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Lansioside D, a new triterpenoid glycoside antibiotic from the fruit peel of *Lansium domesticum* Correa

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Abstract
The major antimicrobial compound, named lansioside D, was isolated from the fruit peel of *Lansium domesticum*. Its structure was elucidated by different spectroscopic methods, including ID and 2D NMR experiments and HR-FABMS analysis. Evaluation of its antimicrobial activity against a wide spectrum of microorganisms showed that it has remarkable activity against the Gram-positive bacteria *Staphylococcus aureus* and *Bacillus subtilis*, and moderate activity against the Gram-negative bacteria *Escherichia coli*. It has no activity against yeasts and molds.

Keywords: Antibiotic, Lansioside D, *Lansium domesticum*, Triterpenoid.

1. Introduction
Lansium domesticum, of the family Meliaceae, is an erect, single-trunked tree growing up to 30 m in height [1]. Commonly known as 'lansones', this plant is native to Southeast Asia and is now found from Southern India to the Philippines. In the Philippines, it is grown mostly in southern Luzon and in northern Mindanao. The fruits are edible and very popular as a dessert. The fruit peels are used as arrow poison, or dried, then burned to drive away mosquitoes [2]. The seeds are used as a febrifuge and vermifuge. The bark is used to treat dysentery and malaria, while the leaf juice is used as eye-drops to dispel inflammation [3].
Only few reports on the bioactive constituents of *L. domesticum* are currently available. The fruit peel was reported to contain the onoceroids lansic acid [4, 5] and lansiosides A, B and C [6, 7]. The seed does not contain these onoceroids but was found to give skeletally new tetranortriterpenoids named dukunolides A-E as its bitter principles [8]. The seeds were also reported to contain antimalarial tetranortriterpenoids identified as domesticulides A-E [9]. The twigs afforded nine new compounds belonging to a rare class of onoceranoid-type triterpenoids exhibiting moderate activity against Gram-positive bacteria [10]. Other compounds isolated from the plant were lansiolic acid and lansionic acid [11].
As part of a program to study Philippine plants being used in folkloric medicine, *L. domesticum* was investigated as a possible source of new antimicrobial compounds. Preliminary antimicrobial assay of the crude methanol extract from the fruit peel showed remarkable activity against *Staphylococcus aureus*. This justified further attempts to isolate and identify the major antimicrobial compound (**1**). The present paper deals with the isolation, structure elucidation and antimicrobial activity of **1**.

2. Materials and methods
2.1 Plant material
L. domesticum fruit peels (~1 kg fresh weight) were obtained from UPLB campus, College, Laguna, Philippines. They were air-dried for one week, yielding 150 g of the dried material.

2.2 Bioactivity-guided fractionation
The dried fruit peels were chopped, soaked in methanol overnight and filtered. The filtrate was dried *in vacuo* below 40 °C. The residue was re-suspended in 100 ml water, then sequentially extracted with *n*-hexane and ethylacetate. The ethyl acetate extract was evaporated to dryness and the residue was then fractionated by silica open column chromatography eluted with solvents of increasing polarity, as follows: toluene → toluene: acetone (9:1) → toluene: acetone (8:2) → toluene: acetone (7:3) → toluene: acetone (6:4) → acetone → methanol.

Medium pressure liquid chromatography (ODS-A, 40 x 2.0 cm column) of the acetone fraction (1.63 g) eluted with 30- 100% CH_3CN at a flow rate of 5 ml/min afforded the major active compound in the 100% CH_3CN fraction (112.4 mg).

2.3 Antimicrobial bioassays

2.3.1 Bioautographic method

The test samples obtained from fractionation were loaded on a silica TLC plate and developed using toluene: acetone: methanol (7:3:1, v/v/v). After development, the TLC plate was freed of the developing solvent, then subjected to bioautography [12]. *S. aureus* was grown overnight in 5 ml of LB medium in a water bath at 37 °C. The bacterial suspension was added to 25 ml of melted LB agar medium and overlaid on the developed TLC plate. The bacteria-seeded plate was then incubated at 37 °C overnight. After incubation, a solution of 12 mM MTT was applied onto the bacteria-covered plate to detect antibacterial activity. Areas with bacterial growth were stained purple with MTT while areas with active compounds appeared as white spots.

2.3.2 Micro-dilution antimicrobial assay

The two-fold serial dilution technique [13] was used to determine the minimum inhibitory concentration (MIC) of the test samples. Three bacteria (*S. aureus*, *Escherichia coli* and *B. subtilis*), 2 yeasts (*Saccharomyces cerevisiae* and *Candida lipolytica*) and 2 molds (*Aspergillus niger* and *Cladosporium herbarum*) were used as test organisms. Bacteria and yeasts were pre-cultured overnight with shaking in LB medium at 37° C and YM medium at 30 °C, respectively. The molds, on the other hand, were grown in PDA medium at room temperature for 2 weeks until a well-expanded fungal mat with spores was formed. The fungal spores were collected by filtration and suspended in a liquid medium containing 100 g/l sucrose, 2.0 g/l KNO_3, 1.0 g/l KH_2PO_4, 0.5 g/l $MgSO_4.7H_2O$ and 0.1 g/l $CaCl_2$.

In a 96-well microtiter plate, 100 µl of bacterial and yeast cultures (1x 10^5 cells/ml) or fungal spore suspension (1x 10^5 spores/ml) were added to 100 µl of test samples prepared in various dilutions starting from 100 µg/ml down to 0.78 µg/ml. Tetracycline was used as the positive control against bacteria while cycloheximide was used as the positive control against yeasts and molds. Methanol was also included as the negative control. The seeded plate was covered and incubated overnight, after which the MIC was noted. MIC is defined as the minimum concentration of the test sample that inhibited microbial growth after a 12-h incubation period.

2.4 Physical and chemical analyses

NMR experiments were performed on a JEOL 400 MHz at 25° C. Mass spectra were obtained using a Shimadzu LCMS QP-8000. HR-FABMS were recorded on a JEOL XR300. IR and UV spectra were measured with a HORIBA FT-210 and Shimadzu UV-1600, respectively.

3. Results and Discussion

The crude methanol extract from the fruit peel of *L. domesticum* showed inhibitory activity against *S. aureus*. Bioautography revealed that the antimicrobial activity of this extract was due to a number of compounds, as indicated by the presence of many inhibition zones that were not stained purple by MTT. A bioassay-guided fractionation was then undertaken to isolate **1** which was the most active compound.

1 was isolated as a white amorphous solid. It was soluble in chloroform, ethyl acetate, acetone, methanol and ethanol. It appeared as a single spot on the TLC plate developed with toluene:acetone:methanol (7:3:1, v/v/v, R_f 0.40) when viewed under UV_{254}, or as a purple spot after spraying with vanillin-sulfuric acid followed by heating to 110 °C for 1 min. Its UV spectrum was observed with two maxima (ε) at 203 (5200) and 250 (1500) nm in methanol. The IR spectrum suggested the presence of hydroxyls (3420 cm^{-1}) and carboxyls (1760 and 1630 cm^{-1}) and exomethylene double bonds (1630 and 890 cm^{-1}). Its molecular formula was analyzed to be $C_{37}H_{58}O_9$ based on the observed molecular ion peak of *m/z* 647 $[M+H]^+$ in FABMS.

The ^{1}H NMR spectrum (Fig.1) showed the presence of 4 quaternary methyl signals at δ_H 0.67 (H-27), 0.80 (H-25), 0.82 (H-30) and 1.02 (H-26), and 2 vinylic methyl signals at δ_H 1.74 (H-29) and 1.78 (H-32). The proton signal at δ_H 3.65 for 3H was assigned to a methoxyl group (H-23). The ^{1}H NMR spectrum also showed the presence of carbinyl protons at δ_H 3.21 (H-3), 3.33 (H-5'a), 3.46 (H-2'), 3.55 (H-3'), 3.66 (H-4'), 3.99 (H-5'b) and 4.40 (H-1'), and olefinic protons at δ_H 4.56 (H-28a), 4.77 (H-31a), 4.82 (H-31b), 4.84 (H-H-28b) and 5.37 (H-15).

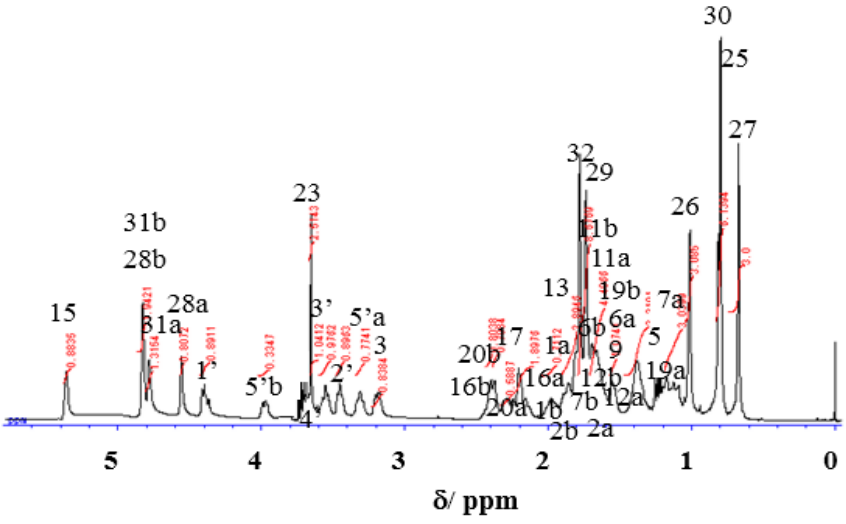

Fig 1: The 400 MHz ^{1}H NMR spectrum of the active compound.

In the ^{13}C NMR spectrum (Fig. 2), a total of 37 carbon atoms were observed, two of which were carboxyls (C-22 at δ_C 174.72 and C-21 at δ_C 178.35). A close inspection of the ^{13}C NMR spectrum by DEPT experiment also revealed the presence of six quaternary carbons at δ_C 38.60 (H-10), 39.06 (H-4), 39.34 (H-18), 135.97 (H-14), 147.63 (H-24) and 148.17 (H-8). All the protons were assigned to their respective carbons by HMQC. The NMR assignments are listed in Table 1. Further comparison of these assignments with those of previously known compounds isolated from *L. domesticum* showed that **1** is a triterpenoid glycoside. In fact, all the ^1H-^1H COSY and HMBC correlations of **1** were consistent with those of lansioside C, a triterpene glycoside previously isolated from the air-dried fruit peel of *L. domesticum*[7]. NOESY experiment also showed that its relative stereochemistry was similar to that of lansioside C. The only difference of **1** with lansioside C is the presence of additional carboxyl (C-22) and methoxyl (C-23) groups. HMBC correlations observed for H-20 and C-22, and H-20 and C-21 indicated that C-22 is connected to C-21. The connection of C-23 to C-22, on the other hand, was established by HMBC correlation observed for H-23 and C-22. Thus the complete structure of **1** was deduced as depicted in Fig. 3, and is being named lansioside D.

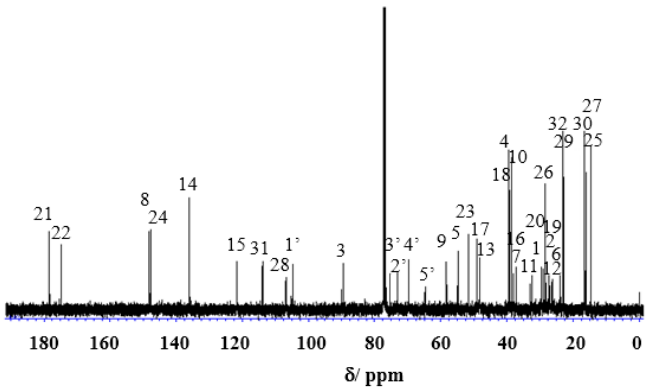

Fig 2: The 400 MHz ^{13}C NMR spectrum of the active compound.

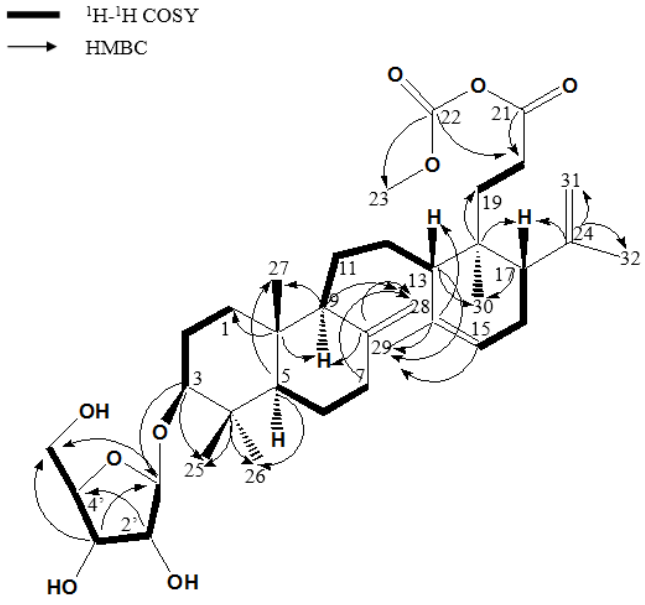

Fig 3: The chemical structure of **1** as deduced from H^1-H^1 COSY, HMQC and HMBC data.

Lansioside D was subjected to antimicrobial assays against a wide spectrum of microorganisms. As shown in Table 2, lansioside D exhibited pronounced activity against Gram-positive bacteria, with an MIC of 31.25 and 15.62 µg/ml for *S. aureus* and *B. subtilis*, respectively. It was moderately active against the Gram-negative *E. coli* (MIC= 250 µg/ml) but was not active against yeasts and molds.

Table 1: ^{13}C and ^1H NMR data of **1** in $CDCl_3$.

Carbon No.	δ_C, ppm	δ_H, ppm	Carbon No.	δ_C, ppm	δ_H, ppm
1	26.08	1.84, 2.21	20	28.93	2.29, 2.40
2	26.47	1.69, 1.89	21	178.35	
3	89.46	3.21	22	174.72	
4	39.06		23	51.60	3.65
5	54.81	1.12	24	147.63	
6	23.81	1.37, 1.74	25	16.11	0.80
7	37.99	1.19, 1.40	26	28.34	1.02
8	148.17		27	14.62	0.67
9	58.38	1.55	28	106.82	4.56, 4.84
10	38.60		29	22.93	1.74
11	29.58	1.66, 1.69	30	16.46	0.82
12	37.14	1.37, 1.79	31	113.90	4.77, 4.82
13	48.30	1.81	32	22.83	1.78
14	135.97		1’	104.82	4.40
15	121.66	5.37	2’	73.10	3.46
16	32.41	1.99, 2.40	3’	75.35	3.55
17	49.12	2.19	4’	69.69	3.66
18	39.34		5’	64.72	3.33, 3.99
19	27.26	1.19, 1.78			

Table 2: Antimicrobial activity of lansioside D.

Test organism	MIC (µg/ml)
Gram-positive bacteria	
***Staphylococcus aureus* IFO 13276**	31.25
***Bacillus subtilis* IFO 3134**	15.62
Gram-negative bacteria	
***Escherichia coli* IFO 3301**	250.00
Yeasts	
***Saccharomyces cerevisiae* IFO 2375**	>1000.00
***Candida lipolytica* IFO 1658**	>1000.00
Molds	
Aspergillus niger	>1000.00
Cladosporium herbarum	>1000.00

Reference antibiotics were tetracycline for bacteria, and cycloheximide for yeasts and molds.

4. Conclusion

Bioassay-guided fractionation of the methanol extract from the fruit peel of *L. domesticum* led to the isolation of a new triterpenoid as the major antimicrobial compound. This compound belongs to the lansiosides and is very similar to lansioside C except for the additional –$COOCH_3$ group, thus was given the name lansioside D. The lansiosides of *L.*

domesticum were previously reported to exhibit other biological activities. Lansioside A was reported to be effective in inhibiting the leukotriene D4 induced contraction of guinea pig ileum [7]. Lansiosides A-C were also reported to be effective in controlling male hormone-type baldness, acne and prostate hypertrophy [14]. It is very likely that lansioside D also possesses similar biological activities.

The remarkable antimicrobial activity of lansioside D against some microorganisms is a positive development. This is especially true considering the need for new antimicrobial compounds due to the rise in the incidence of antibiotic resistance. Development of lansioside D as an antimicrobial drug will need more studies, including safety evaluation to see whether it can really be used by humans without any harmful side effects. This should be very interesting and will be the subject of future research.

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