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THE CONSTITUENTS OF THE LEAVES OF *FICUS BENJAMINA*

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ABSTRACT

Glutinol (3β-hydroxyglutin-5-ene), friedelinol, β-sitosterol, α-amyrin, 6-β-hydroxy stigmast-4-en-3-one, 6-β-hydroxy stigmast-4, 22-dien-3-one and β-sitosterol-3-*O*-glucoside were isolated for the first time from the methanolic extract of the leaves of *Ficus benjamina* (*Moraceae*) cultivated in Egypt. Identification of these compounds has been established by physical and spectral data (UV, IR, MS, ^{1}H-NMR and ^{13}C-NMR, DEPT, HMQC, HMBC) as well as by comparison with authentic samples. Antifungal and antimicrobial activities were carried out and significant results were obtained.

INTRODUCTION

Genus *Ficus* (*Moraceae*) is widely distributed in tropical and subtropical countries and comprises about, 800 species[1], many of them are used for shade purposes (*F. religiosa*) or as ornamental plants (*F. benjamina* L.) while others are well known for their edible fruits (*F. carica* L., *F. sycomarus* L.)[1-3]. Many *Ficus* species have long been used world wide in folk medicine as astringent, carminative, vermicide, hypotensive, anthelmintic and antidysentry[2]. Many recent uses were reported for *Ficus* e.g. ulcer treatment[4], anticancer[5] and antibacterial[6]. Many compounds were isolated from *Ficus* species including, coumarins, phytosterols, triterpenes and flavonoids[7].

Ficus benjamina or weeping fig is a plant used increasingly for indoor decoration that may cause allergic rhinitis and asthma[8,9]. Reviewing the current literature, there is no reports concerning the chemical constituents or the biological activities of this plant except the separation of α-amyrin, bergapten and imperatorin from its latex[10]. This prompted us to investigate the chemical composition of its leaves.

EXPERIMENTAL

Plant material:

The leaves of *Ficus benjamina* were collected from the trees cultivated in Mansoura city, Egypt in March, 2003 and identified by Prof. Dr. Ibrahim Mashaly, Assistant Professor of plant taxonomy, Department of Botany, Faculty of Science, Mansoura University.

General experimental procedures:

Melting points were measured by Hot-Stage melting point microscope (Sybron, USA). UV spectra were measured in methanol using Beckmann DU-7 Spectrophotometer. IR spectra were measured by Nicolet MX-1 FT-IR spectrometer. One- or two-dimensional ^{1}H- and ^{13}C-NMR spectra were run in $CDCl_3$ and DMSO-d_6 at 300 and 500 MHz on JOEL TNM-LA, FT-NMR system, Japan, using TMS as internal standard. EI-MS were recorded by JOEL, GCmate, Japan. Ultra-Violet Lamp 254 and 366 nm (Desaga, Germany). Chromatographic separation was performed using silica gel (E-Merck, Germany). TLC was performed on silica gel GF_{254} (E-Merk, Germany). Authentic samples (α-amyrin and β-sitosterol) were obtained from the Department of Pharmacognosy, Faculty of Pharmacy, Mansoura University. Developed chromatograms were visualized under UV light and by spraying developed plates with 1% vanillin- H_2SO_4 spray reagent followed by heating at 100°C for 10-20 second. For TLC analysis the following solvent systems were used: pet. ether- ether (6:4, system I), chloroform (system II) and chloroform-methanol (9.5:0.5, system III).

Extraction and Isolation:

The air dried powdered leaves (2 Kg) of *F. benjamina* were extracted with (70%) methanol at room temperature by maceration (12 L). The residue left after evaporation of the solvent (280 gm) was diluted with water and successively extracted with pet. ether, chloroform, ethyl acetate and n-butanol (5 L each).

The pet. ether soluble fraction (41.5 gm) was chromatographed over silica gel column (300 gm). Elution was started with pet. ether followed by pet. ether- ethyl acetate mixtures. The eluted fractions (250 ml each) were colleted, concentrated and screened by TLC. Similar fractions were combined together.

Fractions (26-30) eluted with pet. ether- ethyl acetate (90:10) were rechromatographed on a silica gel column to afford compounds **1** (30 mg) and **2** (40 mg), respectively. Fractions (33-40) eluted using pet. ether-ethyl acetate (90:10), were purified on silica gel column to afford compounds **3** (50 mg) and **4** (200 mg) respectively. Fractions (65-76) eluted using pet. ether-ethyl acetate (70:30) were rechroma-tographed over silica gel column to afford compound **5** (25 mg).

The chloroform soluble fraction (5 gm) was chromatographed on silica gel column, using chloroform followed by chloroformmethanol gradient. The eluted fractions (250 ml each) were collected, concentrated and screened by TLC, similar fractions were combined. Fractions (90:10) gave compound **6** (50mg).

Compound 1: colorless small needles (methanol), m.p. 208-210°C, R_f= 0.76 (system I). IR (KBr, γcm^{-1}): 3428 (OH), 1639 and 814 (-C=C-H), 1380 (gem dimethyl). EI-MS m/z: 426 $[M]^+$calculated for ($C_{30}H_{50}O$), 408 $[M-H_2O]^+$, 274 [Fragment a]$^+$and 259 [a-Me]$^+$. ^{1}H-NMR (500 MHz, $CDCl_3$): δ 0.84, 0.95, 0.98, 0.99 ,1.00, 1.09, 1.14 and 1.15 (each 3H, s), 3.46 (1H, br.s.), 5.60 (1H, d, J=6.3). ^{13}C-NMR (Table 1)

Compound 2: Colorless needles (Methanol), m.p. 301-304 °C, R_f= 0.75 (system I). IR (KBr, γcm^{-1}): 3445 (OH). EI-MS m/z: 428 $[M]^+$ calculated for ($C_{30}H_{52}O$), 413 $[M-CH_3]^+$ and 395 $[M-CH_3-H_2O]^+$. ^{1}H-NMR (300 MHz, $CDCl_3$): δ 0.85, 0.93, 0.95, 0.98, 1.00, 1.03 and 1.15 (each 3H, s), 0.92 (3H, d, J=7.5) and 3.70 (1H, br.s). ^{13}C-NMR (Table1)

Compound 3: White crystalline needles (Methanol), m.p. 185-187 °C, R_f= 0.78 (system I). IR (KBr, γcm^{-1}): 3420, 2980, 1650, 1460, 1450, 1380, 1050, 1010, 960, 805.

Compound 4: White crystalline needles (Methanol), m.p. 135-137 °C, R_f= 0.59 (system I). IR (KBr, γcm^{-1}): 3350, 2940, 1530, 1460, 1380, 1360.

Compound 5: Colorless needles (Methanol), R_f= 0.62 (system II). IR (KBr, γcm^{-1}): 3423 (OH), 1680 (α, β-unsaturated C=O), 1630 and 870 (unsaturation). EI-MS m/z: 428 $[M_a]^+$ calculated for $C_{29}H_{48}O_2$, 426 $[M_b]^+$ calculated for $C_{29}H_{46}O_2$. UV (MeOH) λ_{max}: 255 (α, β-unsaturated C=O). ^{1}H-NMR (500 MHz, $CDCl_3$): δ 0.73 (6H, s, H-18$_{a,b}$), 1.37 (6H, s, H-19$_{a,b}$), 0.91 (3H, d, J=6.3, H-21$_a$), 1.04 (3H, d, J=6.3, H-21$_b$), 0.79 (3H, d, J=6.5, H-26$_a$), 0.82 (3H, d, J=6.5, H-26$_b$), 0.81 (6H, d, J= 6.5, H-27$_{a,b}$), 0.85 (6H, m, H-29$_{a,b}$), 4.4 (2H, br.s, H-6$_{a,b}$), 5.80 (2H, s, H-4$_{a,b}$), 5.05 (1H, dd, J=8, 15, H-23$_b$), 5.15 (1H, dd, J=8, 15, H-22$_b$). ^{13}C-NMR (Table 1).

Compound 6: white amorphous powder, m.p. 294-296°C, Rf=0.35 (system III), IR (KBr, γcm^{-1}): 3420, 2940, 1460, 1380, 1080, 1030. ^{1}H-NMR (300 MHz, DMSO-d_6): 0.66 (3H, s, H-18), 0.94 (3H, s, H-19), 0.99 (3H, d, J=6.2, H-21), 0.90 (3H, d, J=5.9, H-26), 0.87 (3H, d, J=5.9, H-27), 0.89 (3H,m, H-29), 4.00 (1H, m, H-3), 5.07 (1H, d, J=5.4, H-1'). ^{13}C-NMR (Table 1).

Antifungal activity

The phytopathogenic fungal strain used was *Aspergillus parasiticus* NRRL 2999 obtained from the National Research Center at El-Doki, Giza, Egypt. The fungus strain was maintained at 5°C until used. The antifungal activity of the alcohol extract of *Ficus benjamina* L. leaves was assayed according to the method described by Farag et al., (1986)[11] and Chkhikvishvili and Gogiya (1995)[12]. Ten-ml portions of potato dextrose agar (PDA)[13] medium were placed in Petri dishes. The spores suspension of the fungus was poured in the center of solid agar surface (control). Different quantities of plant extract were mixed thoroughly with 10 ml of melted PDA medium to give final concentrations of 500, 1000 and 2000 ppm (0.5 mg, 1mg and 2 mg/10 ml of melted PDA medium) then poured into Petri dishes. All plates were incubated at 28±2°C for 7 days after which the fungal growth diameter was estimated compared with control. Experiment was done for three replicates. The effect of the plant extract was estimated by measuring the diameter of the inhibition zone.

***In vitro* antimicrobial activity**

The different extracts of *F. benjamina* and β-sitosterol were tested for their *in vitro* antimicrobial activity against a panel of standard strains of Gram-positive bacteria viz. (*Staphylococcus aureus* IFO 3060 and *Bacillus subtilis* IFO 3007) and Gram-negative bacteria viz. (*Escherichia coli* IFO 3301 and *Pseudomonas aeruginosa* IFO 3445) and the yeast-like pathogenic fungus *Candida albicans* IFO 0583. The primary screening was carried out using the agar disc-diffusion method[14] using Müller-Hinton agar medium. The antibacterial antibiotic ampicillin (100 µg/disc) and the antifungal drug clotrimazole (100 µg/disc) were used as positive standard.

The minimal inhibitory concentration (MIC) for the most active compounds against the same microorganisms used in the primary screening was carried out using the microdilution susceptibility method in Müller-Hinton Broth and Sabouraud Liquid Medium[14]. The MIC of the most active compounds, the antibacterial antibiotic ampicillin and the antifungal drug clotrimazole, were in accordance with the results obtained in the primary screen. The bacterial strains and *Candida albicans* fungus were obtained from the Institute of Fermentation of Osaka, Japan.

Determination of *in vitro* antimicrobial activity

The primary screen was carried out using the agar disc-diffusion method[14] using Müller-Hinton agar medium. Sterile filter paper discs (8mm diameter) were moistened with the compound solution in dimethylsulphoxide of specific concentration (200 µg/disc), ampicillin trihydrate (100 µg/disc) and clotrimazole (100 µg/disc) were carefully placed on the agar cultures plates that had been previously inoculated separately with the microorganisms. The plates were incubated at 37°C and the diameter of the growth inhibition zones were measured after 24 hours in case of bacteria and 48 hours in case of *Candida albicans*. The minimal inhibitory concentration (MIC) for the most active compounds against the same microorganisms used in primary screening were carried out using the microdilution susceptibility method in Müller-Hinton Broth and Sabouraud Liquid Medium[14]. The compounds, ampicillin trihydrate and clotri-mazole were dissolved in dimethylsulphoxide at concentration of 128 µg/ml. The two fold dilutions of solution were prepared (128, 64, 32, ..., 0.5 µg/ml). The microorganism suspentions at 10^6 CFU/ml (colony forming unit/ml) concentrations were inoculated to the corresponding wells. The plates were incubated at 36°C for 24 and 48 hours for the bacteria and *Candida albicans*, respectively. The MIC values were determined as the lowest concentration that completely inhibited visible growth of the microorganism as detected by unaided eye.

RESULTS AND DISCUSSION

The methanolic extract of the air dried leaves was treated as described in experimental. Six compounds were isolated; all of them gave positive Liebermann-Burchard's test indicating their steroidal or triterpenoidal nature.

MS of compound **1** displayed $[M]^+$ at m/z 426 consistent with the molecular formula $C_{30}H_{50}O$ indicating six degrees of unsaturation of a pentacyclic triterpene with one double bond (^{13}C-NMR signals at δ 122.1 and 141.6 ppm). The presence of a secondary hydroxyl group was established by IR, NMR data (3428 cm^{-1}, δ H 3.46 (1H, br. S, H-3), δ C (76.0)). The EI-MS spectrum of **1** showed strong peaks, due to retro-Diels-Alder cleavage of the B-ring, at m/z 274 (fragment a) and 259 characteristic of a triterpene-5-ene skeleton[15]. The melting point and spectral data (IR, EI-MS, ^{1}H-NMR and ^{13}C-NMR) of **1** were in excellent agreement with those reported[16] for **1** as 3*β*-hydroxyglutin-5-ene (glutinol). This is the first report for the isolation of glutinol from the title plant.

Compound **2** was isolated as colorless needles. The molecular formula of compound **2** was deduced as $C_{30}H_{52}O$ from EI-MS fragment at m/z 428 $[M]^+$. ^{13}C-NMR and DEPT spectra showed eight methyl groups, eleven methylene groups, five methine groups and six quaternary carbons. ^{1}H-NMR spectrum showed the presence of seven tertiary methyl singlets (δ 0.85, 0.93, 0.95, 0.98, 1.00, 1.03, 1.15), a secondary methyl doublet at δ 0.92 (d, *J*=7.5) and a hydroxyl methine proton (δ 3.7, 1H, br.s.). These data suggested that compound **2** is almost similar to compound **1** except for the absence of olefinic double bond at C-5/C-6. From the above mentioned data **2** could be identified as friedelinol by comparison to those reported (EI-MS, ^{1}H-NMR and ^{13}C-NMR)[17].This is the first report on isolation of friedelinol in genus *Ficus*.

Compound **3** was identified as α-amyrin by comparison of its m.p. and chromatographic data with authentic sample. α-amyrin has been isolated before from the latex of the plant[10].

Compound **4** was identified as *β*-sitosterol by comparison of its m.p. (mixed m.p.) and chromatographic data with authentic sample as well as cochromatography and undepressed m.p. This is the first report for the isolation of *β*-sitosterol from the title plant.

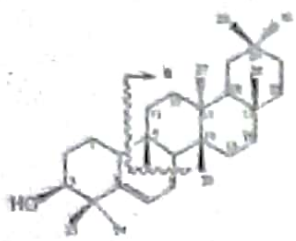
Compound **5** was isolated as colorless needles. UV absorption at 238 nm of **5** indicated a conjugated double bond. Its IR spectrum showed absorbencies for hydroxyl (3423 cm^{-1}), *α,β*-unsaturated ketone (1680 cm^{-1}) and unsaturation (1630 and 870 cm^{-1}). The ^{1}H-NMR and ^{13}C-NMR data suggested that **5** was a mixture of two compounds **5a** and **5b**. ^{1}H-NMR and ^{13}C-NMR data of **5a** and **5b** revealed a close similarity to sitosterol and stigmasterol respectively except to those signals of rings A and B. The ^{1}H-NMR spectrum showed the presence of an enone proton at δ 5.8 (2H, s, H-4$_{(a, b)}$) and the presence of secondary hydroxyl group at δ 4.3 (2H, br.s, H-6$_{(a, b)}$). A ^{13}C-NMR signal at δ 200.6 confirmed the presence of a keto group. In ^{13}C-NMR spectrum four signals were observed in olefinic region. Two carbon signals at 126.4 and 168.6 were attributed to C-4 and C-5 respectively and signals at δ 138.1 and 129.4 were assigned to C-22$_{(5b)}$ and C-23$_{(5b)}$ respectively. The coupling interaction of 6-H (br.s) revealed that the hydroxyl group on this position has *β*-axial orientation rather than *α*-equatorial orientation (as in the second case the 6-*β*H appear as ddd[18] showing axial-axial coupling with 7 *α*H (*J*=12.1 Hz), axial-equatorial coupling with 7-*β*H (*J*=5.6 Hz) and an allylic coupling with 4-H (*J*=1.7 Hz)). On the contrary, the different coupling interactions, particularly the absence of coupling between 6*α*-H and 4-H are characteristic for compounds with *β*-axial hydroxyl group. The structure of **5a** and **5b** were further substantiated by complete assignments of COSY, HMQC and HMBC experiments spectra, which have not been previously reported for them. The substitution pattern of ring A and B was established by 2D-NMR experiments. The ^{1}H-^{1}H COSY spectrum of **5** showed that H-6 (δ 4.3) correlated only with H-7 (δ 2.0). In the HMBC spectrum, the olefinic proton (δ 5.8, H-4) correlated with C-6 (δ 73.3), C-2 (δ 34.3) and C-10 (δ 38.07); H-19 (δ 1.37) correlated with C-1 (δ 38.6), C-9 (δ 53.6); H-6 (δ 4.3) correlated with C-4 (δ 126.4), C-7 (δ 38.6), C-8 (δ 29.8) and C-10 (δ 38.07). ^{1}H-NMR for **5a** and **5b** was in a full agreement with those reported[18] for 6-*β*-hydroxy stigmast-4-en-3-one and 6-*β*-hydroxy stigmast-4,22-dien-3-one respectively. This is the first report for the isolation of these compounds from genus *Ficus*.

Compound **6** was separated as white amorphous powder. It gave positive Liebermann-Burchard's and Molisch's tests indicating its steroidal and glycosidic nature. The glycosidic nature was confirmed from the appearance of anomeric proton doublet at δ 5.07 (*J*=5.4). Attachment of the glycosidic chain at C-3 was indicated by a down field shift (δ 78.4) observed for this carbon. The physical and spectroscopic data of **6** are in full agreement with those reported[19] for *β*-sitosterol-3-O-glucoside. It is worth to note that *β*-sitosterol-3-O-glucoside have not been reported before in *Ficus benjamina*.

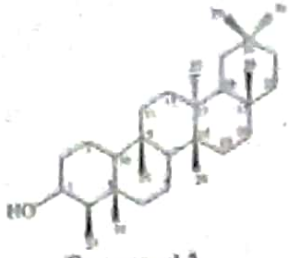
Antifungal activity: alcohol extract of *F. benjamina* showed a moderate antifungal activity against *Aspergillus parasiticus* (54.65% inhibition at concentration of 2000 ppm) (Table 2).

Antimicrobial activity of the different extracts of *F. benjamina* and *β*- sitosterol (200 µg/ 8 mm disc): The chloroform extract showed the greatest activity against *Candida albicans* and *Escherichia coli*. *β*-sitosterol showed a moderate activity against *Escherichia coli* and potent activity against *Pseudomonas aeuroginosa* comparing to the standard Ampicillin (Table 3).

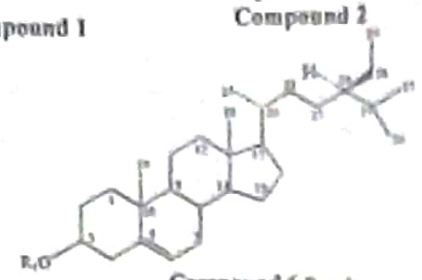
The minimal inhibitory concentrations (MIC, µg/ml) of the different extracts of *F. benjamina* and *β*-sitosterol: The chloroform and ethyl acetate extracts showed the lowest (MIC) against *C. albicans* comparing to alcohol and pet-ether extracts and *β*-sitosterol (Table 4).



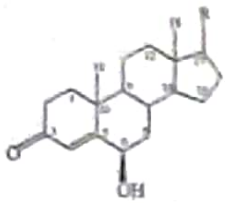
Compound 1



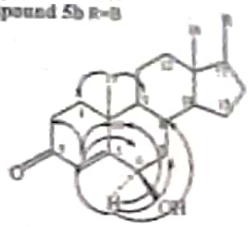
Compound 2



Compound 6 R_1=glc



Compound 5a R=A
Compound 5b R=B



HMBC Correlations of Compound 5

Table (1): ^{13}C-NMR Spectral Data for Compounds 1-5 ($CDCl_3$) and 6 (DMSO-*d6*).

C	1	2	5a		5b	6
1	18.5	17.6		37.2		37.5
2	27.8	35.0		34.3		30.3
3	76.0	72.7		200.6		78.4
4	40.9	49.1		126.4		39.3
5	141.6	37.1		168.6		140.1
6	122.1	41.7		73.3		121.7
7	23.7	15.8		38.6		32.2
8	47.4	53.0		29.8		32.1
9	34.9	38.4		53.6		50.3
10	49.7	61.3		38.1		36.3
11	33.1	35.6		21.1		21.3
12	30.4	30.7		39.6		39.9
13	37.9	39.7		42.5		42.4
14	39.3	38.4		56.1		56.8
15	34.6	32.1		24.2		24.5
16	35.1	36.1	28.27		28.90	28.5
17	30.1	30.1		55.9		56.2
18	43.1	42.8	12.09		12.27	11.9
19	35.1	35.2		19.5		19.2
20	28.3	28.2	36.10		40.50	36.9
21	32.1	32.8	18.80		21.20	19.0
22	39.0	39.3	33.9		138.10	34.2
23	29.0	11.7	26.10		129.40	26.4

Table (1) *continued*

24	25.5	16.4	45.80		51.30	46.0
25	16.3	18.7	29.20		31.90	29.2
26	18.3	20.2	19.90		21.19	19.4
27	19.7	18.7		19.10		19.9
28	32.0	32.3	23.14		24.40	23.4
29	32.4	31.8	12.09		12.34	12.2
30	34.5	35.1	-		-	-
1'						102.4
2'						73.3
3'						78.1
4'						71.6
5'						77.0
6'						62.8

Table (2): Antifungal activity of the alcohol extract of *F. benjamina*

Extract	Growth diameter of *A. parasiticus* (cm) according to concentration*						
	Control	500 ppm	% inhibition	1000 ppm	% inhibition	2000 ppm	% inhibition
Alcohol extract	4.3	2.95	31.39	2.75	36.04	1.49	65.5

* Activities are expressed as the diameter of the inhibition zone (cm). Data are expressed as mean ± standard deviations of triplica determinations.

Table (3): Antimicrobial activity of the different extracts of *F. benjamina* and *β*-sitosterol (200 µg/ 8 mm disc), ampicillin (100 µg/8 mm disc) and clotrimazole (100 µg/ 8 mm disc)

Extracts and Compounds	Diameter of the growth inhibition zone (mm)				
	Sa	*Bs*	*Ec*	*Pa*	*Ca*
Alcohol extract	-	-	11	-	-
Pet-ether extract	-	-	-	-	-
Chloroform extract	-	-	15	-	17
Ethyl acetate extract	-	-	-	-	16
β- sitosterol	-	-	14	14	15
Ampicillin	22	18	21	18	NT
Clotrimazole	NT	NT	NT	NT	21

Sa: Staphylococcus aureus , *Bs: Bacillus subtilis*
Ec: Escherichia coli *Pa: Pseudomonas aeruginosa*
Ca: Candida albicans

(-). Inactive, inhibition zone <10 mm.
(NT). Not tested.

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Table (4): The minimal inhibitory concentrations (MIC, µg/ml) of extracts of *Ficus benjamina* and *β*-sitosterol, ampicillin and clotrimazole.

Extracts and Compounds	Minimal inhibitory concentration (MIC, µg/ml)		
	Ec	*Pa*	*Ca*
Alcohol extract	31.5	-	-
Pet-ether extract	-	-	-
Chloroform extract	15.5	-	7.8
Ethyl acetate extract	-	-	7.8
β- sitosterol	15.5	15.5	15.5
Ampicillin	2	2	NT
Clotrimazole	NT	NT	4

Ec: Escherichia coli., Pa: Pseudomonas aeuroginosa,, Ca: Candida albicans. (NT): Not tested.

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دراسة لمحتويات أوراق نبات فيكاس بنجامينا

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فى هذا البحث تم فصل جلوتينول وفريديلينول وبيتا سيتوستيرول وألفا أميرين و٦- بيتا-هيدروكسى ستيجما-٤-إين-٣-أون و٦- بيتا-هيدروكسى ستيجما-٤،٢٢-داين-٣ أون وبيتا سيتوستيرول-٣-أ-جلوكوزيد من خلاصة الكحول الميثيلى لأوراق نبات فيكاس بنجامينا ل.

المركبات فريديلينول و٦- بيتا-هيدروكسى ستيجما-٤-إين-٣-أون و٦- بيتا-هيدروكسى ستيجما-٤،٢٢-داين-٣ أون تم فصلهم لأول مره من الجنس(فيكاس) وباقى المركبات تم فصلها لأول مره من نبات فيكاس بنجامينا ل ماعدا ألفا أميرين الذى تم فصله من قبل من لبن النبات . وقد تم التعرف على التركيب الدقيق لهذه المركبات بدراسة خواصها الطبيعية بالإضافة إلى مقارنتها بالعينا ت القياسيه وكذلك عن طريق الوسائل الطيفيه المختلفه مثل الأشعه البنفسجيه ودون الحمراء والرنين المغناطيسى بأنواعه البروتونى والكربونى وذا البعدين وكذلك مطياف الكتله.

كما تم دراسة النشاط البيولوجى للخلاصات المختلفة للنبات. ومنها أتضح أن لخلاصة الكلوروفورم تأثير فعال ضد كنديدا ألبيكانس وإشيريشيا كولاى.