TLC to yield 22 mg of compound (**2**) and 18 mg of compound (**3**).

$^{1}$H (1D, 2D, COSY) and $^{13}$C spectrum was recorded using Varian Gemini 200 and 400 MHz machine using $CDCl_3$ as solvent. Peak on H-NMR were recorded as a singlet (s), doublet (d), doublet of doublet (dd), triplet (t), quartet (q), multiplet (m) and/or broad (b) using TMS as reference. DEPT analysis was also perfomed. The chemical shifts were recorded in δ (ppm) and coupling constants, J, in hertz (Hz). A known weight of the sample was dissolved in $CDCl_3$ in a silget sample tube and mixed thoroughly. The solution was transferred into NMR tube and the spectrum recorded. The uncorrected melting points were recorded with open capillary tube using Gallenkamp melting apparatus (Sanyo, West Sussex UK).Visualization of spots on a developed TLC plate was done using long and short wave lengths (λ 366 and 254 nm) on an ENF-240 C/F UV lamp (Spectronics Co., Westbury).

Compound (**1**)
White powder, MP169-173$^{0}$C, IR (KBr) $V_{max}cm^{-1}$: 3595-3510, 3425, 3240, 3163, 2916, 2862, 2515, 1728, 1465-1404, 1265, 1157-1026, 802. $^{1}$H-NMR (Acetone-$d_6$, 200 MHz, δ ppm): 5.32 (1H, m), 5.20 (1H, m), 5.09 (1H, m), 3.38 (1H, m), 1.29 (3H, s), 1.07 (3H, s), 1.02 (3H, s), 0.75 (3H, s), 0.73 (3H, s). $^{13}$C-NMR: 140.7, 138.2, 129.2, 121.6, 71.7, 56.8, 56.0, 51.16, 50.1, 42.2, 40.4, 39.7, 39.6, 37.2, 36.4, 33.9, 31.8, 31.8, 31.6, 28.8, 24.2, 25.3, 21.1, 21.0, 20.0, 19.3, 18.9, 12.1, 11.8.

Compound (**2**)
White crystalline solid, Mp 200-205$^{0}$C, IR (KBr) $V_{max}cm^{-1}$: 3448, 2970-2877, 2723 1735, 1658, 1465, 1365 1249, 1141, 1095, 1018, 902, 825. $^{1}$H-NMR (Acetone-$d_6$, 200 MHz, δ ppm); 5.19 (m), 4.47(m), 2.00(s), 1.95 (d), 1.19(s), 1.14(s), 1.06(s), 1.02(s), 0.93(s), 0.89(d), 0.83(d). $^{13}$C-NMR; 170.1, 139.8, 124.7, 80.4, 59.1, 55.3, 47.8, 47.5, 42.2, 41.6, 40.2, 39.8, 38.5, 37.7, 36.9, 33.8, 32.9, 31.2, 27.70, 26.9, 26.7, 23.7, 23.4, 23.0, 21.0, 20.4, 18.3, 17.3, 16.7, 16.5, 15.4.

Compound (**3**)
White crystalline solid, Mp125-130$^{0}$C, IR (KBr) $V_{max}cm^{-1}$: 3903, 3448, 3301-3085, 2970-2885, 2715, 1736, 1635, 1465, 1373 1250, 1018, 879. $^{1}$H-NMR ($CDCl_3$, 400 MHz, δ ppm) 4.68 (1H,d), 4.56 (1H,s), 4.46 (1H, dd), 2.03 (3H,s), 1.68 (3H,s), 1.02 (3H,s), 0.93(3H,s), 0.85 (3H,s), 0.84 (3H,s), 0.83 (3H,s), 0.78 (3H,s)., $^{13}$C-NMR; 168.9, 150.9, 109.3, 81.0, 55.4, 50.4, 48.3, 43.0, 42.8, 40.0, 38.4, 38.1, 37.8, 35.6, 34.2, 29.8, 29.7, 27.9, 27.4, 25.1, 23.7, 21.3, 20.9, 19.3, 18.2, 18.0, 16.5, 16.1, 16.0, 14.5.

## 3. Results and discussion

Compound (**1**) was isolated from hexane extract as white crystals with a melting point of 174-177$^{0}$C. When the chromatogram was sprayed with *p*-anisaldehyde the spot turned purple, which suggested that compound (**1**) was a terpenoid (Paxton, Dey and Harborne, 1991). The IR spectrum revealed the presence of O-H stretch (3510$cm^{-1}$) C-H stretch (2916$cm^{-1}$), double bond stretch (1465$cm^{-1}$) and O-H deformation (1265$cm^{-1}$).

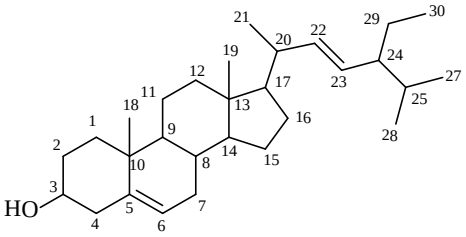
The $^{1}$H-NMR spectrum of compound (**1**) displayed 6 signals that integrated to 3 protons between δ 1.29 and 0.73 characteristic of methyl protons of a tritepenoid. A multiplet centered at δ 3.38 suggested a hydroxylated carbon atom normally C-3. A doublet of doublet at δ 5.32 suggested the presence of olefinic proton and thus a double bond bond involving a quaternary carbon atom. The multiplet centered at δ 5.16 intergrating to two protons suggested the presence of a double bond on the side chain which is identical with the chemical shift of H-22 and H-23 of stigmasterol.

The $^{13}$C-NMR spectrum showed signals at δ 140.7 and 121.2 confirming the presence of a double bond involving a quaternary carbon atom. The signal at δ 138.2 and 129.2 correspond to a double bond on the side chain normally C-22 and C-23. The peak at δ 71.7 indicated the presence of a hydroxyl group assignable to C-3. The $^{13}$C-NMR and $^{1}$H-NMR data of compound (**1**) are in agreement with the data in the literature for β-stigmasterol (Jamal, Yaacob and Din, 2008). The proposed structure, spectral data of compound (**1**) and reported literature for stigmasterol are shown in table 1 below.

Table 1: $^{1}$H-NMR and $^{13}$C-NMR data of β-Stigmasterol(Jamal, et al., 2008) and Compound (**1**)

| Position | β-Stigmasterol | | Compound (**1**) | |
|---|---|---|---|---|
| | $\delta_H$ (COUPLING, J in | $\delta_C$ | $\delta_H$ (COUPLING, J | $\delta_C$ |

| | Hz) | | in Hz) | |
|---|---|---|---|---|
| 1 | | 37.3 | | 37.2 |
| 2 | | 31.7 | | 31.6 |
| 3 | 3.25 (1H, m) | 71.8 | 3.38 (1H, m) | 71.7 |
| 4 | | 42.3 | | 42.2 |
| 5 | | 140.8 | | 140.7 |
| 6 | 5.14 (1H, d, 5.2) | 121.7 | 5.32 (1H, m) | 121.6 |
| 7 | | 31.9 | | 31.8 |
| 8 | | 31.9 | | 31.8 |
| 9 | | 50.2 | | 50.1 |
| 10 | | 36.6 | | 36.4 |
| 11 | | 21.1 | | 21.1 |
| 12 | | 39.7 | | 39.6 |
| 13 | | 42.3 | | 40.4 |
| 14 | | 56.9 | | 56.8 |
| 15 | | 24.4 | | 24.2 |
| 16 | | 28.8 | | 28.8 |
| 17 | | 56.1 | | 56.0 |
| 18 | 1.07 (3H, s) | 12.1 | 1.07 (3H, s) | 12.1 |
| 19 | 1.26 (3H, s) | 19.4 | 1.29 (3H, s) | 19.3 |
| 20 | | 40.5 | | 39.7 |
| 21 | 0.91(3H, s) | 20.9 | 0.73 (3H, s) | 20.0 |
| 22 | 4.62 (1H, m) | 138.3 | 5.20 (1H, m) | 138.2 |
| 23 | 4.61 (1H, m) | 129.4 | 5.09 (1H, m) | 129.2 |
| 24 | | 51.4 | | 51.2 |
| 25 | | 31.9 | | 33.9 |
| 26 | 1.01 (3H, s) | 19.0 | 1.02 (3H, s) | 18.9 |
| 27 | 1.00 (3H, s) | 21.2 | 1.02 (3H, s) | 21.0 |
| 28 | | 25.4 | | 25.3 |
| 29 | 0.97 (3H,s) | 12.0 | 0.75 (3H, s) | 11.8 |




Compound (**1**)

Compound (**2**) was isolated as white needlelike crystals from DCM extract and had a melting point of 200-205$^0$C. When the chromatograph was sprayed with *p*-anisaldehyde the spot turned purple, which suggested that compound (**2**) was a terpenoid(Paxton, et al., 1991). The IR spectrum revealed the presence of C-H stretch (2977cm$^{-1}$), carbonyl group (1735cm$^{-1}$), double bond stretch (1465cm$^{-1}$) and C-O stretch (1249cm$^{-1}$).

The $^1$H NMR spectrum of compound (**2**) showed 7 signals that represented 9 methyl groups. The signal at δ 1.14 (3H), 1.02 (3H), 0.93 (3H), 0.89 (9H) and 0.83 (3H) strongly suggested pentacyclic triterpenoid with 8 methyl groups. The other signals at δ 2.00 (3H) suggested the methyl protons of an acetyl group. The signal centred at δ 5.19 appearing as doublet of doublets an olefinic proton represented and thus a double bond involving a quartenary carbon atom. The signal centred at δ 4.48 suggested a proton attached to an oxygenated carbon atom. Its presence downfield when compared to that of hydroxylated carbon atom suggested the presence of a carbonyl group. The other signals appearing between δ 2.00 and 0.80 represented the methine and the methylene hydrogen atoms of pentacyclic triterpenoid skeleton.

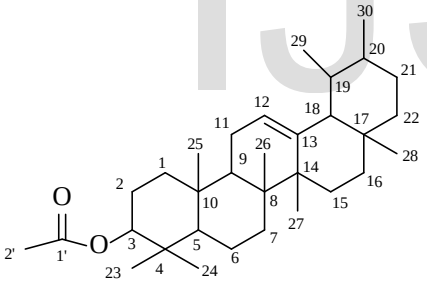
The $^{13}$C-NMR spectrum highly suggested that compound (**2**) was a triterpenoid.The peak at δ 170.1 conforming presence of a carbonyl group. The signals at δ 139.8 and 124.7 represented the carbon atoms of a double bond formed representing the quaternary carbon atom. The signal at δ 80.4 represents an oxygenated carbon atom of pentacyclic triterpenoid normally C-3. The other signals between δ 59.3 and 15.4 represented the methine methylene and quartenary carbon atoms of a pentacyclic triterpenoid.

The spectral data of compound (**2**) closely compared with reported literature for α-amyrin. Compound (**2**) showed 2 extra signals (170.1 and 16.7) in the $^{13}$C-NMR indicating the presence of an acetyl group that was assigned to position 3. This led to the proposal that compound (**2**) was α-amyrin-3-acetate. Table 2 compares $^{13}$C-NMR data of compound (**2**) with that of α-amyrin (Mahato, and Kundu, 1994, Nielsen, et al., 1994).

Table 2: Spectral data for α-Amyrin acetate (Nielsen, et al., 1994) and compound (**2**)

| Position | α - Amyrin | Compound (**2**) | |
|---|---|---|---|
| | $\delta_C$ | $\delta_H$ (COUPLING, J in Hz) | $\delta_C$ |
| 1 | 38.5 | | 38.3 |
| 2 | 27.7 | | 27.2 |
| 3 | 80.4 | 4.47 (1H, dd) | 81.0 |
| 4 | 39.8 | | 38.8 |
| 5 | 55.3 | | 55.4 |
| 6 | 18.3 | | 18.6 |
| 7 | 32.9 | | 32.9 |
| 8 | 40.2 | | 39.9 |
| 9 | 47.8 | | 47.7 |
| 10 | 36.9 | | 37.0 |
| 11 | 23.7 | | 23.4 |
| 12 | 124.7 | 5.18 (1H,dd 4, ) | 124.3 |
| 13 | 139.8 | | 139.3 |

| | | | |
|---|---|---|---|
| 14 | 41.6 | | 42.0 |
| 15 | 27.7 | | 28.7 |
| 16 | 26.7 | | 26.6 |
| 17 | - | | 33.7 |
| 18 | 59.3 | 1.95 (d) | 58.9 |
| 19 | 47.7 | | 39.6 |
| 20 | 47.5 | | 39.6 |
| 21 | 31.2 | | 31.2 |
| 22 | 33.8 | | 41.5 |
| 23 | 30.3 | 0.89 (d) | 28.1 |
| 24 | 16.5 | 0.89 (d) | 15.6 |
| 25 | 15.4 | 0.93 (s) | 15.6 |
| 26 | 23.1 | 1.02 (s) | 16.8 |
| 27 | 23.4 | 0.83 (d) | 23.3 |
| 28 | 28.0 | 1.19 (s) | 28.1 |
| 29 | 17.3 | 1.14 (s) | 17.4 |
| 30 | 20.48 | 1.06 (s) | 21.3 |
| 1′ | 171.3 | - | 170.1 |
| 2′ | 16.4 | 2.00 (3H, s) | 16.7 |




Compound (**2**)

Compound (**3**) was isolated from DCM extract as white crystals and had a melting point of range 125-130$^0$C. When the chromatogram was sprayed with *p*-anisaldehyde the spot turned purple, which suggested that compound (**3**) was a terpenoid (Paxton, et al., 1991). The IR spectrum revealed the presence of C-H stretch (2970cm$^{-1}$), carbonyl group (1736cm$^{-1}$), double bond stretch (1458cm$^{-1}$) and C-O stretch (1250cm$^{-1}$).

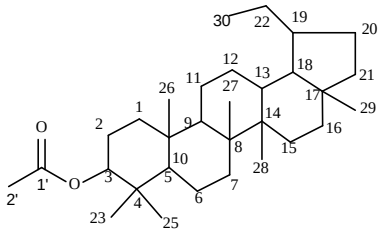
The $^1$H-NMR spectrum had seven singlets signals at δ 1.68 (3H), 1.02 (3H), 0.93 (3H), 0.85 (3H), 0.84 (3H), 0.83 (3H) and 0.74 (3H) suggested that compound (**3**) was a pentacyclic triterpenoid (PCTT) and represented the methyl groups. A more deshielded methyl group appeared at δ 2.03 and suggested presence of an acetyl group. The signal centered at δ 4.47 appearing as doublet of doublets suggested an oxygenated carbon atom. Its presence downfield compared to that of hydroxylated carbon atom suggested the presence of *O*-acteyl group replacing the hydroxyl group. Two broad singlets at δ 4.68 (1H) and 4.56 (1H) indicated the presence of an olefinic group. The other signals appearing between δ 2.37 to 0.78 represented the methine and methylene hydrogen atoms PCTT rings.

The $^{13}$C-NMR spectrum of compound (**3**) strongly suggested that the compound was a triterpenoid. The signal at δ 168.9 represented a carbonyl of an ester group. The signals at δ 150.9 and 109.3 were of a terminal double bond with the former representing the quaternary carbon. A signal at δ 81.0 was for the oxygenated carbon atom normally assignable to C-3. The other signals between δ 55.5 and 14.5 represented the methine, methylene, methyl and quaternary carbon of pentacyclic triterpenoid skeleton. The spectral data compound (**3**) closely compared with that of lupeol acetate. Table 3 below compares the spectral data of compound (**3**) with that of lupeol acetate (Jamal, et al., 2008).

Table 3: Spectral data for Lupeol acetate (Jamal, et al., 2008) and Compound (**3**)

| Position | Lupeol acetate | | Compound (**3**) | |
|---|---|---|---|---|
| | δH (COUPLING, J in Hz) | δC | δH (COUPLING, J in Hz) | δC |
| 1 | | 38.6 | | 38.4 |
| 2 | | 21.7 | | 21.3 |
| 3 | 4.47 (1H, dd, 7.4, 12.0 ) | 81.2 | 4.47 (1H, dd, 8.0, 12.0) | 80.9 |
| 4 | | 38.0 | | 37.8 |
| 5 | | 55.6 | | 55.4 |
| 6 | | 18.4 | | 18.2 |
| 7 | | 34.4 | | 29.8 |
| 8 | | 41.0 | | 40.0 |
| 9 | | 50.5 | | 50.4 |
| 10 | | 37.3 | | 34.2 |
| 11 | | 21.1 | | 20.9 |
| 12 | | 24.0 | | 23.7 |
| 13 | | 36.2 | | - |
| 14 | | 43.0 | | 42.8 |
| 15 | | 25.3 | | 25.1 |
| 16 | | 35.8 | | 35.6 |
| 17 | | 43.2 | | 43.0 |
| 18 | | 48.5 | | 48.3 |
| 19 | | 48.2 | | - |
| 20 | | 151.2 | | 150.9 |
| 21 | | 30.0 | | 29.7 |
| 22 | | 40.2 | | 38.1 |
| 23 | 0.85 (3H, s) | 27.6 | 0.85 (3H, s) | 27.4 |
| 24 | 0.84 (3H, s) | 16.7 | 0.84 (3H, s) | 16.5 |

| | | | | |
|---|---|---|---|---|
| 25 | 1.03 (3H, s) | 16.4 | 1.02 (3H, s) | 16.2 |
| 26 | 0.83 (3H, s) | 16.2 | 0.83 (3H, s) | 16.0 |
| 27 | 0.79 (3H, s) | 14.7 | 0.78 (3H, s) | 14.5 |
| 28 | 0.94 (3H, s) | 18.2 | 0.93 (3H, s) | 18.0 |
| 29 | 4.69 (1H, s), 4.57 (1H, s) | 109.6 | 4.68 (1H, d), 4.56 (1H, s) | 109.3 |
| 30 | 1.69 (3H, s) | 19.5 | 1.68 (3H, s) | 19.3 |
| 1′ | - | 171.3 | - | 169.0 |
| 2′ | 2.05 (3H, s) | 28.2 | 2.03 (3H, s) | 27.9 |




Compound (**3**)

## Acknowledgement

I thank Prof. Paul K. Tarus of Eldoret University, Eldoret, Prof. Alex K. Machocho of Kenyatta University, Nairobi, Christine K. Bii of Kenya Medical Research Institute, Nairobi, Ernest Ruttoh of Kenya National Public Health Laboratory Services, Nairobi and Mr. Karimi of botany department, Kenyatta University; their support was invaluable to this work.

## References

[1] Beentje, H., Adamson, J. and Bhanderi, D., 1994.*Kenya trees, shrubs, and lianas*, National Museums of Kenya, 1994.

[2] Jamal, A., Yaacob, W. and Din, L. B., (2008) *A chemical study on Phyllanthus reticulatus*, Journal of Physical Science, 19, pp. 45-50.

[3] Mahato, S. B. and Kundu, A. P., (1994) *13C NMR spectra of pentacyclic triterpenoids—a compilation and some salient features*, Phytochemistry, 37, pp. 1517-1575.

[4] Nielsen, H. B., Hazell, A., Hazell, R., Ghia, F. and Torssell, K. B., (1994) *Indole alkaloids and terpenoids fromTabernaemontana markgrafiana*, Phytochemistry, 37, pp. 1729-1735.

[5] Paxton, J., Dey, P. and Harborne, J., (1991) *Methods in plant biochemistry*, Academic Press, London, 6, pp. 37.

[6] Pelletier, S. W., 1999.*Alkaloids: chemical and biological perspectives*, Springer, 1999.

[7] Pereira, P. S., França, S. D. C., Oliveira, P. V. a. D., Breves, C. M. D. S., Pereira, S. I. V., Sampaio, S. V., Nomizo, A. and Dias, D. A., (2008) *Chemical constituents from Tabernaemontana catharinensis root bark: a brief NMR review of indole alkaloids and in vitro cytotoxicity*, Química Nova, 31, pp. 20-24.