PHYTOCONSTITUENTS FROM *CALLIANDRA HEMATOCEPHALA* LEAVES AND THEIR BIOLOGICAL ACTIVITIES

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ABSTRACT

Phytochemical investigation of *Calliandra hematocephala* (L` Her) Benth leaves (Leguminosae) resulted in isolation of β-sitosterol (1), Lupeol (2) and dodecanoic acid (3). The compounds were identified by chromatographic (TLC, PC) and spectroscopic analysis (^{1}H NMR and ^{13}C NMR). This is the first report for isolation of compounds 2 and 3 from *Calliandra hematocephala*. All compounds (1-3) showed antifungal activity against *Aspergillus fumigates*, *Penicillium italium* and *Geotricum candidum* .They also showed antimicrobial activity against *Staphylococcus aureus*, *Bacillis subtilis* and *Escherichia coli*.

INTRODUCTION

Calliandra hematocephala (L` Her) Benth. (Family Leguminosae) is a perennial herb, widely distributed throughout tropical and subtropical America, some are present in India and West-Africa(1, 2). Leaves and barks are used in folk medicine as anti-tumor(3), anti-oxidant(3), anti-cholinergic(4), insecticide(5), anti-malarial(6) and astringent(7). Triterpenes(8), flavonoids(9), pipecolic acids(10) and glycolipids(11) have been isolated from *C. hematocephala*. The present paper describes the isolation and identification of three compounds from *Calliandra hematocephala* by nuclear magnetic resonance (NMR) and mass spectroscopy (ESI-MS). These compounds were evaluated for their antimicrobial activities(12).

MATERIAL AND METHODS

General experimental procedures

^{1}H and ^{13}C NMR spectra were performed on a Bruker AMX at 400 MHZ for ^{1}H and 100 for ^{13}C. All NMR spectra were obtained in DMSO-d_6 and $CDCL_3$ using TMS as an internal standard, the observed chemical shift (δ values) are given in ppm, and coupling constants (*J* values) in hertz. TLC was performed on pre-coated plates with a silica gel layer, thickness of 200 m. The ESIMS spectra were measured using a Bruker Bioapex-FTMS with electrospray ionization (ESI). Column chromatographic separation was performed on silica gel 60 (Si gel 60, Merck) and Sephadex LH-20 (Pharmacia). TLC was performed on precoated TLC plates with silica gel 60 F254 (0.2 mm, Merck). Developed chromatograms were visualized by spraying with 1% vanillin-H_2SO_4, followed by heating at 100 ^{0}C for 5 min.

Plant material

Calliandra hematocephala leaves were collected during the flowering stage (April 2011) from Orman garden at Giza. The plant was kindly identified by Engineer Badeia Hassan Aly Dewan, Consultant of Egyptian Flora, Agricultural Museum, Dokki, Giza, Egypt, and by Mrs. Terase Labib, Taxonomist of Orman Garden, Giza, Egypt.

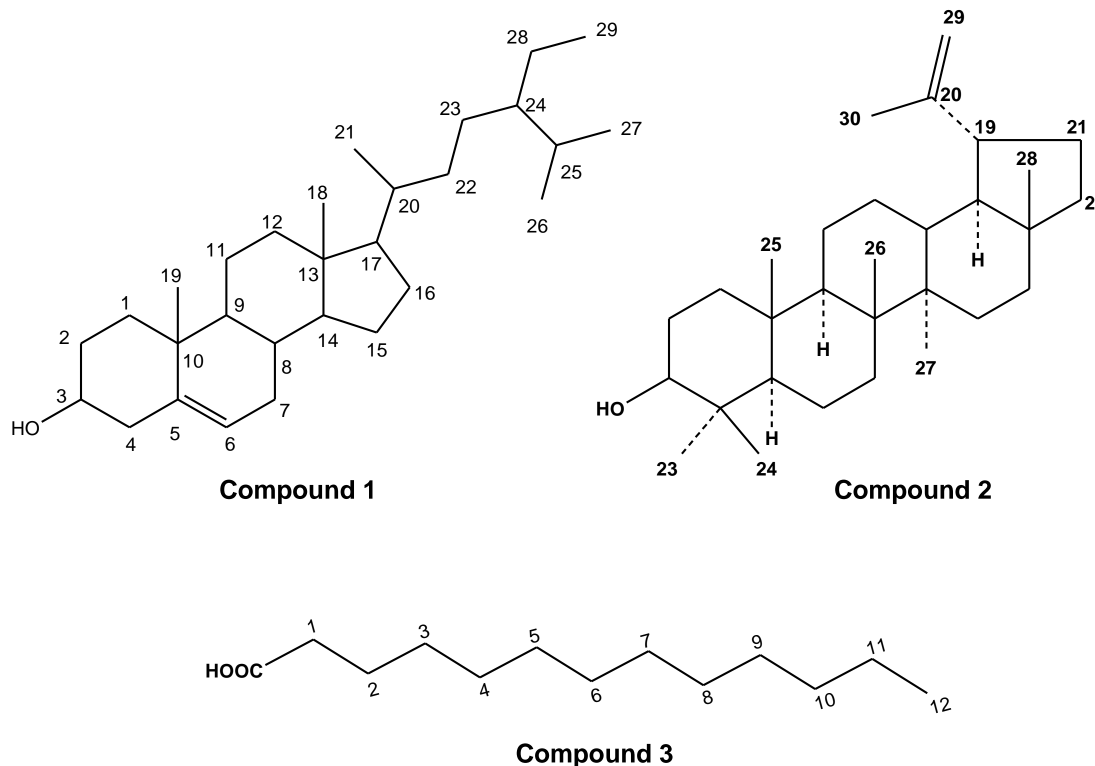
Extraction and isolation

Air-dried powdered leaves of *C. hematocephala* (500g) were subjected to successive extraction with methanol (3Lx4), where 35g dry total extract were obtained. The total extract was subjected to VLC using silica gel for column and different organic solvents starting with petroleum ether, ethyl acetate , *n*-butanol and finally the column was washed out with distilled water to yield four fractions; petroleum ether fraction (4gm), ethyl acetate fraction (5gm), *n*-butanol fraction (6gm) and water fraction (8gm). The petroleum ether fraction (4gm) was subjected to CC using silica gel then finally subjected to Sephadex LH-20 eluted with methanol to yield six sub-fractions of A (200mg), B (350mg), C (500mg), D (450mg), E (100mg) and F (250mg). Sub-fraction D (450mg) was further chromatographed using Sephadex LH-20 and methanol to give two compounds 1 (15mg) and 2 (20mg). Sub-fraction A (200mg) was further purified using Silica gel column eluted with hexane:ethylacetate (95:10, 90:10 and 85:15) followed by re-purification using Sephadex LH-20 and methanol to afford compound 3 (15mg).

Compound 1 [β-sitosterol]: White amorphous powder; gaves positive Lieberman-Burchard test and negative Molish`s test; R_f: 0.46 [TLC, Hexane:Ethyl acetate-80:20]; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.70 (3H, s, H -18), 1.03 (3H, s, H -19), 0.94 (3H, d, *J*=6 Hz, H -21), 0.84 (3H, d, *J*=6 Hz, H -26), 0.82 (3H, d, *J* =6.4 Hz, H -27), 0.86 (3H, t, *J*=7.2 Hz, H -29), 3.54 (1H, m, H-3), 5.37(1H, m, H-6); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 11.87 (C-18), 19.40 (C-19), 19.4 (C-21), 18.79 (C-26), 19.82 (C-27), 11.99 (C-29), 71.82 (C-3), 121.73 (C-6); **EI-MS** *m/z* 414 $[M]^+$.

Compound 2 [Lupeol]: White powder; melting point 213^{0}C; ^{1}H NMR (DMSO-d_6, 400 MHz): δ 0.76, 0.78, 0.89, 0.91, 0.98, 1.23, 1.64 (each 3H, each s, Me×7), 3.16 (1H, *dd*, *J* =5.4, 10.6 Hz, H-3), 4.54 (1H, s, H-29a), 4.68 (1H, s, H-29b); ^{13}C NMR (DMSO-d_6, 100 MHz): δ 150.6 (C-20), 108.9 (C-29), 78.4 (C-3), 55.3 (C-5), 50.3 (C-9), 49.06 (C-18), 47.8 (C-19), 43.01 (C-17), 42.8 (C-14), 40.8 (C-8), 40.5 (C-22), 40.3 (C-13), 40.1 (C-4), 39.9 (C-1), 39.7 (C-10), 39.5 (C-16), 38.9 (C-7), 37.0 (C-21), 35.5 (C-23), 29.6 (C-15), 28.5 (C-12), 27.1 (C-2), 20.8 (C-11), 18.4 (C-30), 18.2 (C-6), 18.0 (C-28), 16.8 (C-25), 16.4 (C-26), 16.0 (C-24), 15.1 (C-27); EIMS *m/z*: 426 $[M]^+$.

Compound 3 [Dodecanoic acid]: White powder; ^{1}H NMR (DMSO-d_6, 400 MHz): δ 0.89 (t, *J*=7.0 Hz, H -12), 2.12 (t, J= 7.5 Hz, H -2), 1.58 (m, H -3), 1.46 (m, H -11), 1.19 (m, H -4-H -9); ^{13}C NMR (DMSO-d_6 100 MHz): 174.6 (C-1), 34.11(C-2), 25.1 (C-3), 29.26-29.47 (C-4-C-9), 31.92 (C-10), 22.64 (C-11) 14.09 (C-12); EIMS *m/z*: 201 $[M+H]^+$.



Compound 1

Compound 2

Compound 3

Antimicrobial and antifungal activities of *Calliandra haematocephala*

Antimicrobial activities of petroleum ether extract of *Calliandra haemtocephala* were investigated in *vitro* against different bacteria and fungi. The following bacterial strains were employed in the screening: Gram-positive bacteria; *Staphylococcus aureus* (RCMB 010028) and *Bacillis subtilis* (RCMB 010067), Gram-negative bacteria; *Escherichia coli* (RCMB 010052) and *Pseudomonas aeruginosa* (RCMB 010043). As fungal strains *Aspergillus fumigates* (RCMB 02568)*, Penicillium italicum* (RCMB 03924)*, Geotricum candidum* (RCMB 05097) and *Candida albicans* (RCMB 05031). Ampicillin, Gentamycin and Amphotericin B were used as reference drugs. The microbial species are environmental and clinically pathogenic microorganisms obtained from Regional Center for Mycology and Biotechnology antimicrobial unit (RCMB), Al-Azhar University.

Determination of Antimicrobial Activity:

Antimicrobial activities of petroleum ether extract of *Calliandra haemtocephala* were detected in *vitro* against the reference drug using the diffusion agar technique[12]. The results were recorded in table 1.

Investigation of lipoidal matter(Saponifiable and Unsaponifiable matters):

The lipoidal matter obtained by extraction of 50 g of the air dried leaves of *Calliandra haematocephala* with n-hexane (3 × 300 ml) was evaporated in vacuo at 40°c to yield (1 g). This residue was kept for preparation of unsaponifiable matter (USM) and fatty acids (FA).

Saponification: The n-hexane extract residue of both *Calliandra haematocephala* was saponified by refluxing with 15 ml of 10% alcoholic K OH for 10 hours. After distillation of the alcohol and dilution with 20 ml H_2O, the unsaponifiable matters of *Calliandra haematocephala* was extracted with ether (3× 20 ml). The combined ether extracts were washed several times with distilled H_2O till completely free from alkalinity, then dehydrated over anhydrous sodium sulphate and filtered. The residue left after evaporation of ether was subjected to GLC for identification of HC and sterol contents. The aqueous mother liquor was acidified with 2 ml conc. H_2SO4 to liberate corresponding free fatty acids. The liberated fatty acids were extracted with ether extract then dehydrated over anhydrous sodium sulphate[13].

GC/ MS analysis of lipoid matter.

Samples were injected under the following conditions
Helium was used as carrier gas at approximately 1 ml/min., pulsed splitless mode. *The solvent delay was 3 min. and the injection size was 1.0 ul. The mass* spectrophotometric detector was operated in electron impact ionization mode an ioning energy of 70 e.v. scanning from m/z 50 to 500. the ion source temperature was 230 ºC and the quadrupole temperature was 150 ºC. The electron multiplier voltage (EM voltage) was maintained 1250v above auto tune. The instrument was manually tuned using perfluorotributyl amine (PFTBA). The GC temperature program was started at 60ºC then elevated to 280ºC at rate of 8 ºC /min, and 10 min. hold at 280ºC the detector and injector temperature were set at 280ºC and 250ºC, respectively. Wiley and Nist 05 mass spectral data base was used in the identification of the separated peaks.

RESULTS and DISCUSSION

Compound 1 [β-sitosterol]: Was obtained as white amorphous powder and gave positive Lieberman-Burchard test(14) and negative Molish's test indicating sterol aglycone. The molecular formula was established as $C_{29}H_{50}O$ from ^{13}C NMR data and **EI-MS** ion peak at *m/z* 414 $[M]^+$. Its ^1H and ^{13}C NMR spectra showed six methyl signals at [δ_H 0.70 (3H, s), δ_C 11.87, Me-18], [δ_H 1.03 (3H, s), δ_C 19.40, Me-19], [δ_H 0.94 (3H, d, *J*=6 Hz), δ_C 19.4, Me-21], [δ_H0.84 (3H, d, *J*=6 Hz), δ_C 18.79, Me-26], [δ_H 0.82 (3H, d, *J* =6.4 Hz), δ_C 19.82, Me-27] and [δ_H 0.86 (3H, t, *J*=7.2 Hz), δ_C 11.99, Me-29], an oxygenated methine signal at [δ_H 3.54 (1H, m), δ_C 71.82, CH-3) and olefinic proton signal at δ_H 5.37 (1H, m, H-6, δ_C 121.73. The chemical shift value at δ_C 71.82 was attributed to the C-3 which is indicated for the presence of 3β hydroxyl group and confirmed that (1) was identified as β-sitosterol(15,16).

Compound 2 [Lupeol]: Was obtained as white powder and gave positive test for steroids and triterpenes(14). Its mass spectrum showed parent molecular ion peak $[M^+]$ at *m/z* 426 which corresponds to the molecular formula $C_{30}H_{50}O$(14). In the ^1H-NMR spectrum of 2, H-3 proton appeared at δ 3.16 (J= 4.5, m) and H-29 olefinic proton showed a multiplet at δ 4.68 and δ 4.54. Seven methyl proton signals also appeared at δ 0.98, 0.91, 0.90, 0.89, 0.87, 0.77 and 0.76 (each 3H, each s, CH_3). These assignments are in good agreement for the structure of lupeol(17, 18).

Compound 3 [Dodecanoic acid]: Was obtained as white amorphous powder. Its mass spectrum showed a peak at *m/z* 201 $[M+H]^+$ corresponding to the molecular formula $C_{12}H_{24}O_2$. The ^1H NMR spectrum displayed a triplet at δ 0.89 for the terminal methyl group. The triplet at δ 2.12 was assigned to the methylene protons adjacent to carbonyl group. The ^{13}C NMR spectrum showed the signal at δ 174.6 assigned for the carboxylic carbon and a signal at δ 14.09 for terminal methyl carbon. Therefore the structure of compound 3 was concluded to be luaric acid (dodecanoic acid)(19, 20).

Results of antimicrobial activity:

The antibacterial and antifungal activities (MIC) of petroleum ether extract were measured *in vitro* against Gram-positive (*Staphylococcus aureus* and *Bacillis subtilis*) and Gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*) bacteria and fungi (*Aspergillus fumigates, Penicillium* italicum*, Geotricum candidum* and *Candida albicans*). The antibacterial activities were compared with those of Ampicillin and Gentamycin while the antifungal activities were compared with that of Amphotericin B. Table 1 showed that, the sample was active against Gram-positive bacteria more than Gram-negative bacteria. The sample has promising antimicrobial activity against *Bacillis subtilis, Staphylococcus aureus, Geotricum candidum*, *Escherichia coli, Aspergillus fumigates and Penicillium italicum*. The sample showed no activity against *Candida albicans* and *Pseudomonas aeruginosa*. The sample under investigation revealed minimum inhibitory concentration comparable to standard (Table 1). At concentration 3.9 (µg/ml) the sample has (MIC) antimicrobial activity against all tested microorganisms except for *Candida albicans* and *Pseudomonas aeruginosa*.

Table (1): Results of antimicrobial activity and MICS (µg/ml) of petroleum ether extract of *Calliandra haematocephala*

Tested microorganisms	Diameter of inhibition zone (mm)		MIC (µg/ml)	
	Sample	ST.	Sample	ST.
Fungi		*Amphotericin B*		*Amphotericin B*
A. fumigates	20.1 ± 0.58	27.3 ± 0.10	3.9	0.24
P. italicum	19.9 ±0.44	21.9±0.12	3.9	0.98
C. albicans	NA	19.8±0. 20	NA	3.9
G. candidum	22.3±0.22	28.7±0.22	0.98	0.06
G + ve bacteria		*Ampicilin*		*Ampicilin*
S. aureus	22.8±0.22	27.4±0.18	0.49	0.12

B. subtilis	24.8±0.22	32.4±0.10	0.12	0.007
G - ve bacteria		*Gentamycin*		*Gentamycin*
P. aeruginosa	NA	17.3±0.15	NA	7.81
E. coli	20.3±0.58	22.3±0.18	3.9	0.49

Well diameter: 6.0 mm …. …. (100 µl was tested), Sample concentration (10mg/ml),

NA: No activity,

Data are expressed in the form of mean ± Standard deviation.

Results of GC/ MS analysis of lipoid matter:

The results of GC/ MS analysis of lipoid matter of *Calliandra haematocephala* revealed that it contains decane, undecane, dodecane, methylhexadecanoate, phytol, phthalate, stigmasterol, β-sitosterol and lupeol with different ranges of concentration. Dodecane and phytol were represented the major components, while stigmasterol and decane were the lowest ones (Table 2).

Table 2: GC/MS analysis of unsaponifiable matter of *C. haematocephala*

NO	GC/MS analysis				
	GC	MS			
	R t	%	Mol. Wt .	B .P	Name
1	3.91	0.02 %	112	83	Ethyl cyclohexane
2	4.90	0.15 %	126	97	Ethyl-methylcyclohexane
3	5.10	0.25 %	128	57	Nonane
4	5.60	0.04 %	114	57	Octane
5	7.18	5.26 %	142	57	Decane
6	8.30	0.14 %	154	55	Cyclodecanone
7	9.28	6.9 %	156	57	Undecane
8	9.97	0.65 %	154	83	Pentylcyclohexane
9	10.52	2.34 %	310	43	Docosane
10	11.23	8.57 %	170	57	Dodecane
11	13.00	0.23 %	184	57	Tridecane

12	14.57	0.30 %	198	55	Tetradecane
13	16.27	0.05 %	212	57	Pentadecane
14	17.77	0.12 %	226	57	Hexadecane
15	18.89	0.04 %	196	55	Cyclotetradecane
16	19.20	o.12 %	240	57	Heptadecane
17	19.60	0.042 %	196	69	Tetradecene
18	20.54	0.82 %	254	57	Octadecane
19	20.67	0.15 %	282	57	Eicos ane
20	21.07	0.21 %	278	68	Neophytadiene
21	21.81	o.74 %	268	57	Nonadecane
22	22.17	8.36 %	270	74	Methyl hexadecanoate
23	23.37	0.14 %	284	74	Methyl heptadecanoate
24	24.20	3.80 %	294	67	Methyl octdecanoate
25	24.46	31.7 %	296	71	Phytol
26	28.38	3.02 %	308	57	Eicosanol
27	38.50	4.04 %	412	55	Stigmaterol
28	39.86	5.40 %	414	414	B- sitosterol
29	41.99	13.12 %	426	207	Lupeol

GC/MS of saponifiable fraction showed that *C. haematocephala* contains Hexadecanoic acid, octadecanoic acid, Phthalic acid and octadecatrienoic acid. Hexadecanoic acid and octadecanoic acid were represented the major components, while Phthalic acid and octadecatrienoic acid were the lowest ones (Table 3).

Table 3: GC/MS analysis of saponifiable matter of C. *haematocephala*

NO	GC/MS analysis				
	GC	MS			
	R t	%	Mol. Wt .	B .P	Name

1	17.31	0.71 %	200	73	Dodecanoic acid
2	17.71	0.083 %	228	88	Dodecanoic acid methyl ester
3	20.09	1.18 %	228	73	Tetradecanoic acid
4	20.47	0.12 %	256	88	Tetradecanoic ethyl ester
5	21.38	0.82 %	242	73	Pentadecanoic acid
6	22.16	2.28 %	270	74	Hexadecanoic acid,methylester
7	22.88	36.89 %	256	73	n-Hexadecanoic acid
8	23.85	0.76 %	270	73	Heptadecanoic acid
9	24.20	1.15 %	294	67	Octadecadienoic methyl ester
10	24.28	1.45 %	292	79	Octadecatrienoic acid
11	24.91	22.76 %	264	57	Octadecadienoic acid
12	27.07	2.15 %	312	88	Octdecanoic acid methyl ester
13	29.02	15.21 %	279	149	Phthalic acid

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مركبات من اوراق نبات كاليندرا هيماتوسيفلا وفاعليتها الحيوية

للسيد الدكتور

السيد محمد السيد الغالى

مـــــــن

قسم العقاقير – كلية الصيدلة – بنين بالقاهرة – جامعة الازهر

تم فصل ثلاث مركبات من خلاصة الأيثير البترولى، مركب منهم من مشتقات استيرولات وهو مركب بيتاسيتوستيرول (١) ومركب من مشتقات التربينات الثلاثيه وهو مركب لوبيول (٢)، و المركب الثالث من مشتقات الاحماض الدهنية وهو حمض الدوديكيونيك (٣) . ويعتبر فصل المركبات ٢، ٣ هو اول مرة من النبات. وقد تم التعرف على تركيبهم الكيميائى على اسس تحاليل اطياف الكتلة والرنين المغناطيسى للبروتونات وذرات الكربون.

وقد وجد ان Diffusion Agar Method كما تم دراسة الفاعلية المضادة للميكروبات لهذه المركبات باستخدام خلاصة الأيثير البترولى لها تا ثير قوى كعامل مضاد للميكروبات.