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NOTE

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Albert Mulianga Makenzi, Lawrence Onyango Arot Manguro and Philip Okinda Owuor

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ABSTRACT

A new oleanane type triterpene characterized as 3α, 24-diacetoxy-12-oleanene **(1)** together with known compounds **2-10**, were isolated from *Annona mucosa* leaf extracts. Their structural elucidation was accomplished using physical and spectroscopic methods. The compounds showed weak to moderate insecticidal activities against stored maize insect pests *Sitophilus zeamais* and *Prostephanus truncatus*.

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Annona mucosa; Annonaceae; sterol; triterpenoids; alkaloid; flavonols; contact toxicity; anti-feedant activity

1. Introduction

Annonaceae is the largest plant family of angiosperms in the order Magnoliales with 135 genera and approximately 2500 species [1]. Despite the great diversity of this genus, it is one of the lesser phytochemically studied tropical plant families [2]. Isoquinoline alkaloids are the characteristic compounds of the Annonaceae family commonly associated with plant defense against herbivorous insects [3]. Extracts from Annonaceae have been tested for control of *Spodoptera frugiperda*, *Plutella xylostella*, *Aedes aegypti* and stored grain insects [4]. Plants from this family including *Annona mucosa* have shown promising biopesticide activities among tropical plants [5]. *Annona mucosa* [synonym: *Rollinia mucosa* (Jacq.) Baill], a native fruit tree of the Amazon and the Atlantic forests where it is popularly known as “Biribá”, grows well in different habitats [6]. The plant is a fast growing tree ranging from 4.0 to

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15.0 m in height with brown hairy twigs. The flowers are 1 to 3 formed together in the leaf axils and the fruit is conical or heart-shaped 15 cm in diameter [7]. In the previous phytochemical analyses of the plant parts, alkaloids, lignans [8] flavonoids and flavonoid glycosides [9] have been isolated and identified. Crude organic solvent extracts of the plant have exhibited antimicrobial [10] and insecticidal activities [11]. This has led to the need to evaluate the potential of *A. mucosa* as a source of phytochemicals with insecticidal properties for the control of stored maize insect pests *S. zeamais* and *P. truncatus*.

In this communication, phytochemical analysis of the leaf extracts of this plant afforded a new compound characterized as 3α,24 diacetoxy-12-oleanene **(1)** together with ten known ones identified as quercetin **(2)** [12], oleanolic acid **(3)** [13], 3β-acetoxytirucallic acid **(4)** [14], 3β-acetoxyursolic acid **(5)** [15], (3 R, 20S)-3α-acetoxy-20-hydroxydammar-24-ene **(6)** [16], 3-oxo-11α-hydroxy-12-ursene **(7)** [17], lupeol **(8),** β-sitosterol **(9)** [18] and 3β-acetoxy-12-oleanene **(10)** [19]. Their structures were established using physical and spectroscopic techniques as well as comparison with literature data. Compounds **1, 3 4, 5, 6, 7** and **8** (Figure 1) are being reported from this plant for the first time. The extracts and isolated compounds showed moderate contact toxicities and anti-feedant activities against *S. zeamais* and *P. truncatus.*

2. Results and discussion

Compound **1** was isolated from the EtOAc leaf extract of *A. mucosa* by repeated medium pressure chromatography on silica gel using solvent system *n*-hexane-EtOAc (4:1) as a white solid compound, R_f =0.46 (solvent system: *n*-hexane-EtOAc, 4:1). It showed a purple color with acidified anisaldehyde after heating on hot plate at 100 °C suggesting the presence of a sterol or a terpenoid derivative. The compound responded positively to Lieberman-Buchard and ceric sulfate tests which further supported the presence of a terpenoid derivative. Its IR spectrum (Figure S 1) determined

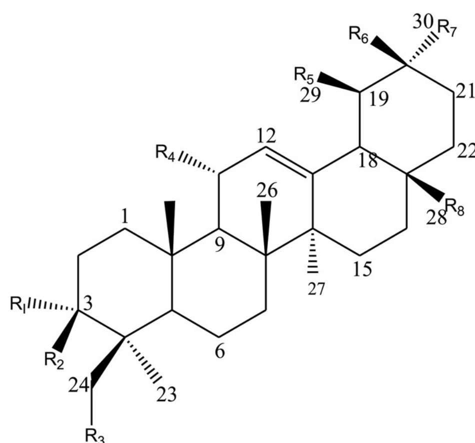

Figure 1. Structures of compounds **1, 3, 5** and **7**.
1 $R_1 = R_3 = OAc$, $R_2 = R_4 = R_5 = H$, $R_6 = R_7 = R_8 = CH_3$
3 $R_1 = R_3 = R_4 = R_5 = H$, $R_2 = OAc$, $R_6 = R_7 = CH_3$, $R_8 = CO_2H$
5 $R_1 = R_3 = R_4 = R_6 = H$, $R_2 = OAc$, $R_5 = R_7 = CH_3$, $R_8 = CO_2H$
7 $R_1 = R_2 = O$, $R_4 = OH$, $R_5 = R_6 = R_8 = CH_3$, $R_7 = R_3 = H$

Table 1. ^{1}H and ^{13}C NMR spectral data of 3α, 24-diacetoxy-12-oleanene (**1**).

C	^{1}H NMR	^{13}C NMR	Multiplicity
1	1.87-1.68 m, 1.11-1.12 m	37.1	CH_2
2	1.68-1.43 m, 1.19-1.12 m	26.9	CH_2
3	4.95 br s	73.4	CH
4		39.8	C
5	1.00-0.96 mm	50.6	CH
6	1.43 – 1.38 m	18.2	CH_2
7	1.43 – 1.38 m, 1.24-1.20 m	32.7	CH_2
8		40.3	C
9	2.00-1.89 m	47.5	CH
10		39.8	C
11	1.90-1.88 m	23.5	CH_2
12	5.20 (t, J = 3.7 Hz)	121.6	CH
13		145.0	C
14		41.7	C
15	1.40-1.38 m, 1.24-1.19 m	26.0	CH_2
16	1.68-1.43m	22.4	CH_2
17		32.4	C
18	2.10-2.00m	47.5	CH
19	1.68-1.60 m	46.8	CH_2
20		31.0	C
21	1.68-1.60 m	34.7	CH_2
22	2.10-1.90 m, 1.12-1.00 m	33.5	CH_2
23	1.00 s	15.7	CH_3
24	4.23 (d, J = 11.0), 3.99 (d, J = 11.5 Hz)	66.7	CH_2
25	0.87 s	15.7	CH_3
26	0.80 s	16.7	CH_3
27	1.24 s	26.0	CH_3
28	0.84 s	28.3	CH_3
29	0.96 s	33.3	CH_3
30	1.00 s	23.6	CH_3
3-CH_3C(O)O-	2.08 s	21.0	CH_3
3-CH_3C(O)O-		171.2	C
24-CH3C(O)O-	2.04 s	21.2	CH_3
24-CH_3C(O)O-		170.5	C

as KBr pellet showed significant absorption bands at 1737.3 and 1248.3 cm^{-1} representing an acetoxy group. The compound was assigned the molecular formula of $C_{34}H_{54}O_4$ as evidenced by HREI-MS ion peak at m/z 526.7902, which suggested eight double bond equivalents, six of which were assigned to the ring of the pentacyclic triterpene skeleton and the remaining two were attributable to a primary and a secondary acetoxy groups in the molecule. The EI-MS daughter ion at m/z 307.0 suggested that both the acetoxy groups were on rings A/B portion of the molecule. The ^{I}H NMR spectrum (Table 1; Figure S 2) of compound **1** showed a vinylic proton peak at δ 5.20 (t, J = 3.7 Hz) assignable to H-12 whereas the relatively downfield oxymethine proton appearing at δ 4.95 (br s, H-3) was attributable to H-3. Also, in the ^{1}H NMR spectrum two geminal methylene protons resonated at δ 4.23 [(d, J_{gem}=11.0 Hz) and 3.99 (d, J_{gem}=11.5 Hz)]. These together with nine methyl groups located on quaternary carbons including two acetoxy moieties centered at δ 0.80, 0.84, 0.87, 0.96, 1.00 × 2, 1.24, 2.04 and 2.08 unambiguously confirmed the presence of Δ^{12}-oleanane skeleton [19]. The ^{13}C NMR spectrum (Table 1; Figure S 3) showed the presence of 34 carbon signals; their multiplicity assignments using DEPT-135 experiments (Figure S 4) established the presence of five methines, eleven methylenes, nine methyls and nine quaternary carbon atoms. The double bond consistent with Δ^{12}-oleanane

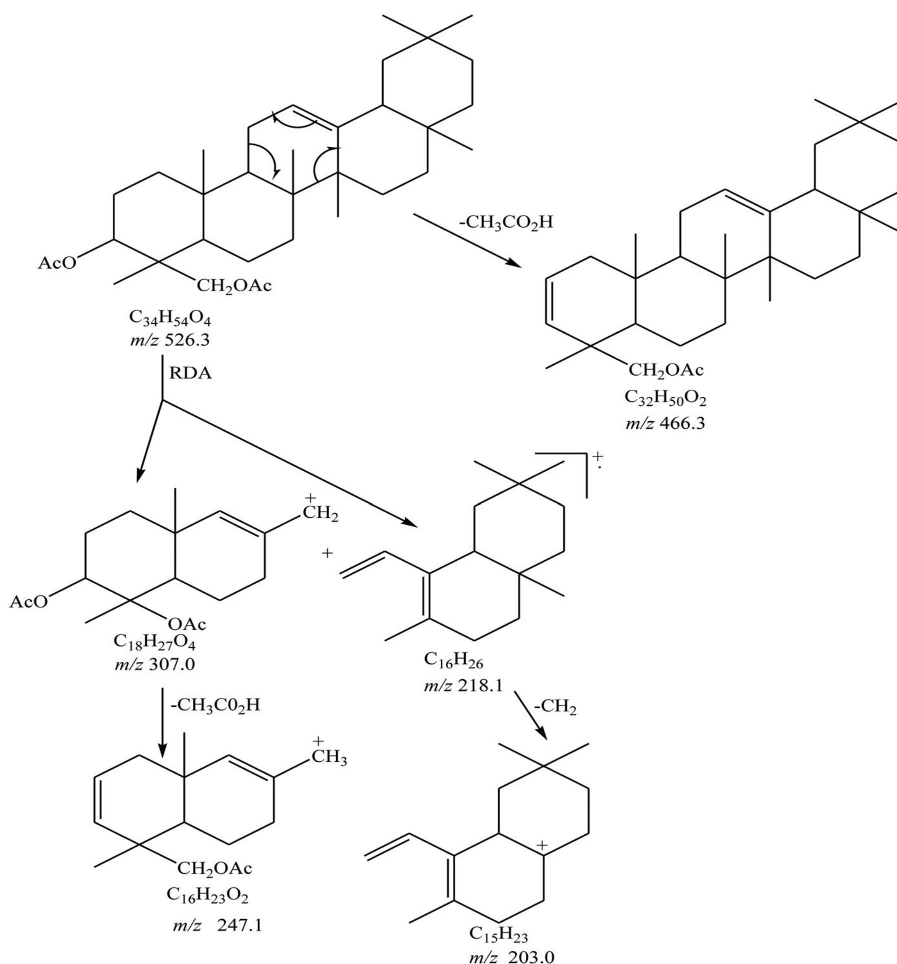

Figure 2. Proposed fragmentation pattern of compound **1** in EI-MS (70 ev).

skeleton exhibited resonance peaks at δ 121.6 (C-12) and 145.0 (C-13), and the down field value of C-13 further supported that the compound is an oleanane derivative rather than an ursane [19]. The foregoing evidences were further supported by significant fragment peaks at *m/z* 307.0 $[C_{18}H_{27}O_4]^+$ and *m/z* 218.1 $[C_{16}H_{26}]^+$ (100%), (Figure 2; Figure S 5) which suggested typical *retro*-Diels-Alder diagnostic fission of 12-oleonene or 12-ursene type triterpene derivatives [18]. Comparison of the ^{1}H and ^{13}C NMR and EI-MS spectral data with those of 3β-acetoxy-12-oleanene [19] revealed close similarity with a notable difference between the two compounds being substitution in ring A in compound **1** as evidenced by replacement of the Me-24 with CH_2OAc group. Also comparing the ^{1}H NMR data of compound **1** with those of synthetically derived urs-12-ene-3α, 24-diol [20] revealed a shift of the hydroxymethylene peaks relatively downfield by approximately 2.40 ppm, further suggesting acetylation at C-24 in compound **1**. The three bond long-range correlations from H-3 to carbons ascribable to C-5 (δ 50.6) and C-24 (δ 66.7) in the HMBC spectrum (Figure 3, Figure S 6) confirmed the presence of acetylated hydroxyl group at C-24, possibly with

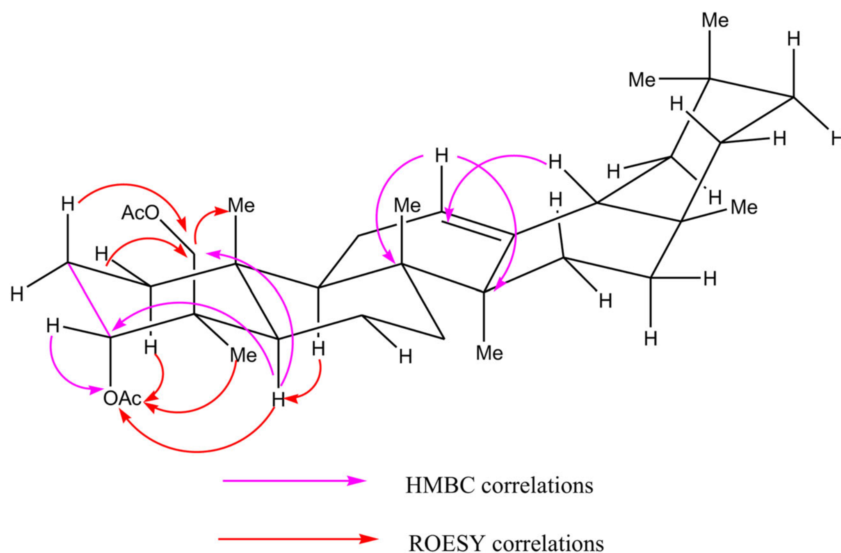

Figure 3. Significant HMBC and ROESY correlations for compound **1**.

β-configuration, a fact substantiated by ^{1}H-^{1}H proximity (ROESY, Figure 3; Figure S 7) correlations between CH_2-24 (δ 4.23) and CH_3-25 (δ 0.87) and in turn with H_{β}-1 (δ 1.68-1.87 m). Similarly, the acetoxy group was suggested to be at C-3 where it was axially oriented as substantiated by the broad singlet peak of equatorially positioned germinal proton which appeared relatively downfield at δ 4.95 [16]. This was further supported by HMBC correlation between H-5 (δ 1.00-0.96 m) and C-3 (δ 73.4). On the other hand, a triplet at δ 5.20 (H-12) correlated with C-8 (δ_c 40.3) and in turn with C-13 (δ_c 145.0) and C-14 (δ_c 41.7) in the HMBC spectrum. On this basis, the position of the double bond was concluded to be between C-12 and C-13, a fact that was corroborated by EI-MS daughter ion at *m/z* 218.0 [$C_{16}H_{26}$] originating from retro-Diels-Alder cleavage. Thus, on the basis of spectroscopic data as well as comparison with various literature data, compound **1** was deduced to be 3α, 24-diacetoxy-12-oleanene.

The management of economic loss caused by *S. zeamais* and *P. truncatus* using botanical pesticides has been documented. In this study the contact toxicity of *n*-hexane, EtOAc and MeOH extracts of *A. mucosa* leaves after 48 h exposure showed good toxicity against both stored grain insect pests. The extracts killed the pests by contact action. The LC_{50} values of the extracts were 96.83 µg/ml, 63.45 µg/ml and 46.43 µg/ml, respectively against *S. zeamais* whereas the LC_{50} values of 99.07 µg/ml, 72.01 µg/ml and 58.80 µg/ml were recorded for *P. truncatus* at the same exposure time (Figure 4). This activity was dependent on concentration and time of exposure of the insects to the extracts. Methanol extract showed promising contact toxicity; however they were lower than those of deltamethrin, a commercial insecticide used as a positive control, which gave LC_{50} of 10.43 and 10.74 μg/ml against *S. Zeamais* and *P. truncatus*, respectively. In order to find bio-molecules responsible for the extracts activity, a total of ten compounds (1-10) were screened (Figure 5) and in the process, it was observed that quercetin (**2**) was the most active with LC_{50} values 29.26 μg/ml and

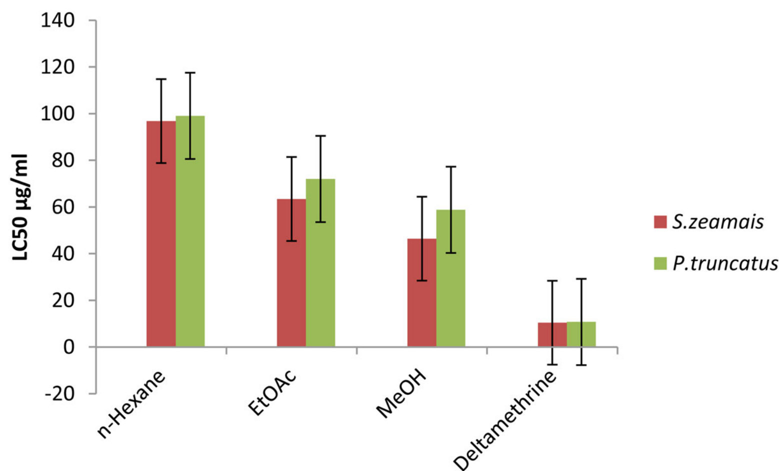

Figure 4. Contact toxicity activities (LC_{50} µg/ml) of `extracts from *A. mucosa* leaves against *S. zeamais* and *P. truncatus*.

24.51 μg/ml, against *S. Zeamais* and *P. truncatus*, respectively. However, the activities were lower than those of deltamethrin.

In addition to contact activities, the antifeedant effect of the three crude extracts of *A. mucosa* leaves against the two stored product insects was also investigated. The *n*-hexane, EtOA and MeOH showed moderate activities against *S. zeamais* with AFI_{50} values of 98.62 µg/ml, 60.32 μg/ml and 46.62 μg/ml, respectively (Figure 6). According to the results, the MeOH extract exhibited stronger anti-feedant activity against the pest than the other two extracts. When compared with positive control azadirachtin which showed an AFI_{50} value of 12.15 μg/ml, the extracts demonstrated weaker anti-feedant activities against the pests. On the other hand, application of the extracts against *P. truncatus* afforded AFI_{50} values of 97.75 μg/ml, 66.05 μg/ml and 49.22 μg/ml, respectively (Figure 6). Similarly, according to the results, the MeOH extract displayed better results than the other extracts. However, they were less active than the reference standard which posted an AFI_{50} value of 12.42 μg/ml.

The results for the anti-feedant assays of isolated constituents (**1-10**) against the two insect species are represented in Figure 7. Quercetin **(2)** showed stronger anti-feedant activity against *P. truncatus* than *S. zeamais* with AFI_{50} values of 20.15 µg/ml and 22.20 µg/ml respectively. On the other hand, oleanolic acid **(3)**, 3β-hydroxytirucallic acid **(4)**, (3 R, 20S)-3-acetoxy-20-hydroydammar-24-ene **(6)** and lupeol **(8)** at the concentration 500 µg/ml, the antifeedant activities were affected by the testing concentrations and the exposure time. Compounds 3β, 24-diacetoxy-12-oleanane **(1)** exhibited antifeedant activities with AFI_{50} values of 42.07 µg/ml and 53.43 µg/ml against *P. truncatus* than *S. zeamais* respectively. On the other hand using 3-oxo-11α-hydroxy-12-ursene **(7)** as the test substance, AFI_{50} values of 57.76 µg/ml and 53.31 µg/ml were observed for *S. zeamais* and *P. truncatus* respectively. This shows that the extracts and isolated constituents from *A. mucosa* possess significant contact toxicity and antifeedant activities against the two stored food product insect pests. Both the extracts and isolated compounds from *A. mucosa* leaves have the potential to control stored grain insect pests. However, for the practical application of the

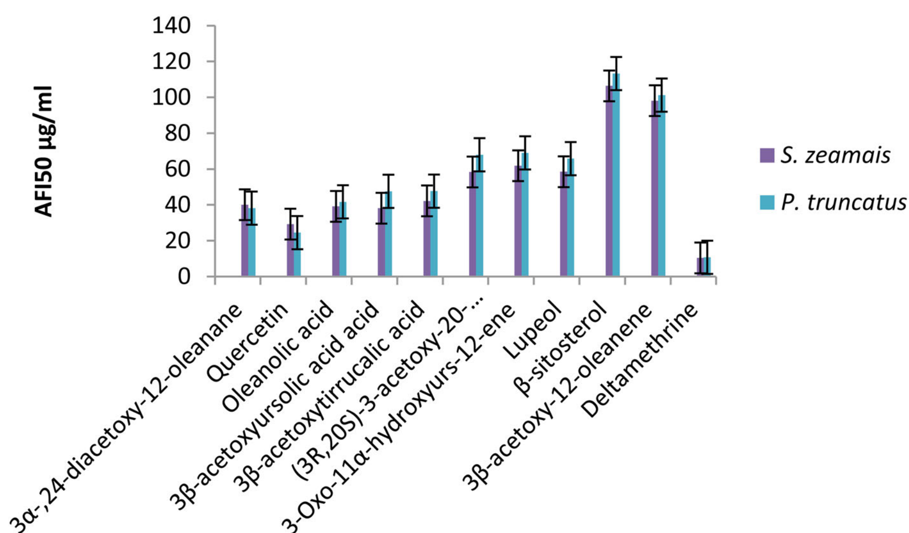

Figure 5. Contact toxicity activities (LC_{50} µg/ml) of isolated compounds from *A. mucosa* leaves against *S. zeamais* and *P. truncatus*.

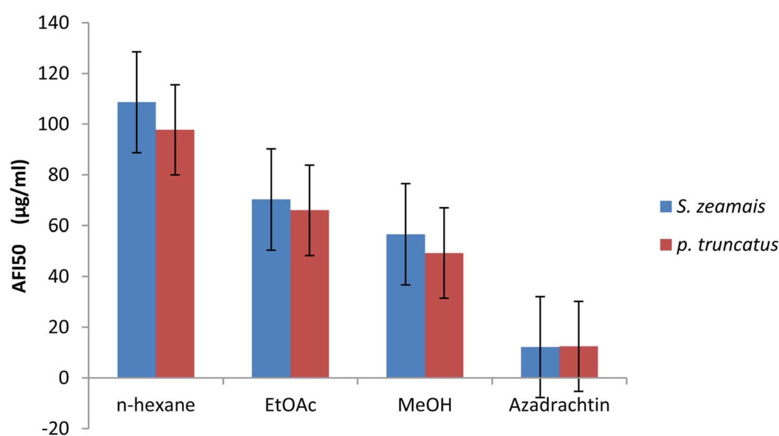

Figure 6. Antifeedant activities (AFI_{50} µg/ml) of extracts from *A. mucosa* leaves against *S. zeamais* and *P. truncatus*.

extracts and the isolated phyto-constituents in stored insect pest control, further studies on the safety and efficacy are necessary.

3. Experimental

3.1. General experimental procedures

Melting points were determined using Gallenkamp melting point apparatus (Manchester, UK). Optical rotation was measured on a Jasco P-1020 Polarimeter (Jasco Corporation, Tokyo, Japan). UV spectra were analysed using a Shimadzu UV-2401A spectrophotometer (Shimadzu corporation, Kyoto, Japan). IR data were

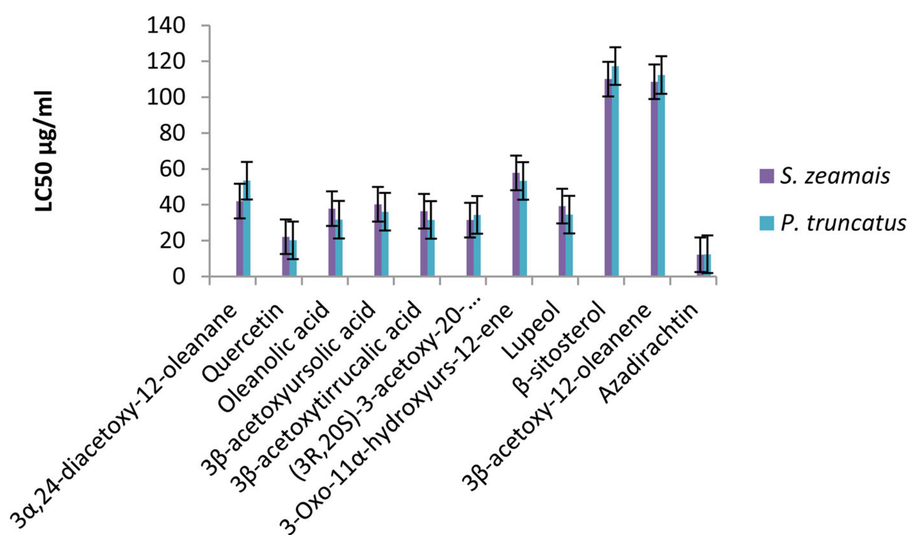

Figure 7. Antifeedant activities (LC_{50} µg/ml) of isolated compounds from *A. mucosa* leaves against *S. zeamais* and *P. truncatus.*

recorded on a Bruker Tensor 27 FTIR spectrophotometer (Bruker Corporation, Bremen, Germany) as KBr pellets. NMR data were measured in $CDCl_3$ and DMSO-d_6 on a JOEL NMR instrument operating at 360 and 90 MHz, respectively. Tetramethylsilane (TMS) was used as zero reference. The mass spectral data were obtained using a Varian MAT 8200 A instrument. Electron-impact (EI) mass spectra (70 eV) were measured on a Hewlett-Packard 5989B mass Spectrometer. A gas chromatography-mass spectrometry (GC-MS) Thermo Finnigan system fitted with a capillary column SPB-5MS (30 m × 0.32 mm i.d., film thickness 0.25 mm) and splitless injection was used; the oven temperature was programmed from 40 to 250 °C at 10 °C/min; injector temperature and ion source temperature were at 250 and 200 °C, respectively; MS full scan was from 50-650 ms; Helium was used as carrier gas. Silica gel 60 (63 – 200 μm, Merck, Darmstadt, Germany) was used for gravity column chromatography (CC). TLC was performed on pre-coated DC Alufolien 60 F254 sheets (Merck, Darmstadt, Germany) and detected by spraying with anisaldehyde spraying agent, UV light and iodine vapor. All solvents used were of analytical grade.

3.2. Plant material

The leaves of *Annona mucosa* (Jacq.) were collected from Kitale, Tranzoia County (Lat: 131’0"S and Long: 37° 16′ 0" E), Kenya, where it grows naturally by Mr. Albert Makenzi. The plant was identified by Mr. Mutiso of Botany Department, University of Nairobi, Kenya where voucher specimens are deposited (Reference No.MU/EA/2015).

3.3. Extraction and isolation

The air dried and pulverized leaves (2.5 kg) were sequentially soaked in *n*-hexane (3 × 4.5 L), CH_2Cl_2 (3 × 4.5 L) and MeOH (3 × 4.5 L), each lasting two days at room

temperature. The extracts were separately filtered and evaporated under vacuum using a rotary evaporator to afford green (23 g), dark green (45 g) and brownish-green (126 g) extracts, respectively.

Silica gel TLC analysis of *n*-hexane extract showed three major spots of R_f values 0.71, 0.56 and 0.45 (*n*-hexane-EtOAc, 4:1), which turned purple with anisaldehyde spraying reagent after heating at 100 °C. A portion of *n*-hexane extract (approx. 20 g) was mixed with 5 g of silica gel in 100 ml of dichloromethane, solvent removed by evaporation under vacuum and the solid mixture chromatographed over silica gel packed column (4.0 × 70 cm, SiO_2, 300 g; pressure ≈1 bar) using *n*-hexane with increasing amount of EtOAc up to 100% of the latter. A total of 170 fractions, each 20 ml were collected and their homogeneity monitored by TLC (solvent systems: *n*-hexane EtOAc, 9:1 and 4:1). The eluants were grouped into four major pools (1-IV) depending on TLC profiles. Fractions 1-35 constituted pool I which upon evaporation of solvent afforded a yellow oily substance that lost color with time and was discarded. Pool II (fractions 37-80, 1.7 g) showed a single spot of R_f 0.71 (solvent system: *n*-hexane-EtOAc, 4:1) which on crystallization (CH_2Cl_2-MeOH, 9:1) gave 3*β*-acetoxy-12-oleanene **(10)** (150 mg) as white crystals. Pool III (fractions 82-120, 1.5 g) also gave a single spot of R_f value 0.56 (eluent: *n*-hexane-EtOAc, 4:1), which on evaporation of solvent followed by crystallization (CH_2Cl_2-MeOH, 9:1) afforded *β*-sitosterol **(9)** (1000 mg). Fractions 121-160 (3.0 g) constituted pool IV, which showed two spots of R_f values 0.56 and 0.45 on TLC using *n*-hexane-EtOAc (4:1) solvent system. The spots were resolved into individual components by repeated column chromatography using *n*-hexane-EtOAc (4:1) to give a further 70 mg of *β*-sitosterol **(9)** and lupeol **(8)** (120 mg).

On the other hand, TLC analysis of EtOAc extract using *n*-hexane-EtOAc (4:1) afforded five spots of R_f values 0.71, 0.56, 0.45, 0.32 and 0.20, respectively which also turned purple using anisaldehyde-sulphuric acid spraying reagent. Change of solvent system to *n*-hexane-EtOAc (3:2) gave three additional spots of R_f values 0.57, 0.47, and 0.41, respectively. Upon spraying the spots with anisaldehyde-sulphuric acid mixture followed by heating they turned purple. Approximately 40 g of the EtOAc extract was adsorbed onto 10 g silica gel and then subjected to column chromatography (4.0 × 70 cm, SiO_2 300 g, and pressure ≈1 bar) using *n*-hexane-EtOAc mixture with increment of 10% of the more polar solvent up to 100% EtOAc and finally with CH_2Cl_2-MeOH (99:1). A total of 323 fractions, each 20 ml were collected. This process afforded pools (V-XI) as determined by TLC profiles [solvent systems: *n*-hexane-EtOAc (4:1, 3:2 and 1:2) and CH_2Cl_2-MeOH (99:1)]. Pool V (fractions 1-30) gave a yellow oily substance which lost color with time and was discarded. Pool VI (fractions 33-60, 0.90 g) showed a single spot R_f 0.71 (eluent: *n*-hexane-EtOAc, 4:1) which upon evaporation of the solvent followed by crystallization in CH_2Cl_2-MeOH (9:1) afforded a further 3*β*-acetoxy-12-oleanene **(10)** in 100 mg. Pool VII (fractions 62-85, 0.5 g) also gave a single spot of R_f value 0.56 (eluent: *n*-hexane-EtOAc, 4:1), which on evaporation of solvent followed by crystallization (CH_2Cl_2-MeOH, 9:1) afforded more *β*-sitosterol **(9)** (105 mg). Fractions 86-130 (2.5 g) constituted pool VIII which exhibited two spots of R_f values 0.56 and 0.45 on TLC using *n*-hexane-EtOAc (4:1) solvent system. Repeated chromatography of this sub-fraction using n-hexane-CH_2Cl_2 (4:1)

led to the isolation of more *β*-sitosterol **(9)** (20 mg) and lupeol **(8)** (46 mg). Pool IX (fractions 133-200, 3.4 g) showed two spots R_f values 0.32 and 0.20 [solvent system: *n*-hexane-EtOAc (4:1)], both turned purple on TLC after spraying with p-anisaldehyde-sulphuric acid mixture spraying reagent followed by heating. The compounds were separated using medium pressure chromatography with *n*-hexane-EtOAc (4:1) to give 3*β*, 24-diacetoxy-12-oleanene **(1)** (R_f=0.32, 65 mg) and 3-oxo-11α-hydroxy-12-ursene **(7)** (R_f=0.20, 71 mg). Pools X and XI (fractions 203-310, 7.0 g) on silica gel TLC analysis using *n*-hexane-EtOAc (3:2) gave three spots of R_f values 0.57, 0.47 and 0.41, respectively and upon repeated chromatographic separation (3.5 × 60 cm, SiO_2, 200 g, pressure ≈ 1 bar) using solvent system *n*-hexane-EtOAc (3:1) followed by the same solvent system in the ratio 3:2 afforded (3 R, 20S)-3-acetoxy-20-hydroxydammar-24-ene **(6)** (R_f=0.57, 45 mg), 3*β*-acetoxyursolic acid **(5)** (R_f=0.47, 85 mg) and 3*β*-acetoxytirucallic acid **(4)** (R_f=0.41, 55.4 mg).

The methanol extract (40 g) was mixed with 20 g of silica gel, dried and then subjected to column chromatography (6.0 × 80 cm; SiO_2 300 g; pressure ≈1 bar), starting with CH_2Cl_2 followed by CH_2Cl_2-MeOH mixture with increasing concentration of the more polar solvent (increment 10%) up to 100% MeOH. A total of 150 fractions, each 50 ml were sampled and their homogeneity determined by TLC (eluent: CH_2Cl_2-MeOH, 99:1, 98:2, 97:3, 95:5, 4:1 and 1:1; *n*-BuOH-HOAc-H_2O, 4:1:5) and those exhibiting similar profiles were combined into two major pools (XII and XIII). Pool XII (fractions 20-35, 4 g) when eluted with CH_2Cl_2-MeOH (99:1) followed by the same solvent (98:2) afforded oleanolic acid **(3)** (R_f = 0.54, 89 mg). Fractions 36-120 (8 g) eluted using CH_2Cl_2-MeOH (95:5) followed by the same solvents in the ratio 9:1 and 4:1 constituted pool XIII and was further repeatedly chromatographically purified using CH_2Cl_2-MeOH (4:1) to give quercetin **(2)** (R_f = 0.34)

3.3.1. 3α, 23-Diacetoxy-12-oleanene (1)

White amorphous powder, m.p. 176-178 °C; $[\alpha]_D^{25}$ +14 ($CHCl_3$, c 0.05); IR ν_{max} (KBr) cm^{-1}: 2848.9, 2814.1, 1737.3, 1457.0, 1376.3, 1248.3, 1191.2, 1028.0, 986.2, 914.5, 823.4, 657.1, 606.0; ^1H and ^{13}C NMR ($CDCl_3$) spectral data: see Table 1; EIMS (70 ev): *mz* (rel. Int.) 526.3 $[M]^+$ (13), 511.2 (1), 468.3 (1), 466.3 (3), 391.2 (1), 307.0 (3), 272.1 (1), 247.1 (4), 218.1 (100), 203.0 (37), 161.0 (100, 135.0 (11), 109.0 (16), 95.0 (17), 68.9 (13), 42.9 (22); HR-MS: *m/z* 526.7902 (calcd for $C_{34}H_{54}O_4$, 526.7901)

3.4. Contact toxicity of plant leaf extracts

This experiment tested the hypothesis that topically applied plant extracts and pure isolate solutions exhibit contact toxicity to *S. zeamais* and *P. truncatus*. The contact toxicities of *A. mucosa* leaf extracts and pure isolates were done according to the known method [21] by topical application using 3rd instar larvae. The extract stock suspensions and pure isolate solutions were prepared immediately prior to the assays by dissolving in acetone to obtain solutions of concentrations 100.0 μg/ml, 300.0 μg/ml, 500.0 μg/ml and 1000.0 μg/ml. The experiments were done in three replicates and for each replicate, 10 larvae were transferred to moist Whatman No. 1 filter paper in a 90 mm disposable Petri dish and chilled for three minutes to reduce their activity

and enable topical treatment to be carried out. Each larvae was separately treated topically with a 0.5 μl droplet of each of the solutions, applied onto the pronotum of an adult weevil, using a Hamilton's syringe (700 series, Microliter TM Hamilton Company, USA). In the negative control treatment, larvae were treated with 1 μl of acetone while in the positive control they were treated with 1 μl of deltamethrine, a commercial insecticide. After topical application, the insects were confined in Petri dishes within metal rings and provided with 5 corn kernels and maintained at $26 \pm 2\,°C$ and $60 \pm 5\%$ relative humidity on a 16:8 (L:D) photocycle for 48 h, after which mortality was assessed. The percentage mortalities were subjected to analysis of variance (ANOVA). The Lethal concentration 50 (LC_{50}) values, the confidence upper and lower limits, regression equations and chi-square ($\chi2$) values were calculated using probit analysis [22].

3.5. Feeding deterrence assay

The activities of the extracts as well as pure isolates were studied using maize flour leaf disk no choice bioassay method [23]. This experiment tested the hypothesis that larval feeding is deterred by plant extracts and pure isolates incorporated in fresh maize flour leaf disks (1350 sq.mm). Crude extracts were tested at 2000.0 µg/ml and for pure isolate concentrations of 100.0, 200.0, 500.0 and 1000.0 µg/ml were used. For no choice tests, a single pre-starved larvae was presented with a flour leaf disk treated with a plant extract diet in 50 mm round Petri dishes. Each extract and concentration treatment combination was tested individually versus the controls. The larvae were inserted into Petri dishes individually on a piece of Whatman N0 1 filter paper (1×1 cm) and placed centrally on portions of treated and control flour leaf disks respectively. For the negative control treatment, 100 μ1 of HPLC grade acetone was added to the diet while for positive control 100 μ1 of azadirachtin was added to the diet. The insects were allowed to feed on treated and control leaf flour disks for twenty four hours. At the end of the experiment, unconsumed area of the leaf flour disk was measured with the aid of a leaf area meter and per cent anti-feedant activity calculated [24] The same procedure was repeated for the pure isolates at the given concentrations. The data were then subjected to analysis of variance. Each experiment was repeated three times. Insect mortality was also recorded.

$$\%\ \text{antifeedant activity} = \frac{[\textit{leaf disc consumed by the insects in control} - \textit{leaf disc consumed by the insect treated}]}{[\textit{Leaf disc consumed by the insect in control} + \textit{leaf disc consumed by the insect in treated}]} \times 100$$

The concentration that deters 50% feeding (AFI_{50}) values, the confidence upper and lower limits, regression equations and chi-square ($\chi2$) values were calculated using probit analysis [22]

3.6. Data analyses

For the initial screening bioassay, data were corrected for mortality in the controls using Abbott's formula:

$$\% \text{ Mortality (adjusted)} = \left[\frac{(\% \text{ AC} - \%\text{AT})}{\%\text{AC}} = \frac{(\% \text{ DT} - \% \text{ DC})\text{X}100}{(100 - \%DC)} \right]$$

Where: AC = alive insects in control; DT = dead insects in treatment; AT = alive insects in treatment; DC = dead insects in control. The results were then normalized using an arcsine transformation. Transformed data were submitted to a randomized complete block analysis of variance (ANOVA) ($P < 0.05$) and differences between treatments were compared using Tukey's test ($P < 0.05$). For the feeding choice assay, the numbers of larvae feeding on extract-treated versus control portions of the diet were compared.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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