Three new compounds from *Erythrina lysistemon* and their antimicrobial, radical scavenging activities and their brine shrimp lethality.

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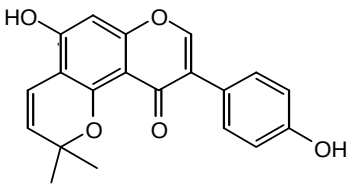
Abstract
Three new compounds, 4',7-dihydroxy-2",2"-dimethylpyrano [5",6":5,6]-isoflavone (**1**), (7*E*) (8,2')- 3,7,9,5',9'-pentahydroxy-4,4'-dimethoxyneolign-7-ene (**2**) and (9*E*,11*Z*) 14-hydroxyoctadecan-9,11-dienoic acid (**3**), along with other known flavonoids, benzenoids, alkaloids and phenylpropanoids were isolated from the twigs, leaves, stem bark, stem wood and flowers of *Erythrina lysistemon*. Their structures were established on the basis of spectroscopic evidence. Some of these compounds have shown high lethality against brine shrimps (*Artemia salina*), moderate radical scavenging ability in the DPPH assay, moderate antifungal activity against *Candida mycoderma*, moderate activity against the Gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*) and weak activity against Gram-negative bacteria (*Escherichia coli*).

Key words: *Erythrina lysistemon*, flavonoids, neolignane, phenolics, radical scavenging, lethality, antimicrobial.

Introduction

Previous studies have shown that *Erythrina lysistemon* (Leguminosae) elaborates erythrinaline alkaloids, some of which are distributed in several parts of this plant [1,2]. The other major group of compounds is the flavonoids, especially prenylated ones, and these compounds are prevalent in the stem and root bark [3,4]. The extracts from this plant have been used in traditional medicine and have also shown antiviral, anticancer and cytotoxic activities [4,5].



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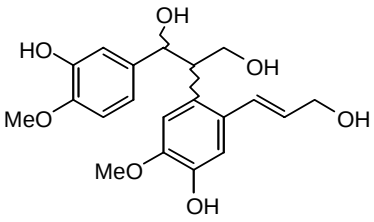

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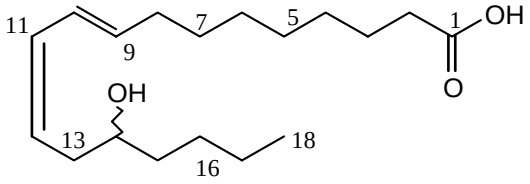

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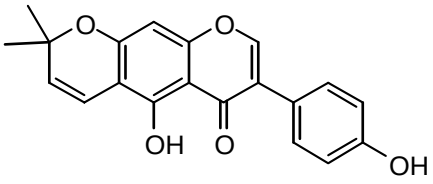

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The present paper reports on the non-erythrina alkaloid contents of the *Erythrina lysistemon* and their lethality against brine shrimp, radical scavenging ability and antimicrobial activities. Three

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new compounds, 4',7-dihydroxy-2",2"-dimethylpyrano [5",6":5,6]-isoflavone (**1**), (7*E*) (8,2')-3,7,9,5',9'-pentahydroxy-4,4'-dimethoxyneolign-7-ene (**2**), the first neolignan in the genus *Erythrina*, and (9*E*,11*Z*) 14-hydroxyoctadecan-9,11-dienoic acid (**3**), have been isolated from this plant. These have been obtained along with other known compounds including the flavonoids daidzein (**4**), genistein (**5**), wighteone (**6**), 4',5,7-trihydroxy-6-(2"-hydroxy-3"-methylbut-3"-enyl)isoflavone (**7**), alpumisoflavone (**8**), derrone (**9**), 6,8-di-prenylgenistein (**10**), erysenegalensein E (**11**)**,** lysistisoflavone [isoerysenegalensein E] (**12**), 2',5,7-trihydroxy-4'-methoxy-5-prenyl isoflavanone (**13**), apigenin (**14**), liquitirigenin (**15**), medicarpin (**16**) phaseollidin (**17**), cristacarpin (**18**), sandwicensin (**19**), 2,4,4'-trihydroxychalcone (**20**). The other non-flavonoid compounds isolated were caffeic acid (**21**), coumaric acid 4-glucoside (**22**), octadecanyl (*E*) ferulate (**23**), 3-methoxy-α,β-dihydrocoumaric acid (**24**), coumaric acid (**25**), the isoquinoline alkaloid precursor norprotosinomenine (**26**), the indole alkaloid hypophorine (**27**), 4-hydroxy-3-methoxybenzaldehyde (**28**), 4-hydroxy-3-methoxybenzoic acid (**29**) and inositol (**30**). Some of these compounds have shown high lethality against brine shrimps, strong radical scavenging ability against DPPH and antifungal activity against the yeast *Candida mycoderma*. Weak activity has also been exhibited against the Gram-negative bacteria (*Escherichia coli)* and moderate activity against Gram-positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus).*

Discussion

The twig (ethyl acetate) and stem wood (chloroform) extracts of *E. lysistemon* were worked up as shown in the experimental to give compounds **1** & **8** and **2** & **3**, respectively.

Compound **1** exhibited a peak at *m/z* 336.1 in its EIMS spectrum and displayed 20 non-equivalent carbon signals in the ^{13}C-NMR spectrum and a molecular formula $C_{20}H_{16}O_5$ was suggested. The ^{1}H- and ^{13}C-NMR spectra showed signals at δ_H 7.94 (1H, s; δ_C 152.9) interpretive of H-2 proton and C-2 carbon resonance of isoflavone [6,7,8]. The peak at 270 nm in the UV (MeOH) spectrum was assigned to the benzoyl moiety (A-ring) of the isoflavone. The ^{1}H-NMR spectrum of this compound further displayed an AA'BB' spin system 7.36 (2H, *dd*, *J* = 8.5, 2.2 Hz) and 6.90 (2H, *dd*, *J* = 8.5, 2.2 Hz)], which was associated with a 4'-hydroxyl substituted ring B of an isoflavonoid. The presence of a dimethylpyrano ring moiety was shown in the same spectrum by the signals [δ_H at δ_H 5.60 (1H, *d*, *J* = 10.1 Hz, H-3"), 6.69 (1H, *d*, *J* = 10.1 Hz, H-4") and 1.45 (6H, *s*, Me). The ^{13}C-NMR spectrum displayed signals at δ_C 128.6 (C-3"), 115.7 (C-4"), 78.4 (C-2") and 28.5 (C-2" Me) representing this group. This was supported by the EIMS spectrum which exhibited a fragment ion peak at *m/z* 321 $[M\text{-}CH_3]^+$, showing a loss of methyl group and typical of 2,2-dimethylchromenes [9]. H-4", resonating at 6.69, correlated with the carbon signals at 159.8 (C-7), 156.8 (C-5) and 105.8 (C-6) in the HMBC spectrum, indicating that this moiety was attached at C-6. The ^{1}H- and ^{13}C NMR spectra for this compound compared well with those of co-isolated compound **8** [5]**.** The absence of the chelated C-5 hydroxyl hydrogen signal indicated that the prenyl group was cyclized to the 5-COH group rather than the 7-COH group contrary to **8**. Further, from the UV spectrum a bathochromic shift of +5 was observed for band II, indicating a free 7-OH [6]. Total assignment for this compound was accomplished by a close examination of the HMBC and HMQC spectra. This data enable compound **1** to be identified as 4,7-dihydroxy-2", 2"-dimethyl-dipyrano [5", 6":5,6]-isoflavone.

Compound **2** did not show the parent ion $[M]^+$ peak expected at *m/z* 376 but that for the $[M\text{-}H_2O]^+$ ion, at *m/z* 358 in it EMS, observed as the highest peak. This may have been due to the

fact that the OH group at C-7' was easily lost to give this fragment ion. Its ^{13}C-NMR spectrum exhibited 20 carbon signals, which were distinguished as two methoxy, two oxymethylene and nine methine (one oxymethine). The following signals were observed, δ_H 4.21 (δ_C 62.8) and δ_H 3.87, 3.77 (δ_C 63.8) [oxymethylenes] δ_H 5.52 (δ_C 88.3) [oxymethine] and δ_H 3.51 (δ_C 54.1) [methane]. The two sets of signals appearing at δ_H 6.77 (δ_C 115.1), 6.84 (δ_C 118.7) and 6.95 (δ_C 109.5) displaying and AMX spin system and δ_H 6.96 (δ_C 115.5) and 6.98 (δ_C 111.1) [singlets], demonstrated the existence of two aromatic rings in **2**. The presence of an olefinic moiety in the molecule was indicated by the signals appearing at δ_H 6.24 ([1H, *ddd*, *J*=15.7, 11.7, 5.9 Hz] δ_C 126.3) and δ_H 6.54 ([1H, *br d*, *J*=15.7 Hz] δ_C 131.0) the size of the coupling constant indicated a *trans*-configuration. The proton signal at δ_H 6.24 correlated (HMBC) with the carbon at δ_C 62.8 (oxymethylene) indicating direct connection to the olefinic moiety. The other olefinic proton resonating at δ_H 6.54 correlated with the carbon signals at δ_C 111.1, 129.3 and 131.5. These observations revealed a C-2, C-4 and C-5 substituted phenylpropanoid system. The methine signal at δ 3.51 correlated with the second oxymethylene carbon (δ 63.8), the oxymethine carbon (δ 88.3) and an aromatic carbon (δ 131.5), while oxymethine proton signal at δ 5.52 correlated with the carbon signals at δ 54.1, 133.5 and 118.7 in the HMBC spectrum. These observations revealed the existence of a second phenylpropanoid system whose C_3 side chain was hydroxylated at positions C-1' and 3' and its C-2' attached to the aromatic ring of the first ArC_3 system. Intense fragment ion peaks at *m/z* 151 and 180 representing cleavage that gives fragments A and B (Figure 3) respectively further strengthens the proposal. Other important HMBC correlations are shown in figure 1. From these observations, **2** was assigned the structure (7'*E*) (8,2')-3,5',7,9,9'-pentahydroxy-4,4'-dimethoxyneolign-7'-ene.

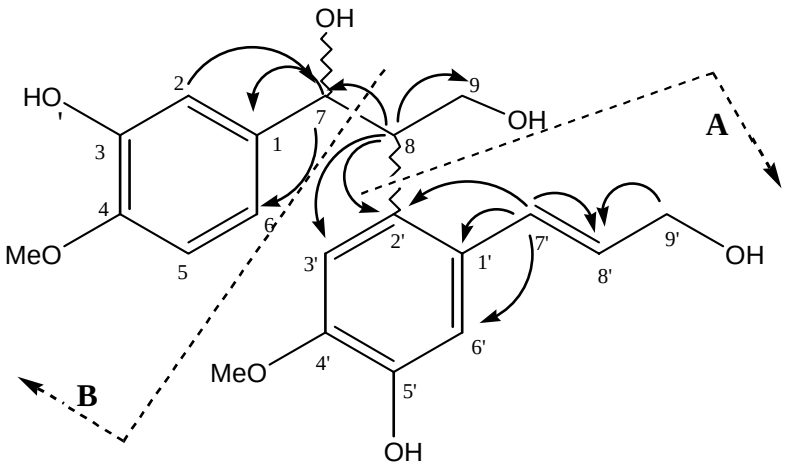

Figure 1: Some important HMBC correlations for compound **2**

Compound **3** was obtained as an oily substance from the stem wood of *E. lysistemon*. Its ^{13}C-NMR data showed eighteen signals out of which one was methyl, eleven were methylenes and five were methines. The EIMS spectrum showed a molecular ion ($[M]^+$) peak at *m/z* 296.2 while

the HRESI-MS gave a sodiated ion ($[M+Na]^+$) exact mass at *m/z* 319.2244 (calc. 296.4449) which assigned for molecular formula $C_{18}H_{32}O_3$. The presence of two double bonds in the molecule was indicated by the signals observed at δ_H6.51 (δ_C126.2), δ_H 5.67 (δ_C 133.2), δ_H 5.44 (δ_C 136.2) and δ_H 5.98 (δ_C 128.2). The signal at δ_C 179.6 was assigned to a carboxylic acid group. Further the ^{1}H and ^{13}C-NMR spectra displayed signals at δ_H 4.18 and δ_C 73.4 (oxymethine). The proton signal δ_H 4.18 (δ_C73.4), showed HMBC correlations with carbons signals at δ_C 25.5, 37.6 and 126.2, while the olefinic proton at δ_H 6.51 correlated with the carbons at δ_C 37.6, 136.1 and 133.2. Close examination of the data showed that the oxymethine group was separated by one methylene group (δ_H 1.56, δ_C 37.6) from the diene moiety. The data available enabled the identification of **3** as (9*E*,11*Z*) 14-hydroxyoctadecan-9,11, -dienoic acid.

Radical scavenging activity of *Erythrina lysistemon* against DPPH

The radical scavenging activities of the crude extracts and isolated compounds were assessed using 2,2-Diphenyl-1-picrylhydrazyl (DPPH). To establish the level of activity, of a given sample, a plot was made of absorbance verses concentration (in μg/ml). The concentration of the sample at which the absorbance at 517 nm decreases to half its initial value was taken as the IC_{50} value of the sample in question. Ascorbic acid was used as the standard and its activity was examined in the same manner.

All the isoflavonoids examined reduced DPPH after 48 hours given the presence of free phenolic hydroxyl groups (Table 3). Compound **5** gave the quickest response amongst this group of compounds (IC_{50} 287 and 149 μg/ml; after 6 and 48 hrs), while Compound **10** exhibited the highest activity (IC_{50} = 55μg/ml; after 48 hrs). The isoflavanone **13** was more active (IC_{50} 240 and 68 μg/ml; after 6 and 48 hrs) than the isoflavones (Table 3).

Table 1: ^{1}H- and ^{13}C-NMR (300 and 75 MHz in $CDCl_3$) data for compounds **1** and **8**

Position	Compound **1***		Compound **8***	
	δ_H	δ_C	δ_H	δ_C
2	7.94 (1H, *s*)	152.9	7.83 (1H, *s*)	153.0
3	-	122.2	-	123.9
4	-	181.4	-	181.4
5	-	156.8		157.2
6	-	105.8	-	106.0
7	-	159.8	-	160.0
8	6.90 (1H, *s*)	95.3	6.36 (1H, *s*)	95.3
9	-	157.7	-	157.7
10	-	106.4	-	106.5
1'	-	124.1	-	123.3
2'	7.36 (1H, *d*, *J*=8.6 Hz)	130.5	7.39 (1H, *d*, *J*=8.3 Hz)	130.7
3'	6.90 (1H, *d*, *J*=8.6 Hz)	115.9	6.89 (1H, *d*, *J*=8.3 Hz)	116.1
4'	-	157.5	-	156.4
5'	6.90 (1H, *d*, *J*=8.6 Hz)	115.9	6.89 (1H, *d*, *J*=8.3 Hz)	116.1
6'	7.36 (1H, *d*, *J*=8.6 Hz)	130.5	7.39 (1H, *d*, *J*=8.3 Hz)	130.7
1"	6.69 (1H, *d*, *J*=10.0 Hz)	115.7	6.75 (1H, *d*, *J*=10.2 Hz)	115.9
2"	5.60 (1H, *d*, *J*=10.0 Hz)	128.6	5.64 (1H, *d*, *J*=10.2 Hz)	128.6
3"	-	78.4	-	78.5
4"	1.45 (3H, *s*)	28.5	-	28.7
5"	1.45 (3H, *s*)	28.5	-	28.7
OH		-	13.13	

Table 2: ^{1}H- and ^{13}C-NMR (300 and 75 MHz in MeOD) data for compound **2**

	δ_H	δ_C
1	-	133.5
2	6.95, *br s*	109.5
3	-	148.3
4	-	148.1
5	6.77, *d*, (8.1)	115.1
6	6.84, *dd*, (8.3, 1.9)	118.7
7	5.52, *d*, (6.3)	88.3
8	3.51, *m*	54.1
9	3.87, *dd*, (14.7, 5.9)	63.8
	3.78, *dd* (14.9, 6.1)	-
1'	-	129.3
2'	-	131.5
3'	6.96, *s*	115.5
4'	-	146.5
5'	-	144.4
6'	6.98, *s*	111.1
7'	6.54, *d*, (15.7)	131.0
8'	6.24, *ddd* (15.8, 11.7, 5.8)	126.3
9'	4.21, *dd*, (15.3, 5.8)	62.8
OMe	3.88, *s*	55.7
	3.82, *s*	55.1

The pterocarpans showed relatively higher radical scavenging ability compared to the isoflavones. Two of these, phaseollidin (**17**) and cristacarpin (**18**) were fast acting showing activity at IC_{50} values of 196 and 226 μg/ml, respectively, after only 30 minutes. After 6 and 48 hours, compounds **17**, **18** and **19** exhibited activities at IC_{50} values 88 & 25, 86 & 74 and 285 & 200 μg/ml, respectively. Comparing **17** and **19**, the methylated pterocarpan is four times less active after 6 hours and after 48 hours its activity is shown to be weaker by a factor of eight. These observations indicated that a free C-9 hydroxyl in pterocarpans is important both in enhancing activity and also the rate of radical scavenging. Also interesting to note in comparing compounds **18** and **19** is the enhancement of activity by the introduction of a hydroxyl at position C-6a.

The neolignan **2** and benzylisoquinoline alkaloid **26** the exhibited the highest activities at IC_{50} values of 78, 32 & 28 μg/ml and 46, 20 &10, respectively, after 30 minutes, 6 and 48 hours, which were higher than those of the standard ascorbic acid. Apart from possessing free phenolic hydroxyl groups, these compounds possess benzyl electronegative atoms (*O* and *N*) groupings, which as in the case of compound **18** could be the reason for their enhanced activities (Table 3).

The leaf extract (MeOH) showed activity after 30 minutes (175 μg/ml). After 6 and 48 hours leaf (MeOH) and the stem wood extracts ($CHCl_3$) showed activity after at 86 & >300 and 80 & 107 μg/ml, respectively. Their activity over those of the other extracts could be explained by the fact their constituents included the pterocarpans and compound **2** and **26** which had shown high radical scavenging strength.

Table 3: Radical scavenging activity of *Erythrina lysistemon* compounds and extracts

Compound	TLC (μg)	Quantitative, (IC_{50}, μg/ml)		
		After 30mins	After 6hrs	After 48 Hrs
Ascorbic acid	<0.1	36	33	33
Leaf (MeOH)	-	175	86	80
Leaf ($CHCl_3$)	-	>300	>300	>300
S/wood ($CHCl_3$)	-	>300	>300	107
S/wood (EtOAc)	-	>300	>300	>300
Flower ($CHCl_3$)	-	>300	>300	>300
Twigs (EtOAc)	-	>300	>300	>300
1	0.5	>300	>300	151
2	<0.1	78	32	28
4	0.5	>300	>300	230
5	0.1	>300	287	149
6	0.5	>300	>300	269
7	1.0	>300	>300	163
8	0.1	>300	>300	192
10	0.1	>300	>300	55
11	5.0	>300	>300	>300
13	0.5	>300	240	68
15	0.1	>300	>300	185
16	1.0	>300	>300	250
17	0.1	196	88	25
18	0.1	226	86	74
19	0.1	>300	285	200
21	0.5	102	82	81
22	5.0	>300	>300	>300
22	10.0	>300	>300	>300
23	0.5	>300	>300	>300
26	5.0	46	20	10
28	0.5	>300	264	141
29	0.5	106	90	58

Antibacterial properties of *Erythrina lysistemon* compounds

Antimicrobial analysis was done as outlined in the experimental section for compounds in appreciable quantities. In the test against the Gram-negative bacteria *Escherichia coli*, the isoflavonoids showed significantly high activities compared to other classes of compounds. The highest activity against this organism was shown by compounds **6**, **7**, **8** and **11** at loadings of 10 μg per spot. These activities were however much lower compared to those observed for the standard which was active at 0.001 μg. Compounds **3**, **4**, **7**, **12**, **17**, **21** and **22** showed quite appreciable activity against the Gram-positive bacteria *Bacillus subtilis* at 5. Hydroxylation on the isoprenyl group seems to increase activity against this organism as shown in compounds **6** & **7** and **11**, **12** & **10** (Table 4). Against the Gram-positive bacteria *Staphylococcus aureus*, compound **4** showed an activity much closer to that of the standard at 1 μg per spot. Flavone **14** showed a higher activity (0.5 μg) than the known antifungal miconazole (1.0 μg) against the yeast *Candida mycoderma*.

Lethality test of *E. lysistemon* extracts and compounds

Both the crude extracts and the pure compounds isolated from *E. lysistemon* were subjected to lethality test using brine shrimps (*Artemia salina*) as outlined in the experimental section. The pterocarpans exhibited extremely high activities against the shrimps. Compounds **17** and **19** had LD_{50} values of 6.9 ppm and 4.7 ppm (Table 4), which were even higher that those exhibited by standards potassium dichromate (25.39 ppm) and the bufadienolide scallaren A (190.85 ppm). The isoflavones also showed relatively high toxicity. Amongst this group of compounds, the highest activities were observed for compounds **1**, **5, 8**, **10** and **12** whose activities were exhibited at LD_{50} values lower than 100 ppm. The benzoic acid derivative **29** however was quite active exhibiting an LD_{50} value of 17.78 ppm. All the crude extracts also showed appreciable activity with the methanol extract of the leaf exhibiting the highest activity (LD_{50} 23.9 ppm). It is from this that the pterocarpan **17** that showed high activity together with isoflavones were isolated and as such must significantly contribute to this observation.

Table 4: Antimicrobial and cytotoxicity activities of compounds isolated from *Erythrina lysistemon.*

Compound	Lethality LC_{50} (μg/ml)	Antimicrobial activity (μg/spot)			
		Escherichia coli	*Bacillus subtilis*	*Staphylococcus aureus*	*Candida mycoderma*
1	19.14	>50	10	>50	10
2	243.00	-	-	-	-
4	97.63	50	5	>50	1
5	18.26	-	-	-	-
6	121.54	10	10	10	5
7	1000.00	10	5	>50	10
8	10.00	10	10	10	50
10	24.65	50	>50	1	5
11	23.10	10	5	>50	10
12	23.10	10	5	>50	10
13	>1000.00	>50	>50	>50	50
14	19.45	>50	>50	>50	0.5
17	6.96	50	5	>50	50
18	-	>50	10	50	10
19	4.74	10	50	50	50
20	-	>50	50	>50	>50
21	363.10	50	5	>50	5
22	41.12	50	10	10	10
23	573.60	>50	>50	>50	>50
26	>1000.00	>50	-	>50	>50
28	19.40	-	-	-	-
29	17.78	>50	-	>50	>50
Chloramphenicol	-	0.001	0.01	0.01	-
Miconazole	-	-	-	-	1

Standards: - Lethality - Scillaren A = 190.85 μg/ml, Potassium Dichromate = 25.39 μg/ml

Experimental section.

General Experimental Procedures: TLC analysis: - silica gel 60 $PF_{254+366}$ pre-coated alumina plates (Merck, 0.25 mm thick). Column chromatography: - silica gel (Merck, 0.100 -

0.0400 mm mesh). Gel filtration: - Sephadex LH-20 on glass columns. Prep-TLC plates (0.5 mm thick): - silica gel (Merck) 60 $HF_{254+366}$ on 20 x 20 cm glass plates. ^{1}H-NMR, (300 or 600 MHz), ^{13}C-NMR (75 or 150 MHz), DEPT, COSY, HMBC, HMQC: - Bruker Avance DXP 300 spectrometer using standard pulse sequences and referenced to residual solvent signal. EIMS: - Finnigan MAT SSQ 7000 single stage quadruple analyzer at 70 eV. The HRESI were obtained from a Bruker Apex III Fourier transform ion cylotron resonance (FT-ICR) mass spectrometer (Bruker Daltonics, Billerica, USA) equipped with an infinity cell, a 7.0 Tesla superconducting magnet (Bruker, karlshule, Germany). UV: - Shimadzu UV-2101PC spectrometer.

Plant Material: The various parts of *E. lysistemon* were collected between July 2001and May 2002 in Gaborone (University of Botswana grounds), Botswana and its voucher specimen (No. EL 0701) is preserved in the Department of Biological Sciences, Faculty of Science, University of Botswana.

Extraction and Isolation: Twigs of *E. lysistemon* were air-dried and ground before being extracted by soaking in EtOAc for 24 hours three times. The resulting extracts were combined and the solvent removed *in vacuo* using rotary evaporator to give 12 g of dark green organic material. This extract was subjected to column chromatography (silica gel) eluting with *n*-Hex/$CHCl_3$ followed by $CHCl_3$/MeOH mixtures with increasing polarities to give 30 eluents *ca* 50 ml, which were combined into six combined fractions A to G, based on TLC profile. The viscous fraction B (eluted with 4% MeOH/$CHCl_3$, 1.5 g) was chromatographed on silica gel column (50 x 3) eluting with the following gradient: $CHCl_3$, MeOH/$CHCl_3$ (1:99, 1:49, 1:19, 1:9, 1:4) and MeOH. A total of 50 fractions of *ca* 50 ml were collected and combined on basis of TLC composition. Residue resulting from the fraction eluted with 1:99 MeOH/$CHCl_3$ (80 mg) was subjected to prep-TLC (1:99 MeOH/$CHCl_3$) followed by purification using Sephadex LH-20 column (40 x 2) to give compounds **1** (20 mg) and **8** (7 mg). Compounds **10** (7 mg), **11** (5 mg) and **12** (4 mg) were obtained from the 1:49 MeOH/$CHCl_3$ eluted fraction (40 mg) through multiple developments on prep-TLC (1:99 MeOH/$CHCl_3$) and cleaning using Sephadex LH-20. Compound **7** (11 mg) was obtained from the fraction eluted with 1:9 MeOH/$CHCl_3$.

4,7-Dihydroxy-2″,2″-dimethylpyrano[5″,6″:5,6]-isoflavanone (1): pale yellow paste, EIMS *m/z* (rel int.) 336 $[M]^+$ (5), 321 $[M\text{-}CH_3]^+$ (11), 302 (3), 286 (24), 270 (24), 254 (100), 243 (4), 216 (3), 197 (4), 153 (15), 137 (60). UV (MeOH) λ_{max} (nm) (log ε)**:** 271 (3.36); + NaOMe: 280; + $AlCl_3$: 281; + $AlCl_3$/HCl: 280; + NaOAc: 276; + NaOAc/H_3OBO_3: 276. ^{1}H-NMR (300 MHz, $CDCl_3$) and ^{13}C-NMR (75 MHz, $CDCl_3$) see Table 1.

Dried powered leaf material (1.67 kg) was soaked successively in $CHCl_3$ and MeOH three times in each case for 24 hours in 5000 ml flasks. The various extracts were combined and concentrated *in vacuo* to obtain 50 and 28 g of green organic material, respectively. The chloroform extract was subjected to gel filtration on Sephadex LH 20, on a column (70 x 5) using 1:1 MeOH/$CHCl_3$ to remove chlorophyll, resulting in 30g of brownish organic material. VLC was employed to separate the various components previously seen on TLC, with elution being carried out using *n*-Hex/$CHCl_3$ and then $CHCl_3$/MeOH while increasing the amounts of $CHCl_3$ and MeOH, respectively, resulting in fractions A to H. Fractions C and D (eluted with 2% and 5% MeOH/$CHCl_3$, respectively) were subjected to gel filtration as above to further remove chlorophyll to give combined fractions C1 & C2 and D1 & D2, respectively. Separation of the components resulting from these fractions was achieved by the use of prep-TLC (5% MeOH/$CHCl_3$) to obtain compound **17** (14 mg, C2) and **6** (31 mg, D2).

The methanol extract was also subjected to flash chromatography eluting with $CHCl_3$/MeOH with increasing amounts of MeOH in $CHCl_3$ to obtain combined fractions A to F. Compounds **26** (14 mg) and **30** (40 mg) were obtained from fractions E and F (8% MeOH/$CHCl_3$ eluent), respectively, through fractional crystallization (solvent $CHCl_3$/EtOAc/MeOH).

Previously dried and ground stem wood material (1.8 kg) was extracted successively with EtOAc and MeOH as described above to obtain 10.5 g and 40.9 g, respectively, of light brown organic material. The EtOAc extract was subjected to step-wise gradient elution chromatography on a column (50 x 5) using *n*-Hex/$CHCl_3$ and then $CHCl_3$/MeOH while increasing the amounts of $CHCl_3$ and MeOH resulting in fractions A to G. Fractions A was subjected to repeated gel filtration using Sephadex LH 20 on a column (30 x 4) to obtain compound **3** (50 mg). Fraction C (eluted with $CHCl_3$) was subjected to gradient elution chromatography using *n*-Hex/$CHCl_3$ and then $CHCl_3$/MeOH (column 30 x 2). Compound **5** (7 mg) was obtained from fractions D by fractional crystallization. The methanol extract was on the other hand treated to flash chromatography (silica gel column; 70 x 6) using $CHCl_3$ then $CHCl_3$/MeOH while increasing MeOH. Through a combination of Column chromatography (silica gel), gel filtration (Sephadex LH-20), preparative-TLC and fractional crystallization compound **19** (16 mg) was obtained from B (eluted with 2% MeOH/$CHCl_3$); **2** (21 mg), **24** (7 mg) and **28** (11 mg), were obtained from fraction C (eluted with 5% MeOH/$CHCl_3$); **15** (5 mg), **16** (8 mg), **20** (17 mg) were obtained from fraction D (eluted with 8% MeOH/$CHCl_3$); **18** (21 mg) was obtained from fraction E (eluted with 8% MeOH/$CHCl_3$).

(7'*E*) (8,2')-3,5',7,9,9'-Pentahydroxy-4,4'-dimethoxyneolign-7'-ene (2): brownish solid, mp 68-71 °C. EIMS *m/z* (rel. int.) 358 $[M-H_2O]^+$ (100), 340 $[M-2H_2O]^+$ (83), 328 (48), 325 (24), 310 (10), 279 (10), 272 (5), 267 (5), 235 (4), 210 (5), 180 (26), 151 (29), 137 (88). ^{1}H-NMR (300 MHz, MeOD) and ^{13}C-NMR (75 MHz, MeOD) see Table 2.

(9*E*,11*Z*) 14-Hydroxyoctadecan-9,11-dienoic acid (3): oily substance, HRESI-MS *m/z* (rel int.) $[M+Na]^+$ 319.2244 (296.4449); EIMS *m/z* (rel int.) 296.2 $[M]^+$ (15), 280 (100), 262 (5), 224 (9), 206 (14), 171 (6), 151 (10), 135 (5), 113 (10), 99 (20), 81 (10); ^{1}H-NMR (300 *M*Hz, $CDCl_3$) 6.51 (1H, *dd*, *J*=15.2, 11.0 Hz, H-12), 5.99 (1H, *t*, *J*=10.9, Hz, H-9), 5.67 (1H, *dd*, *J*=15.5, 7.0 Hz, H-11), 5.44 (1H, *ddd*, *J*=15.4, 10.7, 7.7 Hz, H-10), 4.18 (1H, *dd*, *J*=12.9, 6.3 Hz, H-14), 2.35 (3H, *t*, *J*=7.4 Hz, H-2), 2.18 (1H, *d*, *J*=7.1 Hz, H-8), 1.61 (4H, *m*, H-3), 1.56 (*m*, H-13), 1.33 (15H, *m*, H-4, 5, 6, 7, 15, 16, 17), 0.90 (3H, *t*, *J*=6.6 Hz, H-18). ^{13}C-NMR (75 *M*Hz, $CDCl_3$) 179.6 (C=O), 136.1 (C-11), 133.2 (C-10), 128.2 (C-9), 126.2 (C-12), 73.3 (C-14), 37.6 (C-13), 34.3 (C-2), 32.2 (C-16), 29.7 (C-7), 29.3 (C-5), 29.2 (C-6), 27.9 (C-8), 25.5 (C-15), 25.0 (C-3), 22.9 (C-17), 14.4 (C-18: CH_3).

The stem bark material was air-dried and ground before extraction using chloroform to give 50 g of dark green organic material, which was subjected to separation using column chromatography (silica gel). *n*-Hexane, $CHCl_3$ and MeOH mixtures were used as in the cases above as eluting solvents. The fraction eluted with 1:5 *n*-Hex /$CHCl_3$ was passed through a Sephadex LH 20 column (1:5, MeOH/$CHCl_3$) to obtain compound **23** (34 mg). The column was further eluted with $CHCl_3$ and the resulting combined fraction were separated using prep-TLC (5% MeOH/$CHCl_3$) leading to compound **9** (7 mg). Through multiple developments (x6) on prep-TLC (5% MeOH/$CHCl_3$) compound **13** (30 mg) was isolated from fraction eluted with 4% MeOH/$CHCl_3$.

The butanol extract (50 g) of the flowers of this plant was adsorbed on 50 g silica gel and subjected to flash chromatography and eluted with $CHCl_3$ and $CHCl_3$/MeOH mixtures with increasing polarities. The fraction eluted with 5% MeOH/$CHCl_3$ (2.1 g), was subjected to column chromatography and the fraction resulting from 3% MeOH/$CHCl_3$ was subjected to prep-TLC to obtain compounds **14** (15 mg) and **25** (11 mg). Further elution of the column gave fraction D (1.78g, 7% MeOH/$CHCl_3$) from where it was possible to obtain compound **21** (300 mg) through fractional crystallization (solvent *n*-Hex/$CHCl_3$/EtOAc). Through fractional crystallization it was possible to obtain compounds **22** (860 mg) and **27** (34 mg) from fraction I (2.2 g) and J (300 mg), respectively. These fractions had previously been eluted from the main column using 20% MeOH/$CHCl_3$.

The methanol extract of the pods (42 g) was adsorbed on 42 g silica gel and subjected to column chromatography on a column packed with silica gel (300 g) under $CHCl_3$. Separation was achieved by step wise gradient elution using $CHCl_3$ then $CHCl_3$/MeOH with increasing amounts of MeOH in $CHCl_3$. Compound **4** (24 mg) was obtained from fraction (10% MeOH/$CHCl_3$ eluent, 260 mg) by use of prep-TLC followed by purification using Sephadex LH-20.

Radical scavenging assay using DPPH

2,2-Diphenyl-1-picrylhydrazyl (DPPH), molecular formula $C_{18}H_{12}N_5O_6$, was obtained from Fluka Chimie AG, Bucks. The method used by Kumarasamy *et al*. (2002) [11] and Naik *et al*. (2003) [12] was adopted. Radical scavenging activity was carried out for both crude extracts and pure compounds isolated from *E. lysistemon*. This was done at two levels, first the rapid TLC screening (Qualitative assay) followed by quantitative assay where the reaction of DPPH with the test compounds, indicated by reduction in absorbance was monitored by used of UV at 517nm. In evaluating DPPH inhibition amount, varying amounts (ranging from 0.1 to 50 or 100 μg) of the samples were spotted on TLC after which the plate was sprayed with 0.2% of DPPH solution in methanol. Activity was shown by yellow spots on purple background. On the other hand, for quantitative evaluation of radical scavenging ability, 1.5 ml of 500 μM DPPH in methanol was added to equal volumes of test compounds at concentrations ranging from 0 to 300 μg/ml, mixed well and kept in the dark for 30 minutes after which absorbance was measured. Further measurements were done after 6 and 48 hours. Blank experiment was also carried out to determine the absorbance of DPPH at 0 concentration of sample. A plot was then made of absorbance verses concentration. The concentration of sample in μg/ml at which the absorbance at 517 nm decreases to half its initial value is used as the IC_{50} value of a given compound. Gallic and ascorbic acids were used as the standards and their activities were examine in the same manner.

Lethality Tests.

Samples were prepared by dissolving appropriate quantities of material in chloroform. 5 ml of these solutions were then added to sample vials in triplicates for each of the concentrations 100,10,1 μg/ml (pure sample) and 1000,100,10 μg/ml (Crude extract). The solvent was allowed to evaporate at room temperature over two days after which the samples were dissolved in DMSO (up to 100 μl was added per 5 ml of brine before toxicity affects results). Brine Shrimp eggs were hatched in artificial seawater prepared by dissolving 38 g of sea salt in one litre of de-ionized water. 48 hrs was allowed for eggs to hatch and 10 mature nuplii were then added to each of the vials. This was followed with adjusting the volume with artificial seawater to 5ml per vial. The number of survivors was then counted after 24 hrs.

The LC_{50} were determined graphically from the percent lethality against the log conc. and LC_{50} derived from the best-fit line obtained by linear regression analysis [13].

Antimicrobial test using Agar Overlay Technique.

Microorganisms and culture media.

Strains of the gram-negative bacteria *Escherichia coli* and gram-positive bacteria *Bacillus subtilis* and *Staphylococcus aureus* together with the yeast *Candida mycoderma*, were obtained from anti-microbial culture banks at the Microbiology Department, University of Botswana. The bacteria were broth cultured on Nutrient Agar (NA) while the yeast was maintained on Sabourand Dextrose Agar slants and petri dishes. All these cultures were introduced using a heat sterilized wire loop into 250 ml Erlenmeyer flasks containing 100 ml Nutrient Broth. These were shaken at room temperature on a rotary shaker at 200 rpm for 24 hours to achieve homogenous distribution of the organisms. All media were autoclaved at 120°C for 20 minutes.

Samples tested and inoculums for assay.

The known antibacterial chloramphenical was used as a standard antibacterial agent while miconazole was used as the standard anti-fungal agent. The antibacterium was tested between 0.0001 and 10 μg, while the antifungal was tested between 0.01 and 50 μg. The isolated pure compounds were tested at amounts between 0.1 and 50 μg for both antifungal and antibacterial activity. These amounts were spotted on to glass backed TLC plates coated (25 mm thickness) with silica gel G 60 F_{254} at varying amounts for each sample. The plates were then dried with a hair dryer for complete removal of solvents.

Solid media overlay used for bacteria was Malt Nutrient Agar while Sabourand Dextrose Agar (Oxoid) was used in the case of fungi. The molten agar was maintained at 40°C to keep it from solidifying. 10 ml of nutrient broth containing the microorganisms was seeded in 100 ml of malt nutrient agar. This gave a concentration of approximately 10^7 cells/ml as established by the use of a UV/VIS spectrophotometer, which gave an optical density value at 540nm (OD_{540}) equivalent to 1 [13]. The final concentration in the solid medium was expected to be approximately 10^5 cells/ml. The seeding was done immediately before carrying out the overlay.

Bioautography.

The spotted plates were placed on a hot plate maintained at 35°C while the inoculums was rapidly spread over the TLC plates using a sterile Pasteur pipette. After solidification of the medium, the TLC plates were incubated overnight at 37°C for the bacteria and 25°C for the fungi in polythene boxes lined with moistened chromatography paper. After this the bioautograms were sprayed with an aqueous solution (2.5 mg/ml) of thiazolyl blue (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; MTT) (Fluka) and then incubated for 4 hours at 37°C for the bacteria and 25°C for the fungi. For activity, clear inhibition zones were observed against a purple background [14].

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