Presented at the Research Congress 2013
De La Salle University Manila
March 7-9, 2013

STRUCTURE ELUCIDATION OF TWO NEW PHYTOL DERIVATIVES, A NEW PHENOLIC COMPOUND AND OTHER METABOLITES OF *AVERRHOA BILIMBI*

Gunawan[1], Arlene Cordero[2] and Anamy Paano[1,3]
[1]Chemistry Department, De La Salle University, Manila, [2]University of North Carolina at Greensboro, [3]Author with whom communications should be addressed

Abstract: Methanolic extracts of *A. bilimbi* leaves were obtained by exhaustive extraction with methanol at ambient temperature. DCM and ethyl Acetate extracts were obtained from the alcoholic extract by sequential extraction. The DCM and EtOAc extracts were subjected to repeated flash column chromatography with re-crystallization. Seven isolates were obtained from the extract of leaves. Nam three of which are new compounds. This included squalene (**1**); 3-(6,10,14-trimethylpentadecan-2-yl)furan-2(5H)-one (**2** a new compound); 2,3-bis(2,6,10-trimethylundeca-1,5,9-trienyl)oxirane (**3** a new compound) with negative activity towards α-glucosidase inhibitory assay, Kirby-Bauer antimicrobial assay and DPPH radical scavenging assay]; phytol (**4**); 3,4-Dihydroxyhexanedioic acid (**5**) which was isolated for the first time from a natural source; malonic acid (**6**); and 4,5-Dihydroxy-2-methylenehydroxybenzaldehyde (**7**). Two isolates were obtained from the extract of fruits namely oxalic acid (**8**) [major component] and 2,4-Dihydroxy-6-((4-methylpentyloxy)methyl) benzaldehyde (**9**) [new compound, major component of a reaction that showed high antioxidant activity and moderate activity for α-glucosidase assay]. All these compounds were elucidated from their mass spectral data, 1D NMR (^{1}H, ^{13}C, ^{13}C DEPT NMR) and where necessary, from their 2D NMR (COSY, HSQC, HMBC) data.

Key Words: *Averrhoa bilimbi*; phytol derivative; antioxidant; antidiabetic; natural diacids

1. INTRODUCTION

Averrhoa bilimbi Linn (Oxalidaceae), locally known as Kamias in the Philippines, has been widely used as traditional cure for a number of ailments including *diabetes mellitus*, hypertension, inflammation and bacterial infection. Some studies on the determination of the biological activities of crude and semi purified extracts were reported but no activity study was done on pure isolates(Abbas et al, 2006; Ikram et al, 2009; Ambili et al, 2009; Pushparaj, 2001; Tan et al, 2005; Norhana et al, 2009; Murakami et al, 2000). The reported secondary metabolites of this plant were limited to amino acids, citric acid, vitamins A and C, phenols, sugars, oxalic acid and some volatile compounds (Krishnaiah et al, 2007; Pino et al, 2004; Miean and Suhaila, 2001; Ching and Suhaila, 2001). This study therefore aimed to obtain pure metabolites of the leaves and fruits of this plant and elucidate their structures by spectroscopic analysis. Pure compounds obtained from the samples were also submitted for activity testing but is not covered in this report.

2. METHODOLOGY

Fresh leaves (5 kg) of *A. bilimbi* were air-dried prior to exhaustive extraction with methanol at ambient temperature. The crude methanolic extract was subjected to sequential extraction with hexane, dichloromethane, and ethyl acetate affording extracts LN, LD, and LE, respectively. The remaining methanolic aqueous layer was hydrolyzed with HCl (2M) and re-extracted with ethyl acetate then amyl alcohol to obtain the less polar aglycones giving rise to extracts LMEA and LMA, respectively.

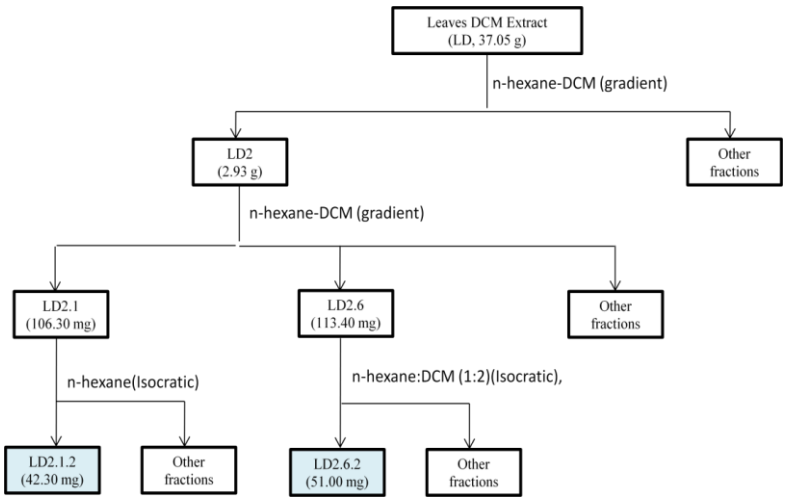
The crude extracts were subjected to flash column chromatography on normal phase silica. Solvents of various polarities were used for gradient and isocratic elution. Selected fractions were purified by re-crystallization and re-chromatography. The purity of the samples were determined from their thin layer chromatograms obtained by multiple development of each plate using various solvent systems

The pure isolates were sent to The University of North Carolina at Greensboro, USA for NMR analysis (^{1}H, ^{13}C, HSQC, HMBC and COSY). ESMS, EIMS and IR data of the isolates were taken at De La Salle University, Manila, Philippines.

The crude extracts and some isolates obtained from *Averrhoa bilimbi* Linn (*A. bilimbi* (L)) were submitted for bioassay tests. The bioassay tests: antioxidant activity assay (DPPH scavenging activity assay), antidiabetic activity (α-glucosidase inhibitory activity assay) and antimicrobial activity (Kirby-Bauer method) were done at the Research Center for Chemistry-Lembaga Ilmu Pengetahuan Indonesia (LIPI) in Indonesia.

3. RESULTS AND DISCUSSION

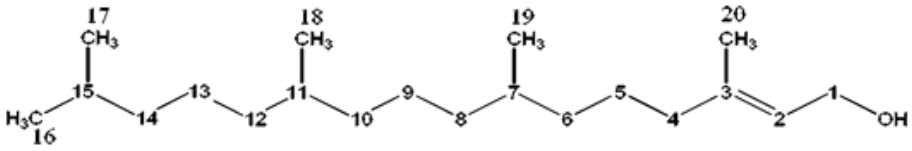
The dichloromethane (DCM) extract from leaves (LD) was fractionated using normal phase silica column chromatography. The column was eluted with combinations of hexane-DCM in increasing polarity. The fractions obtained were pooled together based on their TLC profile affording 8 combined fractions that are mostly impure (Scheme 1). The second (LD2) fourth (LD4) and the sixth (LD6) fractions were subjected to re-column chromatography giving rise to two pure isolates The fractionation of the second fraction is shown in Scheme 1.



Scheme 1 Purification of Isolates LD2.1.2 and LD2.6.2

Isolate LD2.1.2 (42.30 mg; 8.45% or 8.46 mg/kg of *A. bilimbi* fresh leaves) was a pale yellow oil that gave a single spot on TLC (Rf = 0.80, n-hexane) using UV-light then vanillin-sulfuric acid reagent as visualizing agent). The electron impact mass spectrum (EIMS) of isolate LD2.1.2 showed 75.8% match with MS library data for squalene (1). The ^{1}H NMR spectrum of isolate LD2.1.2 was typical of an unsaturated hydrocarbon. Three clusters of ^{1}H NMR resonances were observed at δ 5.11 ppm attributed to olefinic methine (=CH), δ 1.60 – δ 1.69 as signals due to methyl groups (-CH_3) and δ 1.9-2.1 assigned to saturated methines (–CH) and methylenes (-CH_2). The assignment of squalene structure (1) is consistent with the reported ^{1}H and ^{13}C NMR resonances of the known compound squalene (Pogliani et al, 1994).

The second pure isolate of LD2, isolate LD2.6.2, (51.00 mg, Rf = 0.33, hexane)), was a yellow oil. Its ^{1}H NMR spectrum was found to be similar to phytol (2). Four methyl doublet signals were identified between 0.80 ppm and 0.89 ppm and intense resonances related to overlapping methylene and methine units were found between 1.18 ppm and 1.39 ppm. The resonance at 5.32 ppm indicated the presence of a double bond while the doublet at 4.58 ppm indicated the presence of oxygenated methylene group (-OCH_2-). EIMS data of the isolate matched with phytol (2) MS data from the MS library. Hence, the ^{1}H NMR and ^{13}C NMR of phytol (Arigoni et al, 1999) and isolate LD2.6.2 were compared (Table 1)



FNH-I-008

(2) Phytol

Table 1. ^{1}H and ^{13}C NMR Resonances of Phytol (32) and Isolate LD2.6.2

Carbon No.	Chromophore	^{1}H NMR Chem Shifts[a] (ppm)		^{13}C NMR Chem Shifts[b] (ppm)	
		Phytol	LD2.6.2	Phytol	LD2.6.2
1	-C-OH	4.14	4.58	59.39	61.28
2	=CH	5.39	5.32	123.09	118.22
3	=C<	-	-	140.23	142.71
4	$>CH_2$	1.97	1.98	39.85	39.95
5	$>CH_2$	1.40/1,36	1.61	25,12	25.11
6	$>CH_2$	1.24/1.05	1.24	36.65	36.71
7	>CH	1.35	1.36	32.67	32.76
8	$>CH_2$	1.23/1.03	1.24	37.35	37.52
9	$>CH_2$	1.29/1.15	1.61	24.45	24.55
10	$>CH_2$	1.23/1.03	1.04	37.41	37.37
11	>CH	1.35	1.36	32.77	32.88
12	$>CH_2$	1.23/1.03	1.24	37.28	37.44
13	$>CH_2$	1.25	1.61	24.79	24.96
14	$>CH_2$	1.11/1.03	1.13	39.35	39.45
15	>CH	1.50	1.52	27.95	28.07
16	$-CH_3$	0.84	0.85	22.60	22.72
17	$-CH_3$	0.84	0.85	22.69	22.82
18	$-CH_3$	0.83	0.83	19.69	19.79
19	$-CH_3$	0.82	0.82	19.72	19.83
20	$-CH_3$	1.65	1.68	16.14	16.46
-	>C=O	-	-		174.04
-	>CH	-	1.24		32.57

[a] 500MHz, $CDCl_3$, [b] 125 MHz, $CDCl_3$

Most of the ^{13}C NMR resonances of isolate LD2.6.2 is consistent with the ^{13}C NMR chemical shift assignment of phytol except for the extra resonances in isolate LD2.6.2 at 174.04 ppm that corresponds to >C=O and a methine carbon at 32.57 ppm. The ^{13}C NMR resonances of phytol for carbons 1 to 3 (59.39 ppm, 123.09 ppm and 140.23 ppm, respectively) are also slightly shifted to 61.28 ppm, 118.22 ppm and 142.71 ppm, respectively. The slight differences in the ^{13}C NMR resonances of isolate LD2.6.2 and the extra resonances at 174.04 ppm and 32.57 ppm suggest that this compound is not phytol.

The ^{1}H-^{1}H COSY spectrum of the sample showed a correlation of an olefinic proton (5.32 ppm) to an oxygenated methylene group (4.38 ppm, Figure 1). The long range C-H interactions from HMBC spectrum showed a correlation between the carbonyl carbon (174.04 ppm) with the methylene proton (-OCH_2-, 4.58 ppm). The methylene proton (4.58 ppm) was correlated with the quaternary carbon (142.71 ppm) and the methine carbon at 118.22 ppm. The same quaternary carbon was also correlated to a methyl group (1.68 ppm). The methyl protons at 1.68 ppm were in turn correlated to a methine proton at 1.24 ppm in the COSY spectrum. Hence a5-membered lactone is proposed at the beginning of the phytol chain (3). The compound is given the name 3-(6,10,14-trimethylpentadecan-2- yl) furan-2(5H)-one (3). An acetate unit (carbons C-4 and C-5) must have been condensed at positions 1 and 2 of phytol. Some phytol derivatives have been reported (Lou *et al*, 2000) but phytol derivatives with lactone ring like [3-(6,10,14-trimethylpentadecan-2-yl)furan-2(5H)- one] has not been reported. Hence this isolate is new compound.

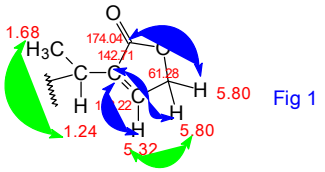
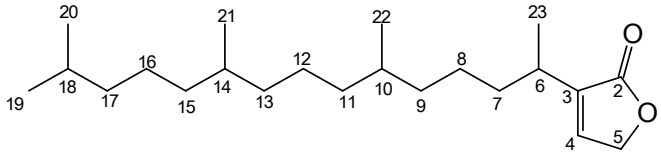


Fig 1

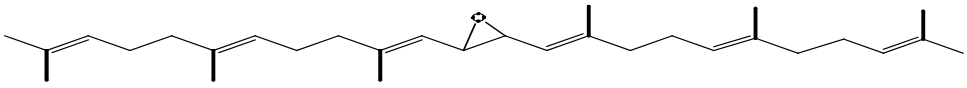
COSY
HMBC



(3) 3-(6,10,14-trimethylpentadecan-2- yl) furan-2(5H)-one (Proposed Structure of LD2.6.2)

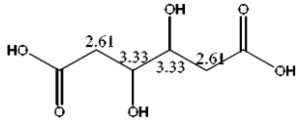
Repeated column chromatography of the fourth fraction of dichloromethane extract (LD4) using combinations of DCM:EtOAc as eluent yielded two pure fractions. Fraction LD4.3.3 (588.50 mg; yellow oil, RF = 0.25, hexane) and fraction LD4.4.3 (291.70 mg, Rf = 0.75 hexane-EtOAc 5:1). The EI mass spectrum of isolate LD4.3.3 matched with that of squalene. It was however noted that isolate LD4.3.3 is more polar (Rf = 0.25, hexane) than squalene (Rf = 0.08, hexane). Their ^{1}H NMR and ^{13}C NMR resonances were then compared. Most of the ^{1}H NMR resonances of isolate LD4.3.3 matched with that of squalene except for the extra ^{1}H NMR resonance at 4.09ppm bonded to 59.01 ppm as shown in the HSQC spectrum. This should complement the absence of ^{1}H and ^{13}C NMR resonances in isolate LD4.3.3 where 2.03 ppm and 28.28 ppm are assigned for C-11 and C-12 of squalene (1). This suggests the oxygenation of C-11 and C-12 of the isolate. This may be in the form of an epoxide or a diol. The presence of an epoxide at C-11 and C-12 is supported by the IR spectrum that shows a weak absorption band at 3033 cm^{-1} (Fleming, 1985). The NMR resonances at 4.09 ppm (^{1}H NMR) and 59.01 ppm (^{13}C NMR) are characteristic of an epoxide rather than a diol (Pogliani et al, 1994).

The C-H and H-H connectivities established from HSQC and COSY spectra of isolate LD4.3.3 confirmed its identity as 2,3-bis (-2,6,10-trimethylundeca-1,5,9- trienyl)oxirane (4). While some epoxides of squalene were previously reported, no epoxide at C-11 and C-12 has been reported. Isolate LD4.3.3 is therefore a new compound. The other fraction, Isolate LD4.4.3, was identified as the known compound phytol (2) from its 1D and 2D NMR spectra and from its mass spectrum.

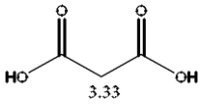


(4) 2,3-bis (-2,6,10-trimethylundeca-1,5,9- trienyl)oxirane (LD4.3.3)

The sixth fraction of the dichloromethane extract of *A. bilimbi* was rechromatographed affording isolates LD6.5.3 and LD6.11.1. Isolate LD6.5.3 was identified from its ^{1}H NMR spectrum as 4-dihydroxyhexanedioic acid (5) also known as 2,5-dideoxyhexaric acid. This acid is commonly formed when cellulose is reacted with alkali (Hranueli et al, 2001). It has also been used as a component of resin and as starting material for photocrosslinkable polyurethane production (Bernhard, Patent). It is also used to relieve constipation, as medication for overactive bladder, as antimuscarinic and as anticholinergic agent (Mehdi , Patent). This is the first time this acid is isolated from a natural source.



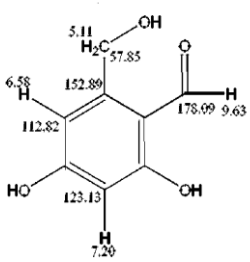
(5)



(6)

Isolate LD6.11.1 was identified as malonic acid (6) from its ^{1}H NMR spectrum and its mass spectrum. Malonic acid is a common acid from fruits. It is an important intermediate in the syntheses of vitamins B1 and B6, barbiturates, non-steroidal anti-inflammatory agents and other numerous pharmaceuticals, agrochemicals, flavors and fragrances compounds (Ohtmer, 1999).

The ethyl acetate extract from *A. bilimbi* leaves was fractionated by column chromatography using combinations of hexane ethyl acetate for gradient elution. The third fraction was rechromatographed affording a pure fraction LMEA3.3 (21.0 mg, Rf = 0.85, n-hexane: EtOAc (2:1, $C_8H_8O_4$ calculated for 191.03148). The ^{1}H NMR spectrum showed a resonance at 9.63 typical of an aldehyde. This was confirmed by the ^{13}C NMR resonance at 178.09 ppm. The doublets at 6.58 ppm (J=4) and 7.20 ppm (J=3.5) are attributed to aromatic protons the are *meta* disposed (Ohtmer, 1999). The ^{13}C and ^{13}C DEPT NMR spectra also showed the presence of a methyleneoxy group at 57.85 ppm. The assignment of the position of the hydroxyl groups on the benzene ring is based on the expected meta disposition of oxygenated substituents formed as a result of the cyclization of polyketides (Niemela and Sjostrom, 1985) and on the J values of the aromatic hydrogens.



(7) 2,4-dihydroxy-6-(hydroxymethylene)benzaldehyde (LMEA3.3)

The structure of isolate LMEA3.3 is therefore proposed as 2,4-dihydroxy-6-(hydroxymethylene)benzaldehyde (7). This structure is in agreement with the electrospray mass spectrum (ESMS) and electron impact (EIMS) mass spectral data of the sample. This compound is the major component of the biologically active extract LE (ethyl acetate extract)

4. CONCLUSION

Seven metabolites were isolated pure from the crude extract of *A.bilimbi*. Their structures were elucidated and were found to be compounds not previously reported from this plant. Two new derivatives of phytol namely 3-(6,10,14-trimethylpentadecan-2-yl)furan-2(5H)-one (3) and 2,3-bis (-2,6,10-trimethylundeca-1,5,9- trienyl)oxirane (4) were identified. A new phenolic compound 4,5-Dihydroxy-2-methylenehydroxybenzaldehyde (**7**) was also elucidated. None of these compounds showed significant activity as antioxidant. α-glucosidase inhibitor and as antimicrobial.

5. ACKNOWLEDGEMENTS

The authors would like to acknowledge the Research Centre for Chemistry-Lembaga Ilmu Pengetahuan Indonesia (LIPI) for the bioassay tests, The University of North Carolina at Greensboro, USA for the NMR experiments and De La Salle University for the use of their research facilities.

6. REFERENCES

Abas, F.; Lajis, N. H.; Israf, D. A.; Khozirah, S.; Kalsom, Y. U. *Food Chemistry*, 2006, 95, 566-573.

Ambili, S.; Subramoniam, A.; Nagarajan, N. S. *Planta Med*, 2009, 75 (1), 55-58.

Arigoni, D.; Eisenreich, W.; Latzel, C.; Sagner, S.; Radykewicz, T.; Zenk, M.H.; Bacher, A. *Proc. Natl. Acad. Sci*. 1999, 96, 1309-1314.

Bernhard, S. *Photocrosslinkable Polyurethanes*. Patent No 2005014682.

Ikram, E. H. K.; Eng, K. H.; Jalil, A. M. M.; Ismail, A.; Idris, S.; Azlan, A.; Nazri, H. S. M.; Diton, N. A. M.; Mokhtar, R. A. M. *Journal of Food Composition and Analysis*, 2009, 22 (5), 388-393.

Ching, L. S.; Suhaila, M. *Journal of Agricultural and Food Chemistry*, 2001, 49 (6),

3101-3105.
Hranueli, D.;Peric, N.; Borovicka, B.; Bogdan, S.; Cullum, J.; Waterman, P.G.; Hunter, I.S. *Food Technol. Biotechnol*. 2001, 39(3), 203-213.64.
Krishnaiah, D.; Sarbatly, R.; Bono, A. *Biotechnology and Molecular Biology Review*, 2007, 1(4), 097-104.
Luo, X.D.; Wu, S.H.; Ma, Y.B.; Wu, D.G.; *Fitoterapia*, 2000,71,492-496.
Mehdi, P. *Theraphy for the Treatment of Desease.* Patent No 2007027675
Miean, K. H.; Suhaila, M. *Journal of Agricultural and Food Chemistry*, 2001, 49 (6), 3106-3112.
Murakami, A.; Ali, A. M.; Salleh, K. M.; Koshimizu, K.; Ohigashi, H. *Biosci.*
Niemela, J.; Sjostrom, E. *Carbohydrate Research*. 1985, 144, 241-249.
Norhana, M. N. W.; Azman, M. N.; Poole, S. E.; Deeth, H. C.; Dykes, G. A.*International Journal of Food Microbiology*, 2009, 136, 88-94.
Ohtmer, K. *Encyclopedia of Chemical Technology*. John Willey and Son: United State of America, 1999.
Pino, J. A.; Marbot, R.; Bello, A. *Journal of Essential Oil Research*, 2004, 16(3), 241-242.
Pogliani, L.; Ceruti, M.; Riddhiardi, G.; Viterbo, D. *Chemistry and Physics of Lipids*. 1994, 70, 21-34.
Pushparaj, P. N.; Tan, B. K. H.; Tan, C. H. *Life Sciences*, 2001, 70, 535-547.
Tan, B. K. H.; Tan, C. H.; Pushparaj, P. N. *Life Sciences*, 2005, 76, 2827-2839.
William, D. H.; Fleming, I. *Spectroscopic Methods in Organic Chemistry*; McGraw Hill: England, 1985. 63.