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Melanogenesis inhibitors from the endophytic fungus *Aspergillus amstelodami*

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Two new compounds, named 3, 4-dimethoxyphenyl-1-O- α -D-ribofuranoside (1) and olean-12-en-23 α , 28 β , 30 β -trioic acid-3-O- β -D-glucopyranoside (2), together with thirteen known compounds, were isolated from the white beans culture of the marine derived endophytic fungus *Aspergillus amstelodami*. Structure elucidation of the new compounds was carried out by one-, two-dimensional spectroscopy, and high resolution electrospray ionization mass. The anti-melanogenic and anti-allergic activity of the isolated compounds were investigated. Compounds (4), (7), (1), (3), (11), (6) and (9) selectively suppressed melanin production in B16 melanoma cells, using arbutin as a positive control. Their IC₅₀ values were 30.8 $\pm$ 5.57, 38.5 $\pm$ 6.08, 52.6 $\pm$ 6.64, 98.0 $\pm$ 1.16, 100.4 $\pm$ 3.05, 112.0 $\pm$ 0.22 and 144.7 $\pm$ 2.35 μ M, respectively, while that of arbutin was 151.7 $\pm$ 1.27 μ M. The tested compounds did not show significant anti-allergic activity in RBL-2H3 cells, as compared to quercetin.

Keywords: 3, 4-Dimethoxyphenyl-1-O- α -D-ribofuranoside • Olean-12-en-23 α , 28 β , 30 β -trioic acid-3-O- β -D-glucopyranoside • B16 melanoma cells • β -Hexosaminidase • *Aspergillus*

Introduction

Natural compounds continue to be the major basis for drug development. Currently, about 50% of drugs in use are derived from natural products.^[1-2] In particular, exploring endophytes for natural compounds increase the probability of chemical and biological novelty.^[3] The aim of this work is to identify the secondary metabolites of the endophytic fungus *Aspergillus amstelodami* AUMC <EGY> and evaluate their biological activity. According to literature, several diketopiperazines were reported from *A. amstelodami*.^[4-8] In this study, in addition to diketopiperazines, we report a new triterpene and a new phenolic ribofuranoside. The pure isolates were tested for their anti-melanogenic and anti-allergic activity.

Melanin is produced by melanocytes, located within the outermost layer of the skin. Hyperactivity of melanocytes and melanin accumulation is involved in many skin disorders such as skin hyperpigmentation, melasma, solar melanosis, and ephelides.^[9] Natural products that can decrease melanin synthesis could be of potential interest for development of medications for hyperpigmentation and related disorders. Numerous secondary metabolites from plant, marine environment and fungi are reported as potent melanogenic inhibitors.^[10] For example, the hydroalcoholic extract of *Spartium junceum* flower^[11] and gagunin D from the marine sponge *Phorbas* sp.^[12] In particular, fungi are a primary source for antimelanogenic compounds. For instance, kojic acid, a fungal secondary metabolite, is a popular antimelanogenic agent that is widely used in skin whitening cosmetics.^[10] Bikoic acid, previously isolated from several endophytes, daedalin A from *Daedalea dickinsii*, dicitrinone B from *Penicillium citrinum*,^[10] and the ethanolic extract from *Antrodia camphorata* mushrooms,^[13] all are evidenced to be potent antimelanogenic candidates. It is worth to mention that diketopiperazines from *Eurotium rubrum*,^[14] and purine derivatives^[15] are previously reported as melanin synthesis inhibitors, which prompted us to investigate the antimelanogenic effect of the isolated compounds.

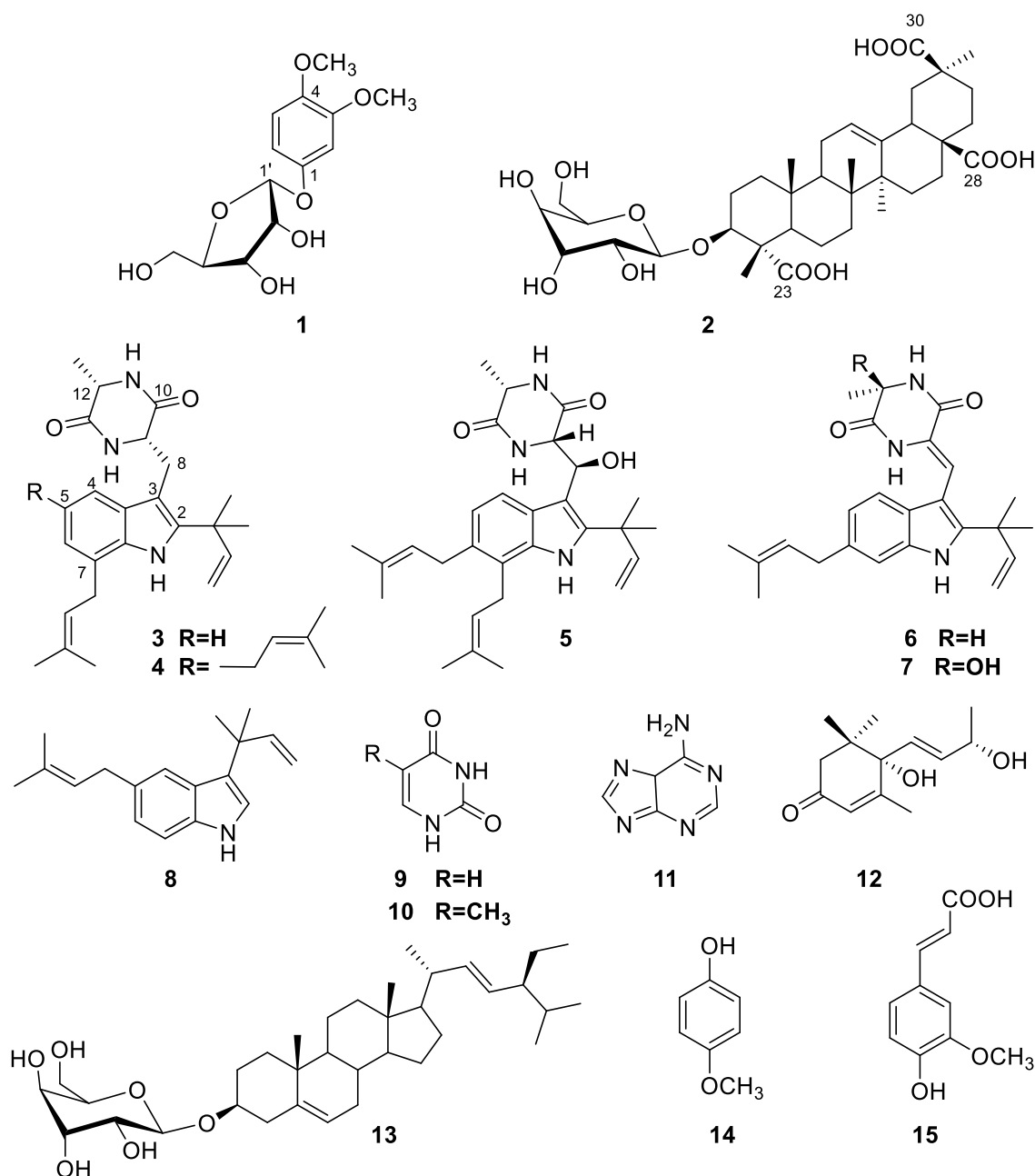
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Allergy can be defined as exaggerated immune reaction towards usually harmless environmental substances, named allergens. Allergy disorders can range from eczema, allergic rhinitis and asthma to the life threatening anaphylaxis.^[16] Allergens stimulate degranulation of basophiles and mast cells and hence the release of allergy mediators that triggers the allergic reaction.^[16] Since β -hexosaminidase is localized in the granules of the mast cells, it is used as a typical marker of mast cell degranulation.^[17] A number of natural products were explored as antiallergic agents, namely, naphthoquinones from *Rhinacanthus nasutus* Kuntze, quinones from *Dioscorea membranacea* Pierre ex Prain,^[16] and flavonoids from *Benchalokawichian*.^[18] To examine the anti-allergic action, we tested the effect of the isolated compounds on β -hexosaminidase release.

Results and Discussion

Isolation and identification of compounds

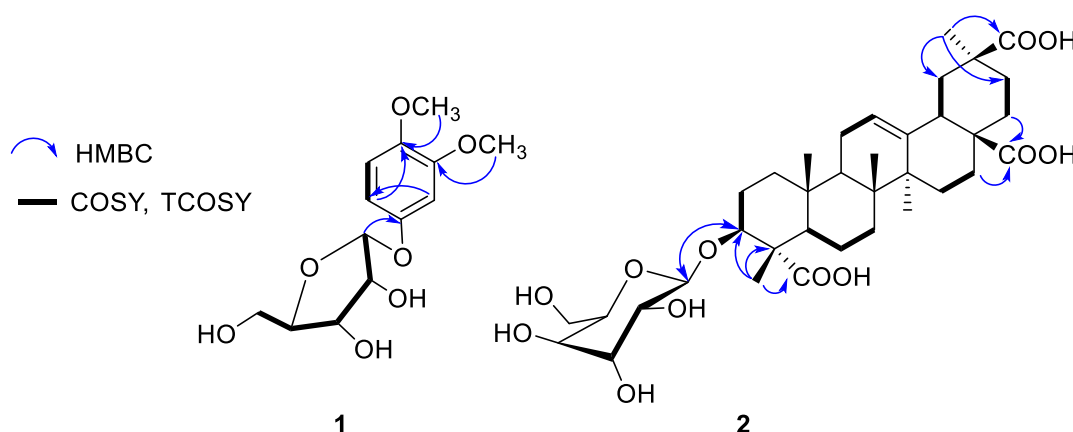
Fifteen compounds (Fig. 1) were isolated from the white beans culture of *A. amstelodami*, including two new compounds (1) and (2).



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Figure 1. Compounds isolated from the white beans culture of *A. amstelodami*.

Compound (1) was assigned to the molecular formula $C_{13}H_{18}O_7$ based on the positive molecular ion peak at m/z 309.1009 $[M+Na]^+$ (calcd 309.0950). The ^1H - and ^{13}C -NMR data (Table 1) showed the presence of an ABX system concluded from the splitting pattern of the aromatic protons at $\delta_{(H)}$ 6.66 (d, 2.7, H-2), 6.84 (d, 8.8, H-5), and 6.56 (d, 8.7, 2.7, H-6). The ^{13}C -NMR spectrum showed additional five sugar signals at $\delta_{(C)}$ 101.2, 71.4, 69.3, 86.0 and 61.6, typical for a ribofuranoside moiety.^[19] The assignment of the sugar moiety was further confirmed by ^1H - ^1H COSY, and HMBC (Fig. 2). After acid hydrolysis, the TLC pattern was found to be identical to authentic ribose. The anomeric proton signal at $\delta_{(H)}$ 5.45 (d, 4.5) suggested the α -anomeric orientation of the sugar. The connectivity between the sugar moiety and the aglycone part was established by the HMBC correlation from the anomeric proton H-1' to C-1 (151.6). Thus, compound (1) was identified as 3, 4-dimethoxyphenyl-1- O - α -D-ribofuranoside. According to literature, 3, 4-dimethoxyphenyl-1- β -D-glucoside^[20] was previously reported, but this is the first report for the glycoside incorporating D-ribose as a sugar moiety.

**Figure 2.** Key HMBC, COSY and TCOSY correlations of compounds (1) and (2).

Compound (2) was assigned to the molecular formula $C_{36}H_{54}O_{12}$ based on the positive molecular ion peak at m/z 701.3600 $[M+Na]^+$ (calcd 701.3513). Analysis of 1D and 2D NMR data revealed the presence of thirty six carbon signals. The oxygenated carbon signals at $\delta_{(C)}$ 103.4, 74.3, 76.5, 70.0, 76.3 and 61.3 were assigned to a β -glucopyranoside moiety. This was confirmed by the anomeric proton signal at $\delta_{(H)}$ 4.36 (H, d, 7.8), correlated through HSQC to C-1' (103.4). The sugar moiety was further confirmed by TLC against authentic glucose after hydrolysis. The remaining thirty carbon signals were assigned to a triterpene skeleton; the olefinic carbon signals at $\delta_{(C)}$ 122.4 and 144.8 were consistent with an oleanene type. The ^1H -NMR spectrum exhibited only five tertiary methyl groups. The HMBC correlations from CH_3 -24 to C-3 (85.8) and C-23 (184.6), from CH_3 -29 to C-30 (184.3), and from H-22 to C-28 (184.3) suggested the presence of three carboxylic functions at C-23, C-28 and C-30. The HMBC correlation from H-3 to C-1' and from H-1' to C-3 proved that the glucose residue is linked at C-3. The ^{13}C -NMR data of the aglycone were in full agreement with those reported for gypsogenic acid-3- O - β -D-glucoside,^[21] except for the replacement of CH_3 -30 with a carboxylic function. The stereochemistry was established based on the coupling constant and the ROESY correlations. The coupling constant of H-3 (12, 4.2) indicated the β -orientation of the hydroxyl function,^[22] also confirmed from the ROESY correlations of H-3 to H-1', H-2 α , and H-5 α . The β -orientation of CH_3 -24 was decided based on its ROESY correlation to H-2 β , meanwhile the α -orientation of CH_3 -29 was concluded from its correlations to H-19 α . The chemical shift of CH_3 -29 ($\delta_{(C)}$ 28.3) also confirmed the α -orientation as compared to literature data ($\delta_{(C)}$ 28.1).^[23] Based on the above results compound 2 was concluded to be olean-12-en-23 α , 28 β , 30 β -trioic acid-3- O - β -D-glucopyranoside.

The known compounds were identified as echinulin (3),^[24-25] tardioxopiperazine B (4),^[26] arestrictins A (5),^[27] neochinulin D (6),^[5, 8] variecolorin O (7),^[24] claudine A (8),^[28] uracil (9),^[29] thymine (10),^[30] adenine (11),^[31] blumenol A (12),^[32] stigmasterol-3- O - β -D-glucoside (13),^[33] mequinol (14),^[34] and ferulic acid (15).^[35] Apart from echinulin^[25] and neochinulin D^[5], these compounds are reported for the first time from *A. amstelodami*.

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Table 1. ^1H - and ^{13}C -NMR data of compounds (1) and (2) (δ in ppm, J in Hz).

Compound 1 ^a			Compound 2 ^b		
Position	$\delta_{\text{(C)}}$	$\delta_{\text{(C)}}$	Position	$\delta_{\text{(C)}}$	$\delta_{\text{(C)}}$
1		151.6	1	1.07, 1.61	38.4
2	6.66 (H, d, 2.7)	103.0	2	1.87	24.8
3		149.3	3	4.05 (H, dd, 12, 4.2)	85.8
4		143.8	4		53.5
5	6.84 (H, d, 8.8)	112.5	5	1.56	51.3
6	6.56 (H, dd, 8.7, 2.7)	107.4	6	1.22	20.5
3 OCH ₃	3.72 (3H, s)	55.4	7	1.19	32.3
4 OCH ₃	3.69 (3H, s)	56.0	8		39.4
1'	5.45 (H, d, 4.5)	101.2	9	1.66	48.1
2'	4.01 (H, m)	71.4	10		36.0
3'	3.9 (H, dd, 6.4, 3.2)	69.3	11	1.66, 1.88	23.0
4'	3.96 (H, dd, 7.5, 4)	86.0	12	5.35 (br t, 3.6)	122.4
5'	3.45 (2H, m)	61.6	13		144.8
			14		41.5
			15	1.81, 0.99	27.8
			16	1.64, 1.85	25.0
			17		46.4
			18	2.89 (H, dd, 13.8, 3)	43.0
			19	1.47, 1.92	43.7
			20		44.4
			21	1.92, 1.16	31.1
			22	1.5, 1.76	34.5
			23		184.6
			24	1.09 (3H, s)	12.0
			25	0.94 (3H, s)	14.9
			26	0.82 (3H, s)	16.5
			27	1.16 (3H, s)	25.0
			28		184.3
			29	1.06 (3H, s)	28.3
			30		184.3
			1'	4.36 (H, d, 7.8)	103.4
			2'	3.08 (H, dd, 9, 7.8)	74.3
			3'	3.33 (H, m)	76.5
			4'	3.24 (H, m)	70.0
			5'	3.24 (H, m)	76.3
			6'	3.65 (H, dd, 12, 4.8)	61.3
				3.81 (H, dd, 12, 1.8)	

^a measured in DMSO-d₆ ^b measured in CD₃OD

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Anti-melanogenic activity

The isolated compounds were preliminary tested, at final concentration of 100 μ M, for their effect on cell viability and melanin production in B16 melanoma cells, using arbutin as a positive control.^[36] Compounds that showed significant decrease in melanin production with no or little cytotoxicity to cells, as compared to control, are considered optimum melanogenic inhibitors. Hence the ratio of melanin production to cell survival was calculated as an indication of anti-melanogenic activity (Fig. 3), to exclude compounds that their inhibitory effects may be caused by their cytotoxicities. In this context, compounds (4), (7), (1) and (6) were superior to arbutin, meanwhile, (9), (11), (3) and (8) showed comparable activity. These compounds were further investigated and their IC_{50} values were determined (Table 2). Compound (8) was not further investigated due to limited amount.

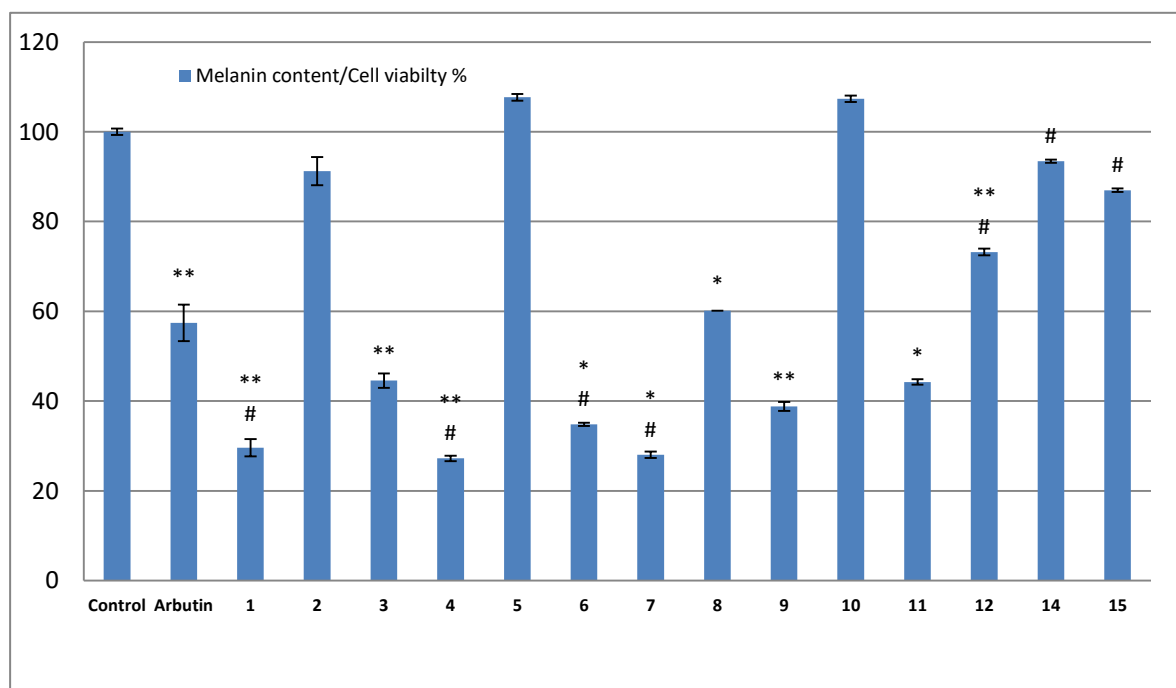


Figure 3. The effect of the isolated compounds on melanin content and cell viability in B16 cells. Blue bars show the ratio of melanin content to cell viability (%) as compared to untreated control. Data are expressed as the mean $\pm$ S.D (n=3) of three independent experiments. Arbutin was used as positive control. All compounds were used at 100 μ M. Significant difference was determined by the Student's t-test (compared to control: *p < 0.05 and **p < 0.01, compared to quercetin: #p < 0.05 and ##p < 0.01).

Apart from compounds (3)^[14] and (9),^[37] the antimelanogenic activity of the tested compounds is described for the first time. In terms of IC_{50} values, compounds (4), (7) and (1) were the most active, $IC_{50} = 30.8 \pm 5.75$, 38.5 ± 6.08 and 52.6 ± 6.64 μ M, respectively, followed by (3), (11) and (6), $IC_{50} = 98.0 \pm 1.16$, 100.4 ± 3.05 and 112.0 ± 0.22 , respectively, meanwhile (9) was the least active compound, $IC_{50} = 144.7 \pm 2.35$. Notably, the tested compounds were found to be more potent inhibitors of melanin production than arbutin, the positive control, $IC_{50} = 151.7 \pm 1.27$. The IC_{50} values for cell viability indicated that tested compounds selectively inhibited melanin production with no effect on cell viability.

The diketopiperazines (4) and (7) were the most active, their IC_{50} values were about fifth and fourth of that of arbutin, respectively. Since they selectively suppressed melanin production at much lower concentrations than arbutin with no effect on cell viability ($IC_{50} > 100$), they could be recommended as potential candidates for whitening cosmetics. According to literature, several diketopiperazines such as echinulin, alkaloid E7, isoechinulin B, and C are previously reported^[14] to inhibit melanin synthesis.

Compound (1) was one order of magnitude less active, its IC_{50} value was about third of that of arbutin. Despite the simple structure, a previous study reported that a polyhydroxy tetrahydrofuran skeleton with an aromatic moiety in the anomeric position is an optimum lead for tyrosine kinase inhibitors,^[38] which can explain the interesting activity of the compound.

Chem. Biodiversity

Remarkably, the ribose moiety allows the compound to mimic the ATP structure and position the aromatic moiety at the adenine site, hence efficiently blocks tyrosine kinase.^[38]

Meanwhile the other diketopiperazines (3) and (6) as well as the purine (11) were less active; still their IC₅₀ values were significantly lower than arbutin. For the diketopiperazine skeleton, a previous structure activity relationship study^[14] reported the importance of the prenyl groups at C-2, C-5 and C-7 for activity, which is in full accordance with (4) being the most active among the diketopiperazines. The higher activity of (7) vs (3) may be attributed to the sp² carbon at C-8, previously suggested in the same study.^[14] Meanwhile, comparison of (7) to (6) indicated that a hydroxyl group at C-12 greatly enhanced the activity.

It is worth to mention that purine skeleton is reported as a central scaffold for development of tyrosine kinase inhibitors,^[39] which may explain the activity of compound (11).

The pyrimidine (9) was the least active compound among the tested compounds; its IC₅₀ value was comparable to arbutin. Uracil (9) in particular was previously evidenced to block the expression of melanin synthesis related genes.^[37]

The above results seemed to indicate that compounds (4), (7), (1), (3), (11), (6) and (9) could be lead natural compounds for development of new anti-melanogenic drugs with improved safety margin, since they selectively suppressed melanin production with no effect on cell viability. Also their IC₅₀ values were lower than arbutin, the positive control. Our results and literature indicated that the diketopiperazine skeleton is favorable for antimelanogenic activity, where compounds (3), (4), (6) and (7) all are diketopiperazines that showed considerable activity. Meanwhile (1) and (11) possess an optimum skeleton for development of tyrosine kinase inhibitors,^[38, 39] which may give an idea about their mechanism of action. According to literature, the ribose moiety of the new compound (1) has an essential role in activity. There is no doubt that the isolated compounds inhibited melanogenesis, however in the future more studies should be undertaken to clear out their mechanism in inhibition of melanin synthesis.

Table 2. The effect of the tested compounds on melanin content and cell viability in B16 melanoma cells.

Compound	IC ₅₀ (μM) for melanin content	IC ₅₀ (μM) for cell viability
1	52.6 ± 6.64*	> 100
3	98.0 ± 1.16*	> 100
4	30.8 ± 5.75**	> 100
6	112.0 ± 0.22*	> 200
7	38.5 ± 6.08**	> 100
9	144.7 ± 2.35	> 200
11	100.4 ± 3.05*	> 100
Arbutin	151.7 ± 1.27	> 500

IC₅₀ represents compound concentration causing a 50 % inhibition. Data are expressed as mean ±SD of three independent experiments each done in triplicate. The statistical significance was analyzed using Student's t-test (*p < 0.05 and **p < 0.01 as compared with arbutin).

Anti-allergic activity

The anti-allergic activity of the isolates was investigated in RBL-2H3 at 100 μM using quercetin as a standard anti-allergic compound (Fig. 4).^[40] In the same context, the ration of hexosaminidase to cell viability was calculated (Fig. 4). Compounds (6) and (11) significantly decreased β-hexosaminidase release as compared to control with no significant cytotoxicity to cells. However, their anti-allergic effects were significantly lower than quercetin. According to literature, none of the tested compounds was previously reported to inhibit β-hexosaminidase release. Even though, some oleanane triterpenes are reported as inhibitors of β-hexosaminidase release^[41-42], compound (2) did not show significant activity.

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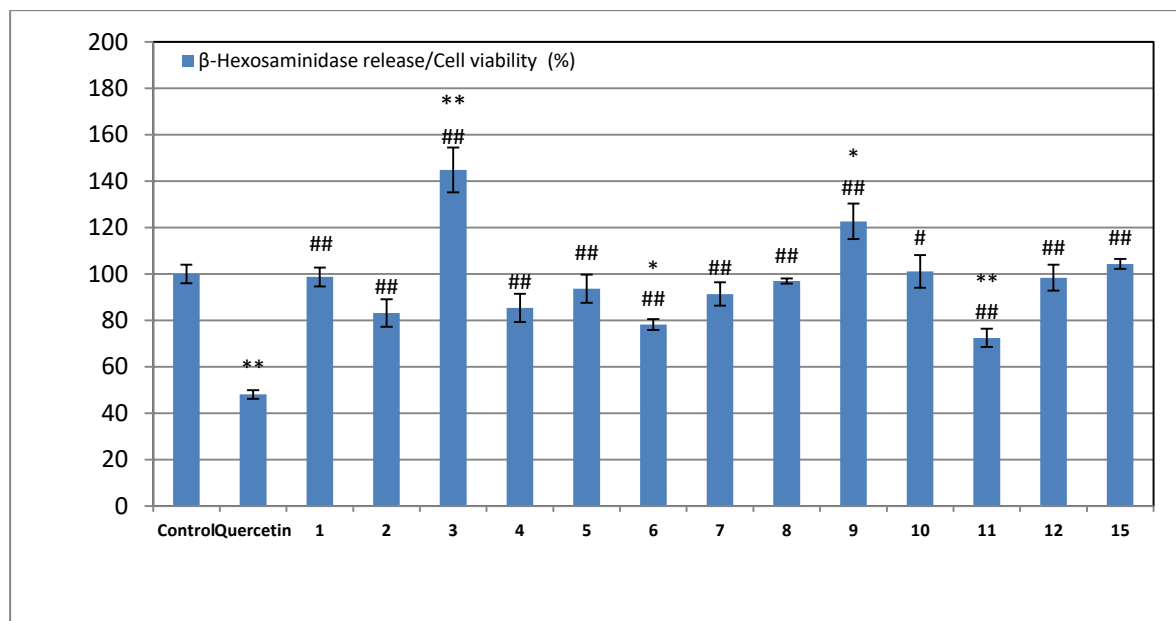


Figure 4. The effects of the isolated compounds on β -hexosaminidase release in RBL-2H3 cells. Blue bars show the ratio of β -hexosaminidase release to cell viability (%) as compared to untreated control. Data are expressed as the mean $\pm$ S.D (n=3) of three independent experiments. Quercetin was used as positive control. All compounds were tested at 100 μ M. Significant difference was determined by the Student's t-test (compared to control: *p < 0.05 and **p < 0.01, compared to quercetin: #p < 0.05 and ##p < 0.01).

Conclusion

Fifteen compounds were isolated from the white beans culture of *A. amstelodami*. Including two new compounds (1) and (2), and eleven compounds that are reported for the first time from the fungus. The anti-melanogenic and anti-allergic activity of these compounds were described for the first time. The results suggested that compounds (1), (3), (4), (6), (7), (9) and (11) are promising lead natural compounds for development of new medications for hyperpigmentation. Meanwhile, the tested compounds did not show significant anti-allergic activity as compared to positive control.

Experimental Section

General Experimental Procedures

1D and 2D-NMR measurements were carried out on a Varian INOVA (600 MHz for ^1H and 150 MHz for ^{13}C) and (400 MHz for ^1H and 100 MHz for ^{13}C). Chemical shifts (δ) are expressed in ppm with reference to the residual solvent signal. Coupling constants (J values) are given in Hz. FT-IR measurements were carried out using Thermo Fisher Scientific iS10 spectrometer equipped with ATR (Attenuated Total Reflection) accessory. HR-ESIMS were measured using a Bruker microTOF mass spectrometer. Column chromatography was carried out using Silica gel G 60-230 (Merck, Germany), Sephadex LH-20 (Sigma-Aldrich, Missouri, USA) and reversed phase silica gel (Rp-C₁₈, Bakerbond octadecyl C₁₈, 40 μ m) (Phillipsburg, NJ, USA), RP HPLC using Cosmosil AR-II, 250 x 10 mm i.d and 250x 4.6 mm i.d using a Jasco PU2089 gradient pump and PU2075 UV/VIS detector. Thin-layer chromatography was carried out using silica gel 60 F₂₅₄ aluminum plates (Merck, Germany). Authentic sugar samples were obtained from the Department of Pharmacognosy, Faculty of Pharmacy, Mansoura University. Acid hydrolysis was done according to the method described by Timité et al.^[43]

Fungal Material

The leaf parts of the mangrove plant *Avicennia marina* were collected from kilo 17, Safaga, Red Sea, Egypt in October 2017. Fungal material was isolated from the petiole segments according to the standard procedure described before.^[44] The fungus was identified as *Aspergillus amstelodami* AUMC <EGY> by PCR using the universal fungal primers ITS1 and ITS4 (GenBank accession No. LC471409). The fresh mycelia grown on malt plates (Oxoid Ltd, Basingstoke, Hants, UK) were inoculated into 14 flasks (1L), each preloaded with autoclaved white beans (100 g beans and 110 ml water). The fermentation was carried out for 28 days under static conditions at 25°C.

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Extraction and Isolation

The fungal culture was extracted with EtOAc (3 x 500 ml). The extract was concentrated and suspended in H₂O, then defatted with hexane and exhaustively extracted with EtOAc. The EtOAc extract was concentrated to afford a white precipitate (184 mg) and the remaining mother liquor was concentrated to dryness (1.65 g). The mother liquor was fractionated over CC (SiO₂, Hexane/EtOAc 100: 0 to 0: 100, then EtOAc/MeOH 100: 0 to 75:25) to afford 13 fractions (Fr. 1 -13). Fr. 10 (22.7 mg, eluted with Hexane/EtOAc 0: 100) was subjected to semi preparative HPLC (10 %CH₃CN containing 0.1 % HCOOH, isocratic, 15 min, 254 nm, 2.5 ml/min, Cosmosil 5C18 ArII, 250 x 10 i.d.mm) to yield compound 1 (1.3 mg). Fr. 13 (9.9 mg, eluted with EtOAc/MeOH 75: 25) was subjected to CC (Rp18, MeOH/H₂O, gradient) to afford 4 sub-fractions (13-1 to 13-4). Fr. 13-3 (3.1 mg, eluted with MeO/H₂O 90:10) was finally purified over CC (SiO₂, CH₂Cl₂/MeOH 96: 4) to afford compound 2 (1.7 mg).

3, 4-Dimethoxyphenyl-1-O- α -D-ribofuranoside (1). Colorless amorphous. *R_f*=0.47 (CH₂Cl₂/MeOH, 9.5/0.5, silica gel). IR (ATR): 3370, 2922, 2850, 1598, 1513, 1453, 1379, 1354, 1229, 1028, 534.¹H- (600 MHz) and ¹³C-NMR (150 MHz): see Table 1. HR-ESI-MS (pos.): 309.1009 ([M+Na]⁺, C₁₃H₁₈O₇Na⁺, calc. 309.0950).

Olean-12-en-23 α , 28 β , 30 β -trioic acid-3-O- β -D-glucopyranoside (2). Colorless amorphous. *R_f*=0.28 (CH₂Cl₂/MeOH, 8/2, silica gel). IR (ATR): 3389, 2923, 2870, 1551, 1464, 1391, 1361, 1077, 1021.¹H- (600 MHz) and ¹³C-NMR (150 MHz): see Table 1. HR-ESI-MS (pos.): 701.3600 ([M+Na]⁺, C₃₆H₅₄O₁₂Na⁺, calc. 701.3513).

Cell culture

Dulbecco's Modified Eagle's medium with low glucose (DMEM) (Wako, Japan) and Eagle's minimal essential medium (EMEM) (Wako, Japan) were used for B16 melanoma and for RBL-2H3 cells, respectively. The culture medium was supplemented with 10 % fetal bovine serum (FBS) (GE healthcare, UK) and 1 % Penicillin-Streptomycin Solution (Wako, Japan) and incubated at 37 °C in a humid atmosphere containing 5 % CO₂.

Measurement of melanin contents in B16 melanoma cells

Melanoma assay was performed according to Arung et al.^[45] The confluent culture of B16 melanoma cells was rinsed with phosphate-buffer saline (PBS) (Wako, Japan), detached using 0.25% trypsin/EDTA (Wako, Japan). The cells were seeded in a 24-well plate (1.0 x 10⁵ cells/well) and maintained at 37 °C in a humid atmosphere containing 5 % CO₂ for 24 h. Test samples were added to B16 melanoma cells and incubated for 72 h. Cells were washed with PBS, collected in a 1.5 mL tube using 0.25% trypsin/EDTA. The supernatant of B16 melanoma cell was completely removed and then cell lysis was performed by heating for 10 min at 100 °C in 1N NaOH to dissolve the melanin. The cell lysate was transferred to a 96-well microplate, and the absorbance was measured at 405 nm using a microplate reader (MTP-310, Corona Electric, Japan).

Measurement of the release of β -hexosaminidase assay in RBL-2H3 cells

β -Hexosaminidase release was measured according to the procedure previously described.^[46] RBL-2H3 cells were grown in 96-well plates (5 x 10⁴ cells /well) at 37 °C, in 5 % CO₂ for 24 h. Cells were washed with Tyrode's buffer (pH 7.2) and then incubated with the test compounds diluted in Tyrode's buffer at 37 °C and 5% CO₂ for 60 min. Cell degranulation was stimulated by addition of 100 μ L of 1mg/mL A23187 (1 % v/v) (Sigma-aldrich, USA) diluted in Tyrode's buffer at 37 °C and 5% CO₂ for 3 h. The cell supernatant (50 μ L) and *p*-nitrophenyl-N-acetyl- β -glucosaminide (50 μ L) diluted in 0.1 M citric acid buffer (pH 4.5) were mixed in 96-well plate and then incubated for 3 h at room temperature in dark. The enzyme–substrate reaction was stopped by addition of 0.1 M sodium bicarbonate buffer (pH 10). The absorbance was measured at 405 nm using a microplate reader (MTP-310, Corona Electric, Japan).

MTT assay for cell viability

To assess the cell viability, RBL-2H3 cells or B16 melanoma cells were seeded in a plate at 37°C under humidified air containing 5% CO₂. After incubation with tested compounds, 0.1 mL of fresh media and 0.02 mL of 5 mg/mL MTT solution were added to each well. Incubation was continued for 4 h, the culture media was discarded, 40 mM HCl-isopropanol was added to each well. The absorbance was measured at 570 nm using a microplate reader. To validate the results, MTT assay was run for compounds in absence of cells, all compounds did not react with MTT.

Supplementary Material

Supporting information for this article is available on the WWW under <http://dx.doi.org/10.1002/MS-number>.

Author Contribution Statement

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Marwa Elsbaey performed compounds isolation and NMR data analysis, Marwa Elsbaey and Amal Sallam participated in isolation, culturing and identifying the fungal material, and manuscript preparation, Mohamed El-Metwally collected the plant material, Maki Nagata performed the cell experiments and statistical analysis, Chiaki Tanaka performed high resolution mass measurements, Kuniyoshi Shimizu and Tomofumi Miyamoto provided NMR measurements and supervised the whole work.

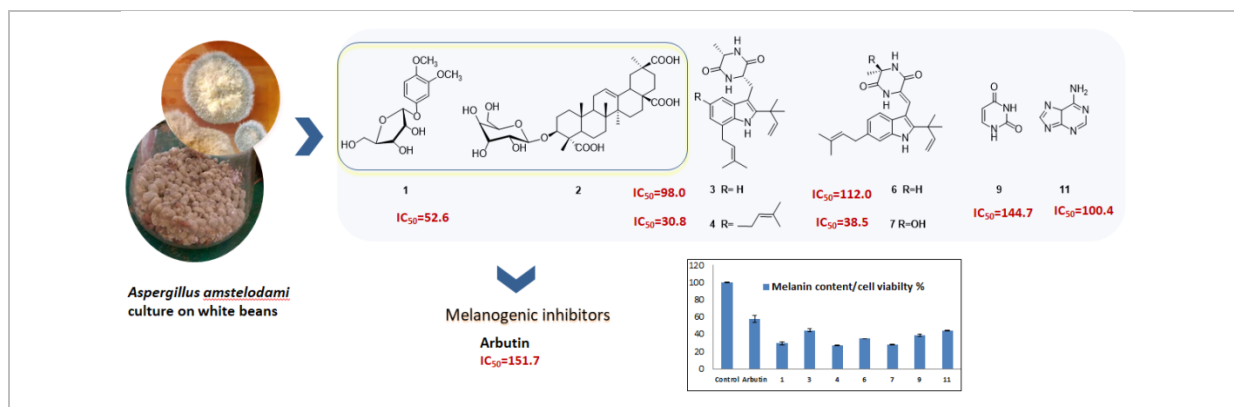
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Twitter Text

Two new compounds along with thirteen known compound were isolated from the endophytic fungus *Aspergillus amstelodami*. The isolated diketopiperazines showed promising anti-melanogenic activity.

Supporting Information

Melanogenesis inhibitors from the endophytic fungus *Aspergillus amstelodami*

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Kuniyoshi Shimizu^d and Tomofumi Miyamoto^b

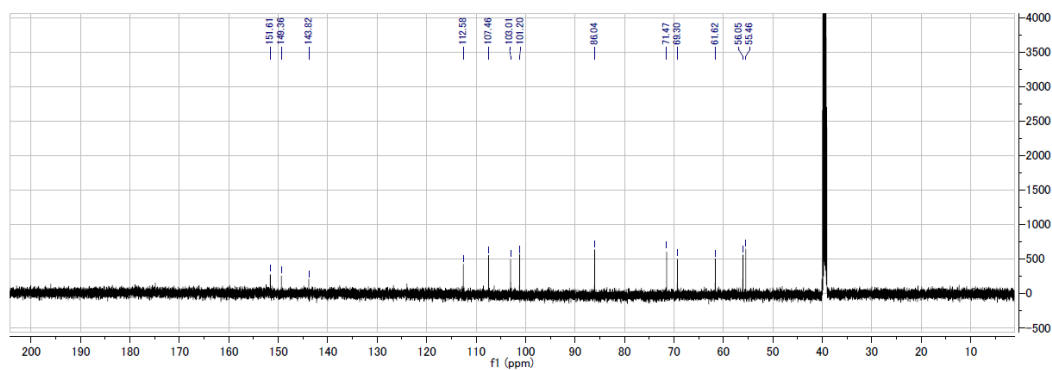
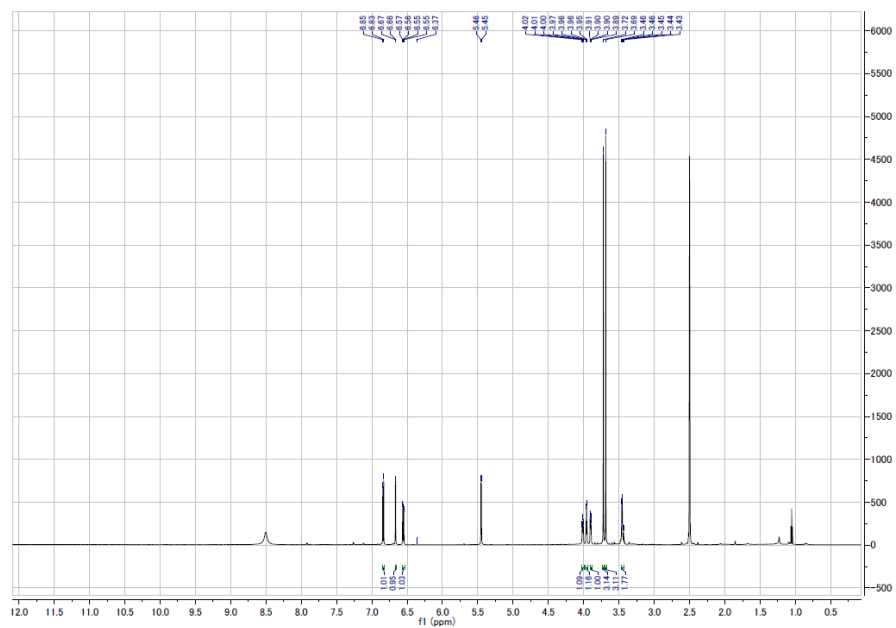
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Content	Page
Figure S1: ^1H -NMR spectrum of compound 1 in DMSO- d_6 (600 MHz).	2
Figure S2: ^{13}C -NMR spectrum of compound 1 in DMSO- d_6 (150 MHz).	2
Figure S3: COSY of compound 1 in DMSO- d_6 .	3
Figure S4: HSQC of compound 1 in DMSO- d_6 .	3
Figure S5: HMBC of compound 1 in DMSO- d_6 .	4
Figure S6: HR-ESIMS of compound 1.	4
Figure S7: IR (ATR) spectrum of compound 1.	5
Figure S8: ^1H -NMR spectrum of compound 2 in CD_3OD (600 MHz).	6
Figure S9: ^{13}C -NMR spectrum of compound 2 in CD_3OD (150 MHz).	6
Figure S10: COSY of compound 2 in CD_3OD .	7
Figure S11: TCOY of compound 2 in CD_3OD .	7
Figure S12: ROESY of compound 2 in CD_3OD .	8
Figure S13: HSQC of compound 2 in CD_3OD .	8
Figure S14: HMBC of compound 2 in CD_3OD .	9
Figure S15: HR-ESIMS of compound 2.	9
Figure S16: IR (ATR) spectrum of compound 2.	10
Figure S17: Known compounds in literature related to compound 2.	11
Table (S1): The ^{13}C -NMR data of the aglycone part of compound 2 as compared to literature data.	12
Table (S2): Results of MTT assay for compounds (without cells).	13
Molecular identification of fungal isolates	14
Figure S18: Phylogenetic tree based on ITS sequences of <i>Aspergillus amstelodami</i> (AUMC 13926) aligned with closely related sequences accessed from the GenBank with 100% similarity.	15
Figure S19: Detailed isolation procedure for the fungal metabolites.	36



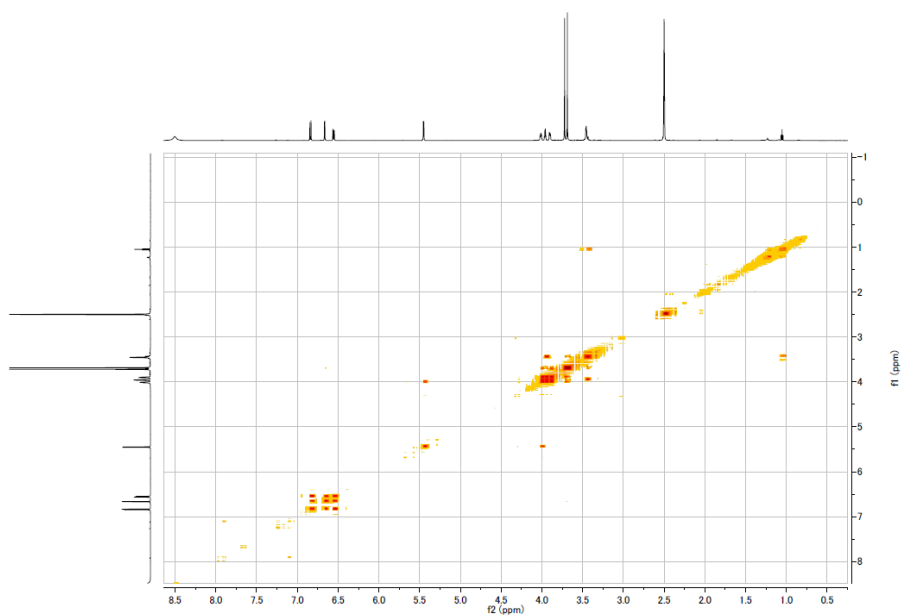


Figure S3: COSY of compound 2 in DMSO-d6.

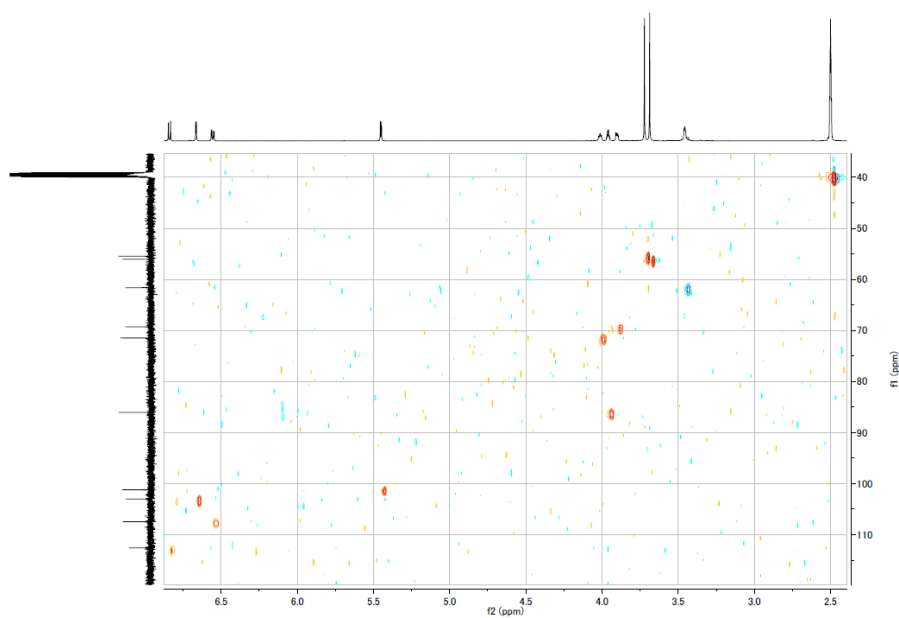


Figure S4: HSQC of compound 2 in DMSO-d6.

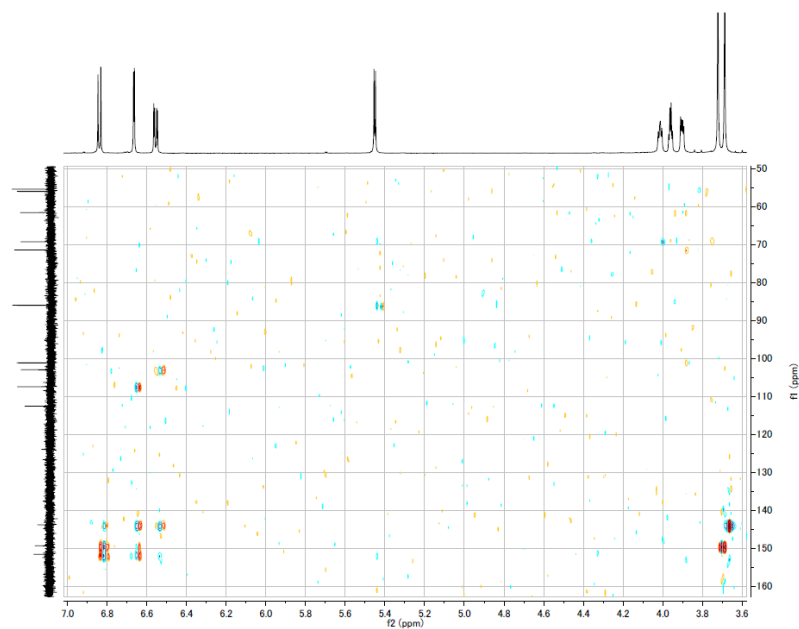


Figure S5: HMBC of compound 1 in DMSO-d6.

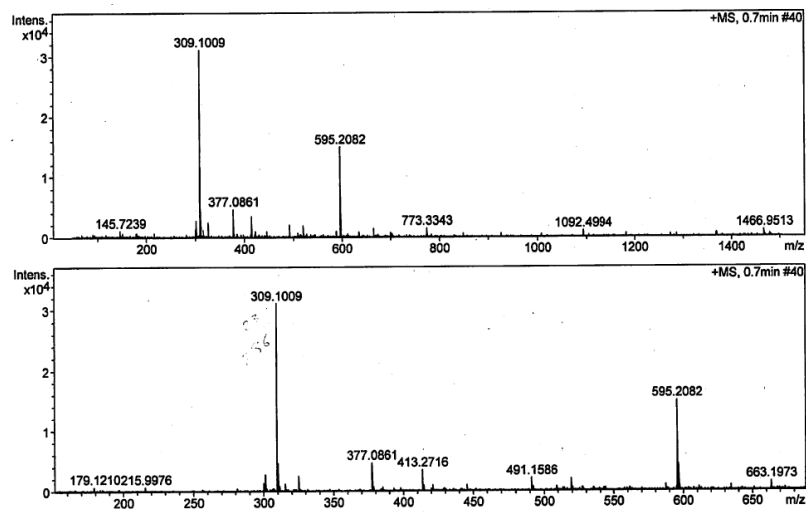


Figure S6: HR-ESIMS of compound 1.

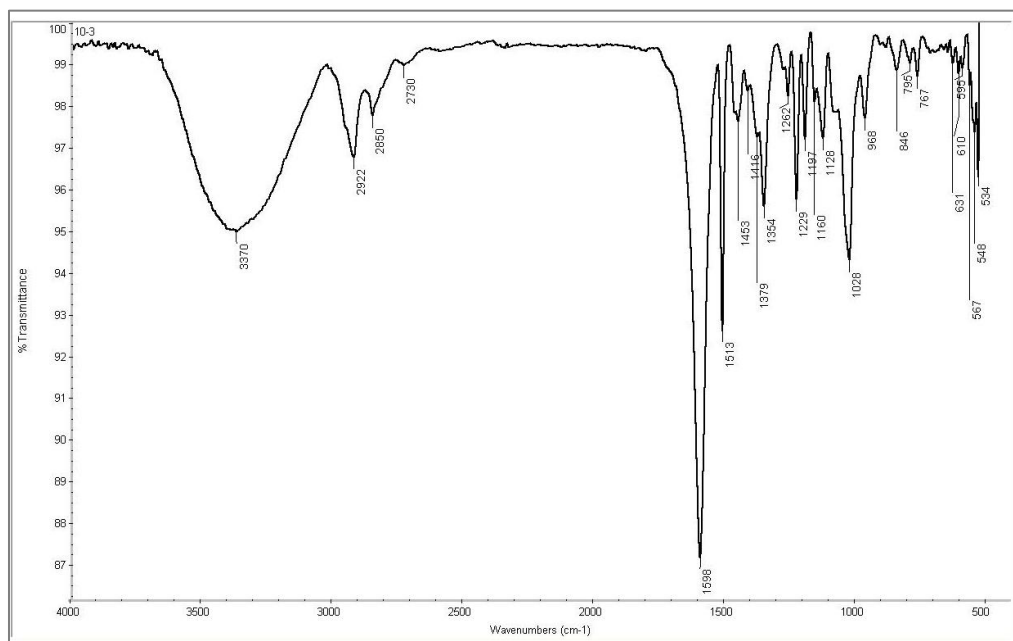


Figure S7: IR (ATR) spectrum of compound 1.

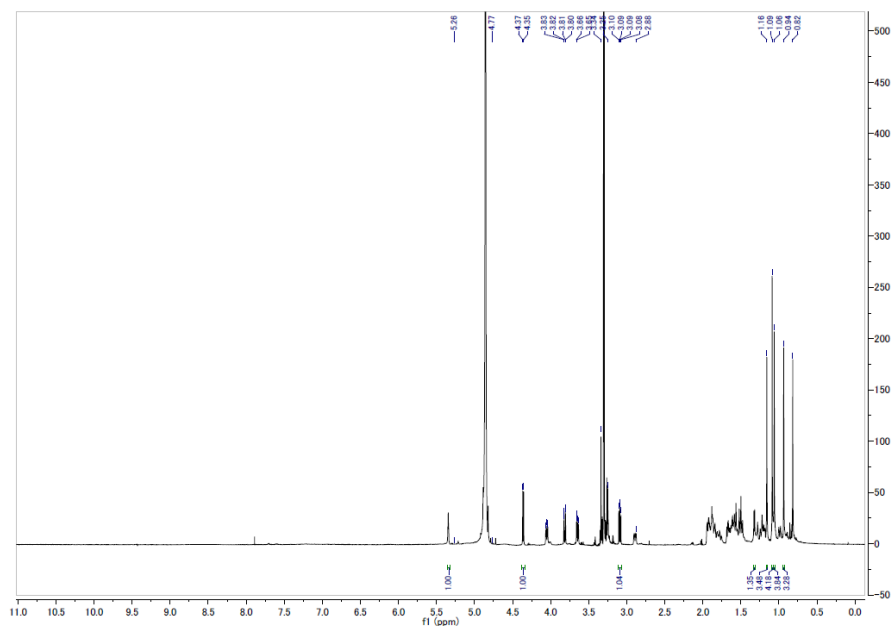


Figure S8: ¹H-NMR spectrum of compound in CD₃OD (600 MHz).

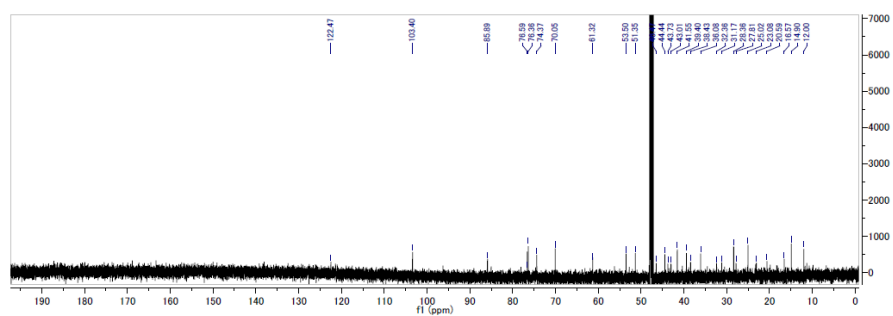


Figure S9: ¹³C-NMR spectrum of compound 2 in CD₃OD (150 MHz).

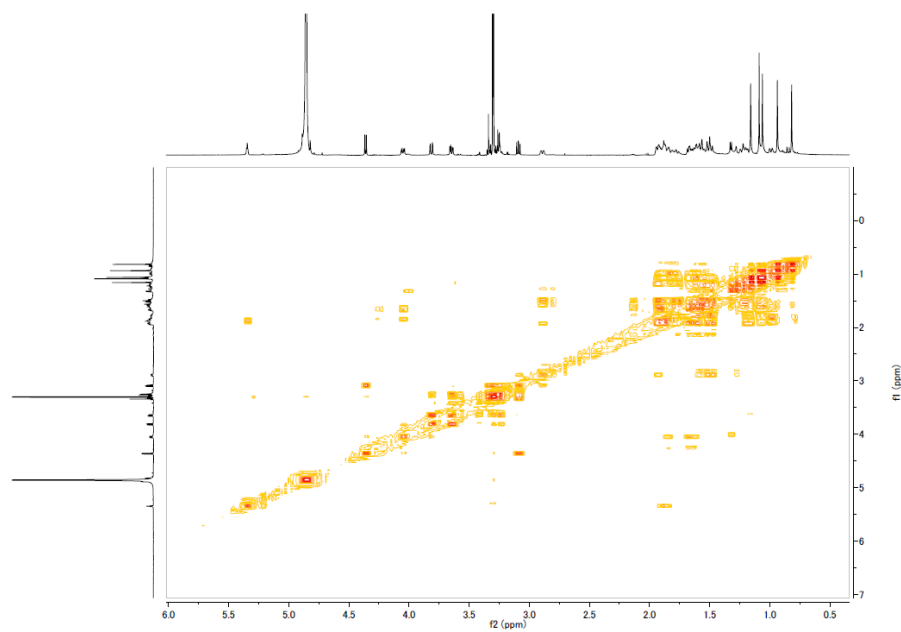


Figure S10: COSY of compound 2 in CD₃OD.

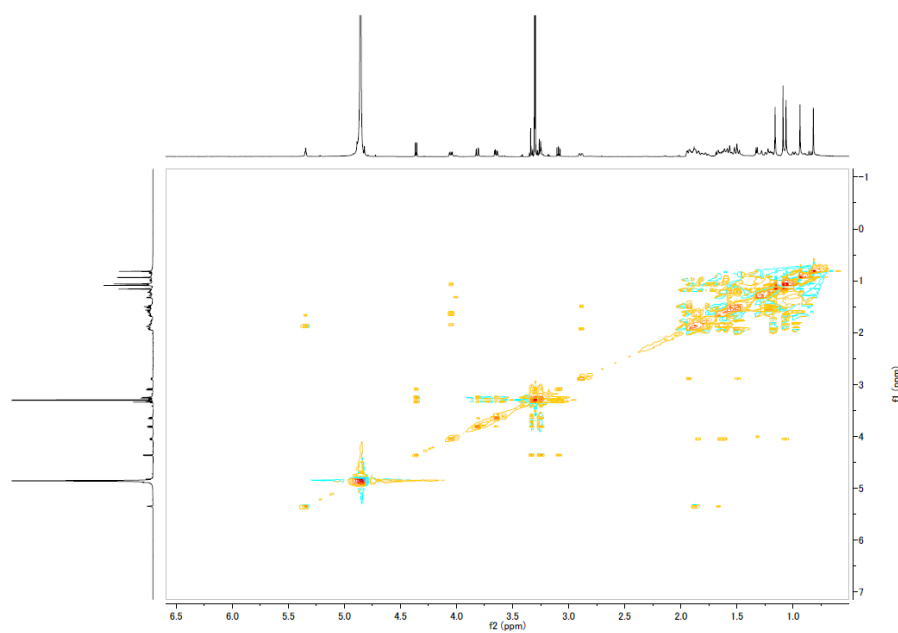


Figure S11: TCOSY of compound 2 in CD₃OD.

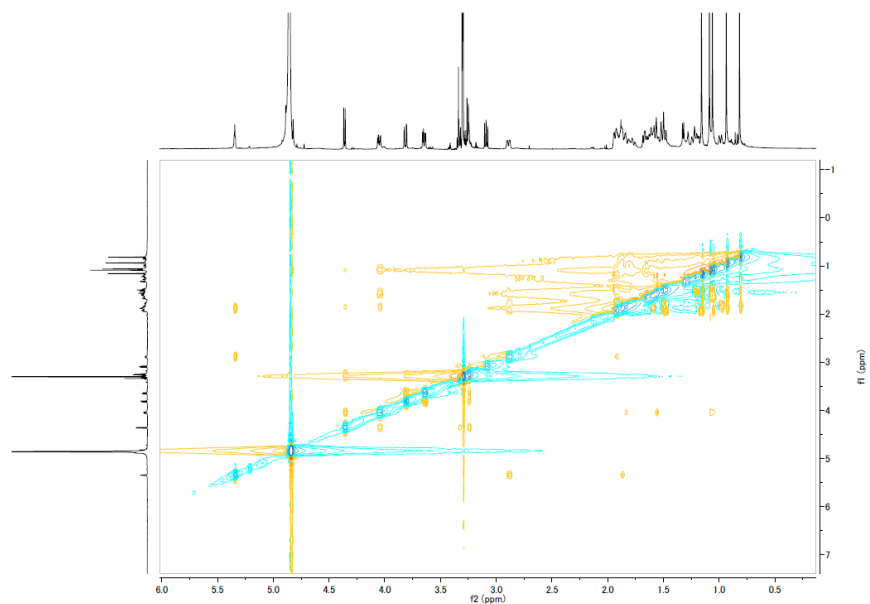


Figure S12: ROESY of compound 2 in CD₃OD.

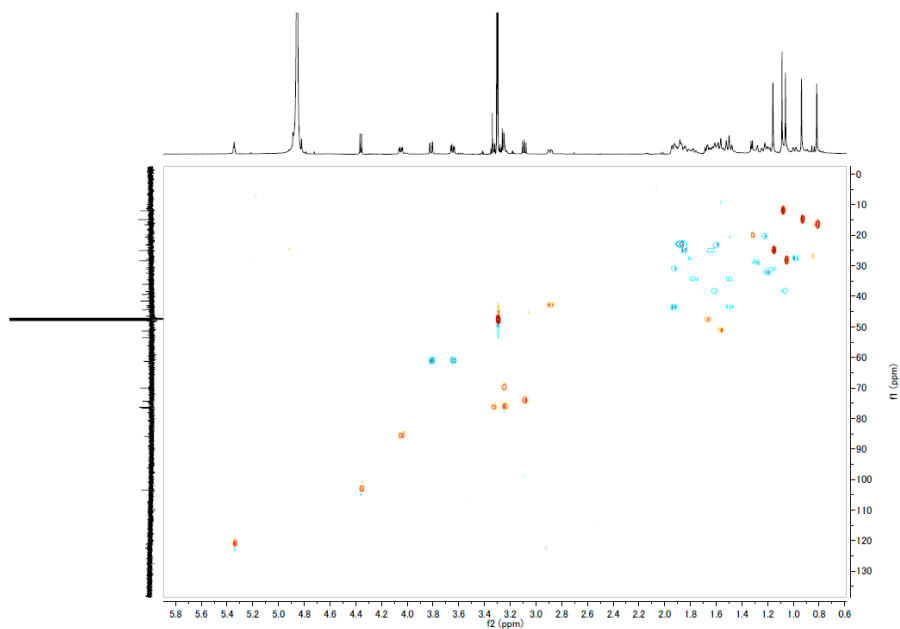


Figure S13: HSQC of compound 2 in CD₃OD.

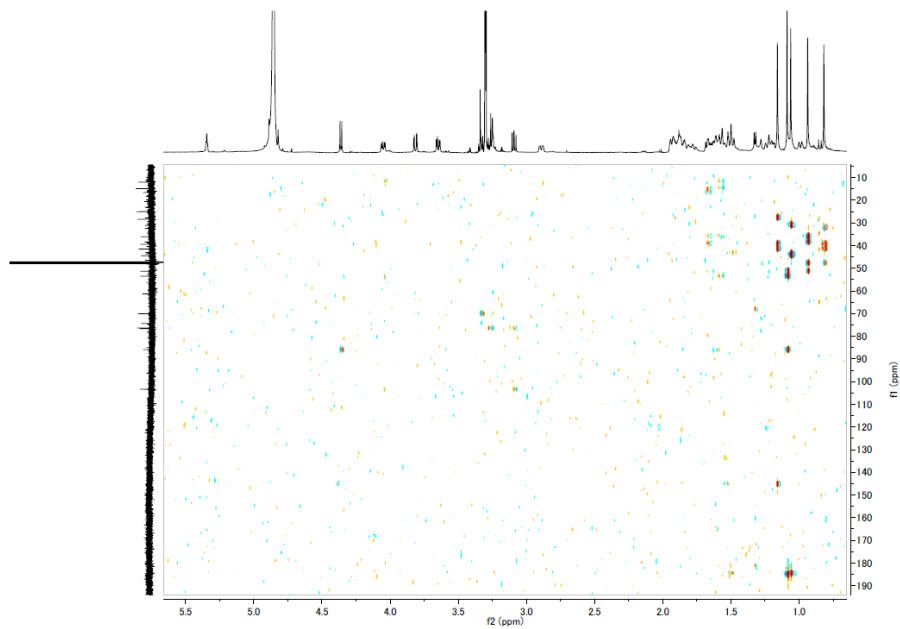


Figure S14: HMBC of compound 2 in CD₃OD.

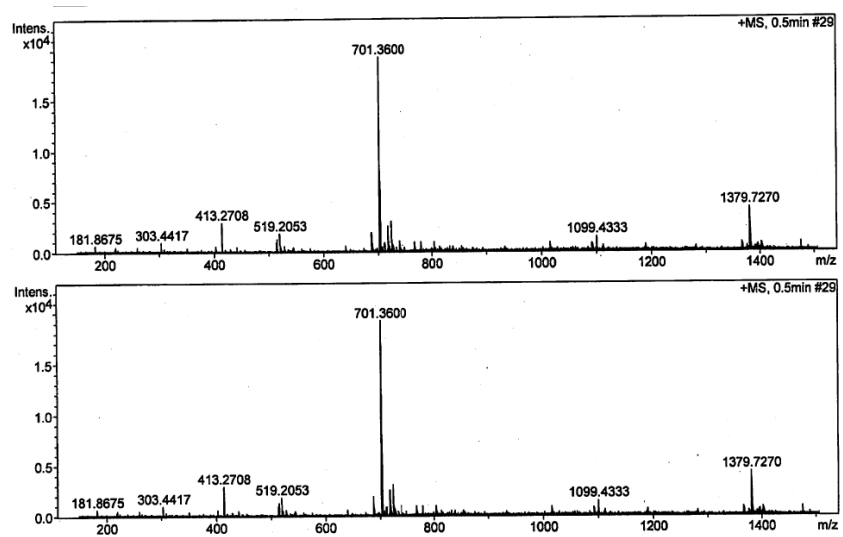


Figure S15: HR-ESIMS of Compound 2.

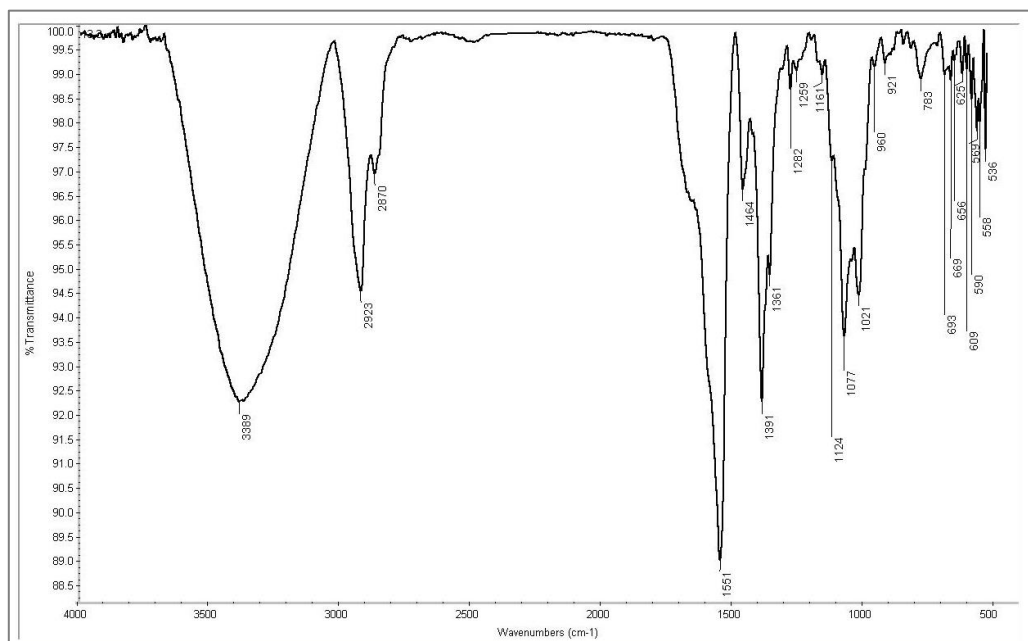
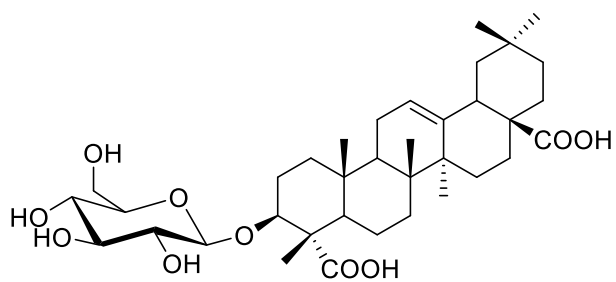
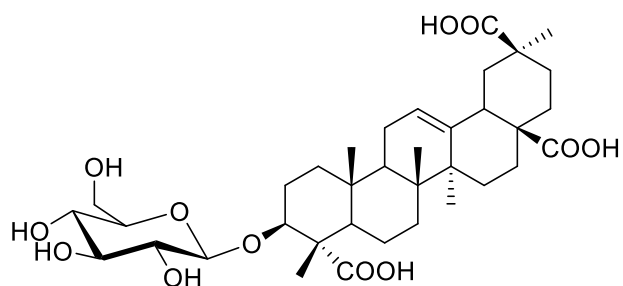


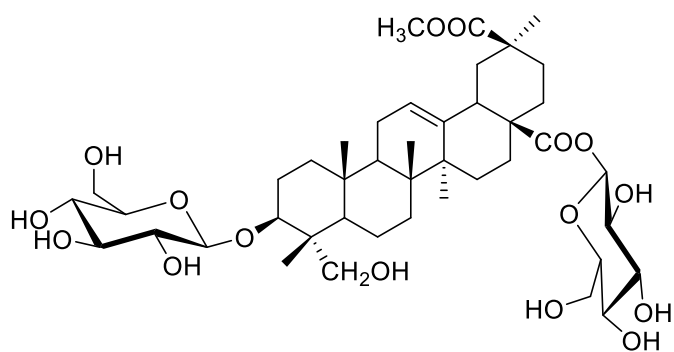
Figure S16: IR (ATR) spectrum of compound 2.



Gypsogenic acid-3-O- β -D-glucopyranoside



Compound 2



Diplocisin

Figure S17: Known compounds in literature related to compound 2.

Table (S1): The ^{13}C -NMR data of the aglycone part of compound 2 as compared to literature data.

	Compound 2	Gypsogenic acid-3-O- β -D-glucopyranoside ¹	Diplocisin ²
Position	$\delta(\text{C})$ in CD_3OD	$\delta(\text{C})$ in $\text{C}_5\text{D}_5\text{N}$	$\delta(\text{C})$ in $\text{C}_5\text{D}_5\text{N}$
1	38.4	35.6	38.7
2	24.8	26.1	25.9
3	85.8	84.9	82.1
4	53.5	53.2	43.4
5	51.3	52.0	47.6
6	20.5	21.2	18.3
7	32.3	32.9	32.8
8	39.4	40.0	39.9
9	48.1	48.2	48.1
10	36.0	36.6	36.9
11	23.0	23.7	23.8
12	122.4	122.3	-----
13	144.8	144.8	143.8
14	41.5	41.9	42.0
15	27.8	28.2	28.3
16	25.0	23.7	23.6
17	46.4	46.6	46.5
18	43.0	42.1	43.2
19	43.7	46.6	42.4
20	44.4	30.9	44.0
21	31.1	34.1	30.6
22	34.5	33.2	34.0
23	184.6	180.4	64.7
24	12.0	12.7	13.7
25	14.9	15.8	16.2
26	16.5	17.2	17.5
27	25.0	26.1	26.1
28	184.3	180.1	175.9
29	28.3	33.2	28.1
30	184.3	23.7	176.8

MTT assay

In order to validate cell viability measurement by MTT assay, MTT assay was run for compounds without cells, all compounds does not react with MTT reagent.

Table (S2): Results of MTT assay for compounds (without cells).

Compound	Absorbance-blank
Control (DMSO)	0.0240±0.001
1	0.0227±0.001
2	0.0227±0.002
3	0.0210±0.001*
4	0.0197±0.002*
5	0.0173±0.002*
6	0.0253±0.001
7	0.0273±0.001
8	0.0273±0.001
9	0.0237±0.001
10	0.0263±0.002
11	0.0223±0.002
12	0.0227±0.001
15	0.0287±0.002

Data are expressed as the mean ± S.D (n=3) of three independent experiments Significant difference was determined by the Student's t-test (compared to control: *p < 0.01).

Molecular identification of fungal isolates:

1. Methods

The fungal strain was cultivated on Czapek's yeast extract agar with 20% sucrose (CY20S) medium at 28° C for 7 days.³ The culture was sent to the molecular Biology Research Unit, Assiut University for DNA extraction using Patho-gene-spin DNA/RNA extraction kit provided by Intron Biotechnology Company, Korea. The fungal DNA was then sent to SolGent Company, Daejeon South Korea for polymerase chain reaction (PCR) and gene sequencing. PCR was performed using two universal fungal primers ITS1 (forward) and ITS4 (reverse) which were incorporated in the reaction mixture. Primers have the following composition: ITS1 (5' - TCC GTA GGT GAA CCT GCG G - 3'), and ITS4 (5'- TCC TCC GCT TAT TGA TAT GC -3'). The purified PCR product (amplicon) was reconfirmed using a size nucleotide marker (100 base pairs) by electrophoreses on 1% agarose gel. Then these bands were eluted and sequenced with the incorporation of dideoxynucleotides (dd NTPs) in the reaction mixture. Each sample was sequenced in the sense and antisense directions using ITS1 and ITS4 primers.⁴ Sequences were further analyzed using Basic Local Alignment Search Tool (BLAST) from the National Center of Biotechnology Information (NCBI) website. Phylogenetic analysis of sequences was done with the help of MegAlign (DNA Star) software version 5.05.

2. Results

Sequencing data:

Sequencing data of rRNA gene covering 18S (partial sequence), internal transcribed spacer-1 (ITS1), 5.8S ribosomal RNA gene, and internal transcribed spacer-2 (ITS2), (complete sequence) and 28S ribosomal RNA gene (partial sequences) are shown below:

Identification: *Aspergillus amstelodami* (AUMC 13926)

Number of nucleotides: 527 letters

Sequencing data:

TGCGGAAGGATCATTACCGAGTGCGGGCCCTCTGGGTCCAACCTCCCATCCGTGTCTATCT
GTACCCTGTTGCTTCGGCGTGGCCACGGCCCGCCGGAGACTAACATTTGAACGCTGTCTGA
AGTTTGCAGTCTGAGTTTTTAGTTAAACAATCGTTAAACTTTCAACAACGGATCTCTTGG

TTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAATTAATGTGAATTGCAGAATTCAGT
 GAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGGCATGCCTGTCC
 GAGCGTCATTGCTGCCCTCAAGCACGGCTTGTGTGTTGGGCTTCCGTCCCTGGCAACGGGG
 ACGGGCCCAAAGGCAGTGGCGGCACCATGTCTGGTCCTCGAGCGTATGGGGCTTTGTCAC
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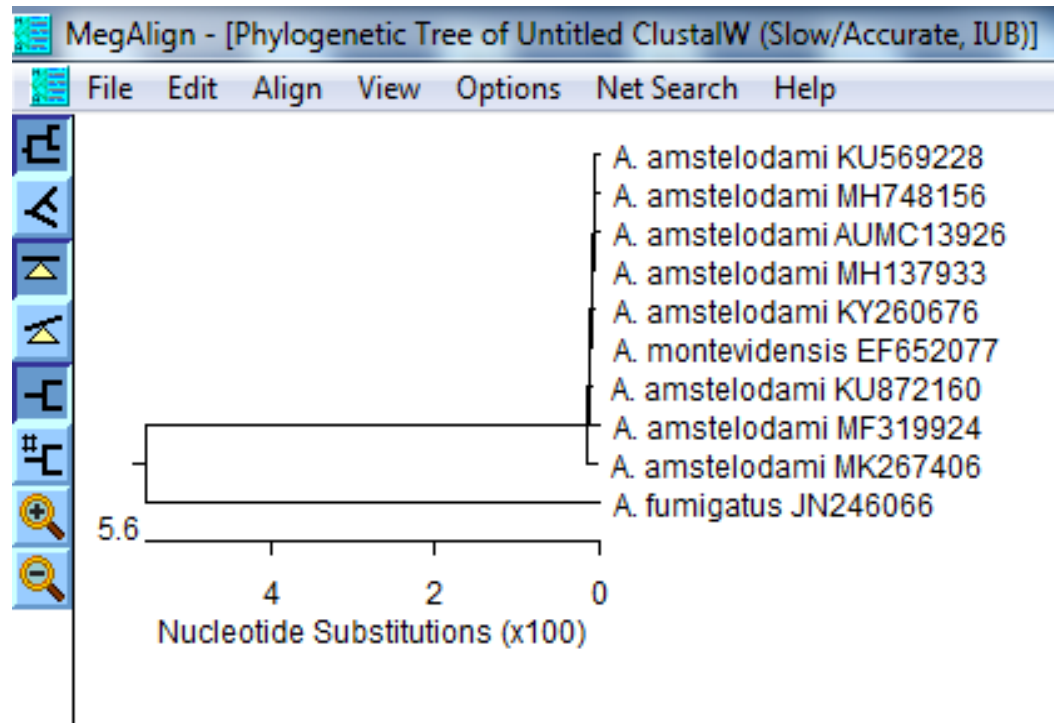


Figure 18S : Phylogenetic tree based on ITS sequences of *Aspergillus amstelodami* (AUMC 13926) aligned with closely related sequences accessed from the GenBank with 100% similarity. *Aspergillus fumigatus* was included as an outgroup strain.

Closely related data of fungal strains accessed from the GenBank:

***Aspergillus amstelodami* isolate E20327 ITS small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence**

GenBank: MK267406.1

FASTA Graphics

Go to:

LOCUS MK267406 578 bp DNA linear

PLN 12-DEC-2018

DEFINITION *Aspergillus amstelodami* isolate E20327 ITS small subunit ribosomal

RNA gene, partial sequence; internal transcribed spacer 1, 5.8S

ribosomal RNA gene, and internal transcribed spacer 2, complete

sequence; and large subunit ribosomal RNA gene, partial sequence.

ACCESSION MK267406

VERSION MK267406.1

KEYWORDS .

SOURCE *Aspergillus amstelodami*

ORGANISM *Aspergillus amstelodami*

Eukaryota; Fungi; Dikarya; Ascomycota;

Pezizomycotina;

Eurotiomycetes; Eurotiomycetidae; Eurotiales;

Aspergillaceae;

Aspergillus.

REFERENCE 1 (bases 1 to 578)

AUTHORS Buehler,A.J., Evanowski,R.L., Wiedmann,M. and Martin,N.H.

TITLE Short communication: ITS sequence-based characterization of fungal isolates from multiple yogurt facilities-a case study

JOURNAL Unpublished

```

REFERENCE      2   (bases 1 to 578)
AUTHORS        Phillips,M.D.
TITLE          Direct Submission
JOURNAL         Submitted (06-DEC-2018) Food Science, Cornell
University, 352
                Stocking Hall, Ithaca, NY 14853, USA
COMMENT        ##Assembly-Data-START##
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sequencing
                ##Assembly-Data-END##
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RNA, internal
                transcribed spacer 1, 5.8S ribosomal RNA,
internal
                transcribed spacer 2, and large subunit
ribosomal RNA"
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   241 cgcagcgaaa tgcgataatt aatgtgaatt gcagaattca gtgaatcatc gagtctttga
   301 acgcacattg cgccccctgg tattccgggg ggcatgcctg tccgagcgtc attgctgccc
   361 tcaagcacgg cttgtgtgtt gggcttccgt ccctggcaac ggggacgggc ccaaaaggca
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   481 cagctggcag ctagcctcgc aaccaatctt ttaaccagg ttgacctcgg atcaggtagg
   541 gatacccgct gaacttaagc atatcaataa gcggagga
//=====

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***Aspergillus amstelodami* strain BT26 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence**
GenBank: MH137933.1

[FASTA](#) [Graphics](#) [PopSet](#)

[Go to:](#)

LOCUS MH137933 527 bp DNA linear
PLN 06-OCT-2018

DEFINITION *Aspergillus amstelodami* strain BT26 internal
transcribed spacer 1,
partial sequence; 5.8S ribosomal RNA gene and
internal transcribed
spacer 2, complete sequence; and large subunit
ribosomal RNA gene,
partial sequence.

ACCESSION MH137933

VERSION MH137933.1

KEYWORDS .

SOURCE *Aspergillus amstelodami*

ORGANISM *Aspergillus amstelodami*
Eukaryota; Fungi; Dikarya; Ascomycota;
Pezizomycotina;
Eurotiomycetes; Eurotiomycetidae; Eurotiales;
Aspergillaceae;
Aspergillus.

REFERENCE 1 (bases 1 to 527)

AUTHORS Midilli,B. and Abaci Gunyar,O.

TITLE Researching of gene region responsible for
ribotoxin production in
Aspergillus species isolated from animal feed in
the market

JOURNAL Unpublished

REFERENCE 2 (bases 1 to 527)

AUTHORS Midilli,B. and Abaci Gunyar,O.

TITLE Direct Submission

JOURNAL Submitted (28-MAR-2018) Biology, Ege University,
Genclik Caddesi,
Izmir 35040, Turkey

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sequencing
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spacer 1, 5.8S
                    ribosomal RNA, internal transcribed
spacer 2, and large
                    subunit ribosomal RNA"
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    61  tgtaccctgt tgcttcggcg tggccacggc ccgccggaga ctaacatttg aacgctgtct
    121  gaagtttgca gtctgagttt ttagttaaac aatcggttaa actttcaaca acggatctct
    181  tggttccggc atcgatgaag aacgcagcga aatgcgataa ttaatgtgaa ttgcagaatt
    241  cagtgaatca tcgagtcctt gaacgcacat tgcgccccct ggtattccgg ggggcatgcc
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    361  acggggacgg gcccaaaagg cagtggcggc accatgtctg gtcctcgagc gtatggggct
    421  ttgtcaccgc ctcccgtagg tccagctggc agctagcctc gcaaccaatc tttttaacca
    481  ggttgacctc ggatcaggta gggataccgc ctgaacttaa gcatatc
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```

***Aspergillus montevidensis* isolate NRRL 108 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence**

GenBank: EF652077.1

[FASTA](#) [Graphics](#) [PopSet](#)

[Go to:](#)

LOCUS EF652077 1111 bp DNA linear

PLN 05-APR-2016

DEFINITION *Aspergillus montevidensis* isolate NRRL 108 18S ribosomal RNA gene,

partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA

gene, and internal transcribed spacer 2, complete sequence; and 28S

ribosomal RNA gene, partial sequence.

ACCESSION EF652077

VERSION EF652077.1

KEYWORDS .

SOURCE *Aspergillus montevidensis*

ORGANISM *Aspergillus montevidensis*

Eukaryota; Fungi; Dikarya; Ascomycota;

Pezizomycotina;

Eurotiomycetes; Eurotiomycetidae; Eurotiales;

Aspergillaceae;

Aspergillus.

REFERENCE 1 (bases 1 to 1111)

AUTHORS Peterson,S.W.

TITLE Phylogenetic analysis of *Aspergillus* species using DNA sequences

from four loci

JOURNAL Mycologia 100 (2), 205-226 (2008)

PUBMED [18595197](#)

REFERENCE 2 (bases 1 to 1111)

AUTHORS Peterson,S.W.

TITLE Direct Submission

JOURNAL Submitted (04-JUN-2007) Microbial Genomics and Bioprocessing

Research Unit, National Center for Agricultural
Utilization

Research, 1815 North University Street, Peoria,
IL 61603, USA

FEATURES	Location/Qualifiers
source	1..1111 /organism="Aspergillus montevidensis" /mol_type="genomic DNA" /isolate="NRRL 108" /db_xref="taxon:1173306" /note="teleomorph: Eurotium amstelodami"
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misc_RNA	13..153 /product="internal transcribed spacer 1"
rRNA	154..310 /product="5.8S ribosomal RNA"
misc_RNA	311..475 /product="internal transcribed spacer 2"
rRNA	476..>1111 /product="28S ribosomal RNA"

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121 ttgcagtctg agtttttagt taaacaatcg ttaaaacttt caacaacgga tctcttggtt
181 ccggcatcga tgaagaacgc agcgaaatgc gataattaat gtgaattgca gaattcagtg
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421 acccgctccc gtaggtccag ctggcagcta gcctcgcaac caatcttttt aaccagggtg
481 acctcggatc aggtagggat acccgctgaa ctaagcata tcaataagcg gagggaaaaga
541 aaccaaccgg gattgcctca gtaacggcga gtgaagcggc aagagctcaa atttgaaatc
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661 aagtgtctcg gaacgggcca tcggagaggg tgagaatccc gtctgggacg ggggtgccgc
721 gtccatgtga agctccttcg acgagtcgag ttgtttggga atgcagctct aaatgggtgg
781 taaatttcat ctaaagctaa atactggccg gagaccgata gcgcacaagt agagtgatcg
841 aaagatgaaa agcactttga aaagagagtt aaacagcacg tgaaattgtt gaaaggggaag
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   1081 acggacgctg gcataatggt cgtaaacgac c
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***Aspergillus amstelodami* isolate PT5 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence**

GenBank: MH748156.1

FASTA Graphics PopSet

Go to:

LOCUS MH748156 544 bp DNA linear

PLN 20-AUG-2018

DEFINITION *Aspergillus amstelodami* isolate PT5 small subunit ribosomal RNA

gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence.

ACCESSION MH748156

VERSION MH748156.1

KEYWORDS .

SOURCE *Aspergillus amstelodami*

ORGANISM *Aspergillus amstelodami*

Eukaryota; Fungi; Dikarya; Ascomycota;

Pezizomycotina;

Eurotiomycetes; Eurotiomycetidae; Eurotiales;

Aspergillaceae;

Aspergillus.

REFERENCE 1 (bases 1 to 544)

AUTHORS Subhadarsini,S., Kamalraj,S. and Jayabaskaran,C.

```

TITLE      Direct Submission
JOURNAL    Submitted (11-AUG-2018) Department of
Biochemistry, Indian
           institute of science, CV Raman Road, Bangalore,
Karnataka 560012,
           India
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sequencing
           ##Assembly-Data-END##
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RNA, internal
                           transcribed spacer 1, 5.8S ribosomal RNA,
internal
                           transcribed spacer 2, and large subunit
ribosomal RNA"
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     301 cctgtccgag cgtcattgct gccctcaagc acggcttggt tgttgggctt ccgtccctgg
     361 caacggggac gggcccaaaa ggcagtggcg gcacatgtc tggctctcga gcgatgggg
     421 ctttgtcacc cgctccgta ggtccagctg gcagctagcc tcgcaaccaa tctttttaac
     481 caggttgacc tcggatcagg tagggatacc cgctgaactt aagcatatca ataagacgga
     541 ggaa
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***Aspergillus amstelodami* strain BT11 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence**
GenBank: MH137918.1

FASTA Graphics PopSet

Go to:

LOCUS MH137918 529 bp DNA linear
PLN 06-OCT-2018

DEFINITION *Aspergillus amstelodami* strain BT11 internal
transcribed spacer 1,
 partial sequence; 5.8S ribosomal RNA gene and
internal transcribed
 spacer 2, complete sequence; and large subunit
ribosomal RNA gene,
 partial sequence.

ACCESSION MH137918

VERSION MH137918.1

KEYWORDS .

SOURCE *Aspergillus amstelodami*

ORGANISM *Aspergillus amstelodami*

Eukaryota; Fungi; Dikarya; Ascomycota;

Pezizomycotina;

Eurotiomycetes; Eurotiomycetidae; Eurotiales;

Aspergillaceae;

Aspergillus.

REFERENCE 1 (bases 1 to 529)

AUTHORS Midilli,B. and Abaci Gunyar,O.

TITLE Researching of gene region responsible for
ribotoxin production in
 Aspergillus species isolated from animal feed in
the market

JOURNAL Unpublished

REFERENCE 2 (bases 1 to 529)

AUTHORS Midilli,B. and Abaci Gunyar,O.

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TITLE      Direct Submission
JOURNAL    Submitted (28-MAR-2018) Biology, Ege University,
Genclik Caddesi,
            Izmir 35040, Turkey
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sequencing
            ##Assembly-Data-END##
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                               /country="Turkey"
                               /collected_by="Bahar Midilli"
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spacer 1, 5.8S              ribosomal RNA, internal transcribed
spacer 2, and large         subunit ribosomal RNA"
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            421  ttgtcaccgg ctcccgtagg tccagctggc agctagcctc gcaaccaatc tttttaacca
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***Aspergillus amstelodami* isolate GCO10 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence**
GenBank: MH748161.1

FASTA Graphics PopSet

Go to:

LOCUS MH748161 558 bp DNA linear
PLN 20-AUG-2018

DEFINITION *Aspergillus amstelodami* isolate GCO10 small
subunit ribosomal RNA
 gene, partial sequence; internal transcribed
spacer 1, 5.8S
 ribosomal RNA gene, and internal transcribed
spacer 2, complete
 sequence; and large subunit ribosomal RNA gene,
partial sequence.

ACCESSION MH748161

VERSION MH748161.1

KEYWORDS .

SOURCE *Aspergillus amstelodami*

ORGANISM *Aspergillus amstelodami*

Eukaryota; Fungi; Dikarya; Ascomycota;

Pezizomycotina;

Eurotiomycetes; Eurotiomycetidae; Eurotiales;

Aspergillaceae;

Aspergillus.

REFERENCE 1 (bases 1 to 558)

AUTHORS Subhadarsini,S., Kamalraj,S. and Jayabaskaran,C.

TITLE Direct Submission

JOURNAL Submitted (11-AUG-2018) Department of
Biochemistry, Indian

 institute of science, CV Raman Road, Bangalore,
Karnataka 560012,
India

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Sequencing Technology :: Sanger dideoxy
sequencing
##Assembly-Data-END##
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                        /country="India: Rameshwaram"
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541 tatcaataag cggaggaa
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***Aspergillus amstelodami* strain SW173 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence**

GenBank: KY260676.1

FASTA Graphics PopSet

Go to:

LOCUS KY260676 552 bp DNA linear

PLN 24-OCT-2017

DEFINITION *Aspergillus amstelodami* strain SW173 18S

ribosomal RNA gene,

partial sequence; internal transcribed spacer 1,
5.8S ribosomal RNA

gene, and internal transcribed spacer 2, complete
sequence; and 28S

ribosomal RNA gene, partial sequence.

ACCESSION KY260676

VERSION KY260676.1

KEYWORDS .

SOURCE *Aspergillus amstelodami*

ORGANISM *Aspergillus amstelodami*

Eukaryota; Fungi; Dikarya; Ascomycota;

Pezizomycotina;

Eurotiomycetes; Eurotiomycetidae; Eurotiales;

Aspergillaceae;

Aspergillus.

REFERENCE 1 (bases 1 to 552)

AUTHORS Kumari,R. and Ghosh,A.K.

TITLE Fungal diversity analysis of stored wheat grains

JOURNAL Unpublished

REFERENCE 2 (bases 1 to 552)

AUTHORS Kumari,R. and Ghosh,A.K.

TITLE Direct Submission

JOURNAL Submitted (28-NOV-2016) Department of
Biotechnology, Indian

```

Institute of Technology, Kharagpur, Medinipur,
West Bengal 721302,
India
COMMENT ##Assembly-Data-START##
Assembly Method      :: Sequencher v. 5.3
Sequencing Technology :: Sanger dideoxy
sequencing
##Assembly-Data-END##
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                        /country="India"
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rev_name: ITS4"
                        misc_RNA    <1..>552
                        /note="contains 18S ribosomal RNA,
internal transcribed spacer 1, 5.8S ribosomal RNA, internal
transcribed spacer 2, and 28S ribosomal RNA"
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   481  tctttttaac caggttgacc tcggatcagg tagggatacc cgctgaactt aagcatatca
   541  ataagcgag ga
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***Aspergillus amstelodami* isolate BAB-6506 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence**

GenBank: MF319924.1

FASTA Graphics

Go to:

LOCUS MF319924 554 bp DNA linear PLN 29-JUL-2017

DEFINITION *Aspergillus amstelodami* isolate BAB-6506 small subunit ribosomal

RNA gene, partial sequence; internal transcribed spacer 1, 5.8S

ribosomal RNA gene, and internal transcribed spacer 2, complete

sequence; and large subunit ribosomal RNA gene, partial sequence.

ACCESSION MF319924

VERSION MF319924.1

KEYWORDS.

SOURCE *Aspergillus amstelodami*

ORGANISM *Aspergillus amstelodami*

Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina; Eurotiomycetes; Eurotiomycetidae; Eurotiales; Aspergillaceae; *Aspergillus*.

REFERENCE 1 (bases 1 to 554)

AUTHORS Verma,R., Fulekar,M.H., Dantroliya,S., Bhargava,P., Soni,S. and Bagatharia,S.B.

TITLE ITS1 and ITS2 ribosomal RNA full gene profiling of isolates from

Microbial Repository of Biodiversity Gene Bank of Gujarat State

Biotechnology Mission

JOURNAL Unpublished

REFERENCE 2 (bases 1 to 554)

AUTHORS Verma,R., Fulekar,M.H., Dantrolia,S.,
 Bhargava,P., Soni,S. and
 Bagatharia,S.B.
 TITLE Direct Submission
 JOURNAL Submitted (13-JUN-2017) Gujarat Biodiversity Gene
 Bank, Gujarat
 State Biotechnology Mission, 9th floor, 11th
 Block, Udyogbhavan,
 Gandhinagar, Gujarat 382010, India
 COMMENT ##Assembly-Data-START##
 Sequencing Technology :: Sanger dideoxy
 sequencing
 ##Assembly-Data-END##
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 /country="India"
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 RNA, internal
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 internal
 transcribed spacer 2, and large subunit
 ribosomal RNA"
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 61 tccgtgtcta tctgtaccct gttgcttcgg cgtggccacg gcccgccgga gactaacatt
 121 tgaacgctgt ctgaagtttg cagtctgagt ttttagttaa acaatcggtt aaactttcaa
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 301 ggggggcatg cctgtccgag cgtcattgct gccctcaagc acggcttggtg tgttggtgctt
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***Aspergillus amstelodami* strain KDH15 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence**

GenBank: KU569228.1

FASTA Graphics

Go to:

LOCUS KU569228 535 bp DNA linear

PLN 08-AUG-2016

DEFINITION *Aspergillus amstelodami* strain KDH15 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence.

ACCESSION KU569228

VERSION KU569228.1

KEYWORDS .

SOURCE *Aspergillus amstelodami*

ORGANISM *Aspergillus amstelodami*

Eukaryota; Fungi; Dikarya; Ascomycota;

Pezizomycotina;

Eurotiomycetes; Eurotiomycetidae; Eurotiales;

Aspergillaceae;

Aspergillus.

REFERENCE 1 (bases 1 to 535)

AUTHORS Nur Amalina,K. and Latiffah,Z.

TITLE Characterization and mycotoxin production of *Aspergillus* spp from groundnuts (*Arachis hypogaea*)

JOURNAL Unpublished

REFERENCE 2 (bases 1 to 535)

```

AUTHORS    Nur Amalina,K. and Latiffah,Z.
TITLE      Direct Submission
JOURNAL    Submitted (21-JAN-2016) School of Biological
Sciences, Universiti
            Sains Malaysia, Minden, Pulau Pinang 11800,
Malaysia
COMMENT    ##Assembly-Data-START##
            Assembly Method          :: MEGA v. 5.2
            Sequencing Technology    :: Sanger dideoxy
sequencing
            ##Assembly-Data-END##
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spacer 2, and 28S
                                     ribosomal RNA"
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121 tgtctgaagt ttgcagtctg agtttttagt taaacaatcg taaaactttt caacaacgga
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421 gggctttgtc acccgctccc gtaggtccag ctggcagcta gcctcgcaac caatcttttt
481 aaccaggttg acctcggatc aggtagggat acccgctgaa cttaagcata tcaat
//=====

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***Aspergillus amstelodami* strain KL21 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence**

GenBank: KU872160.1

FASTA Graphics

Go to:

LOCUS KU872160 534 bp DNA linear PLN 30-MAY-2016

DEFINITION *Aspergillus amstelodami* strain KL21 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S

ribosomal RNA gene, partial sequence.

ACCESSION KU872160

VERSION KU872160.1

KEYWORDS .

SOURCE *Aspergillus amstelodami*

ORGANISM *Aspergillus amstelodami*

Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina; Eurotiomycetes; Eurotiomycetidae; Eurotiales;

Aspergillaceae; *Aspergillus*.

REFERENCE 1 (bases 1 to 534)

AUTHORS Nur Amalina,K. and Latiffah,Z.

TITLE *Aspergillus amstelodami* isolated from groundnuts (Arachis hypogaea)

JOURNAL Unpublished

REFERENCE 2 (bases 1 to 534)

AUTHORS Nur Amalina,K. and Latiffah,Z.

TITLE Direct Submission

JOURNAL Submitted (07-MAR-2016) School of Biological Sciences, Universiti

Sains Malaysia, Minden, Pulau Pinang 11800, Malaysia

COMMENT ##Assembly-Data-START##

Assembly Method :: MEGA v. 5

Sequencing Technology :: Sanger dideoxy sequencing

##Assembly-Data-END##

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transcribed	spacer 1, 5.8S ribosomal RNA, internal
transcribed spacer	2, and 28S ribosomal RNA"

ORIGIN

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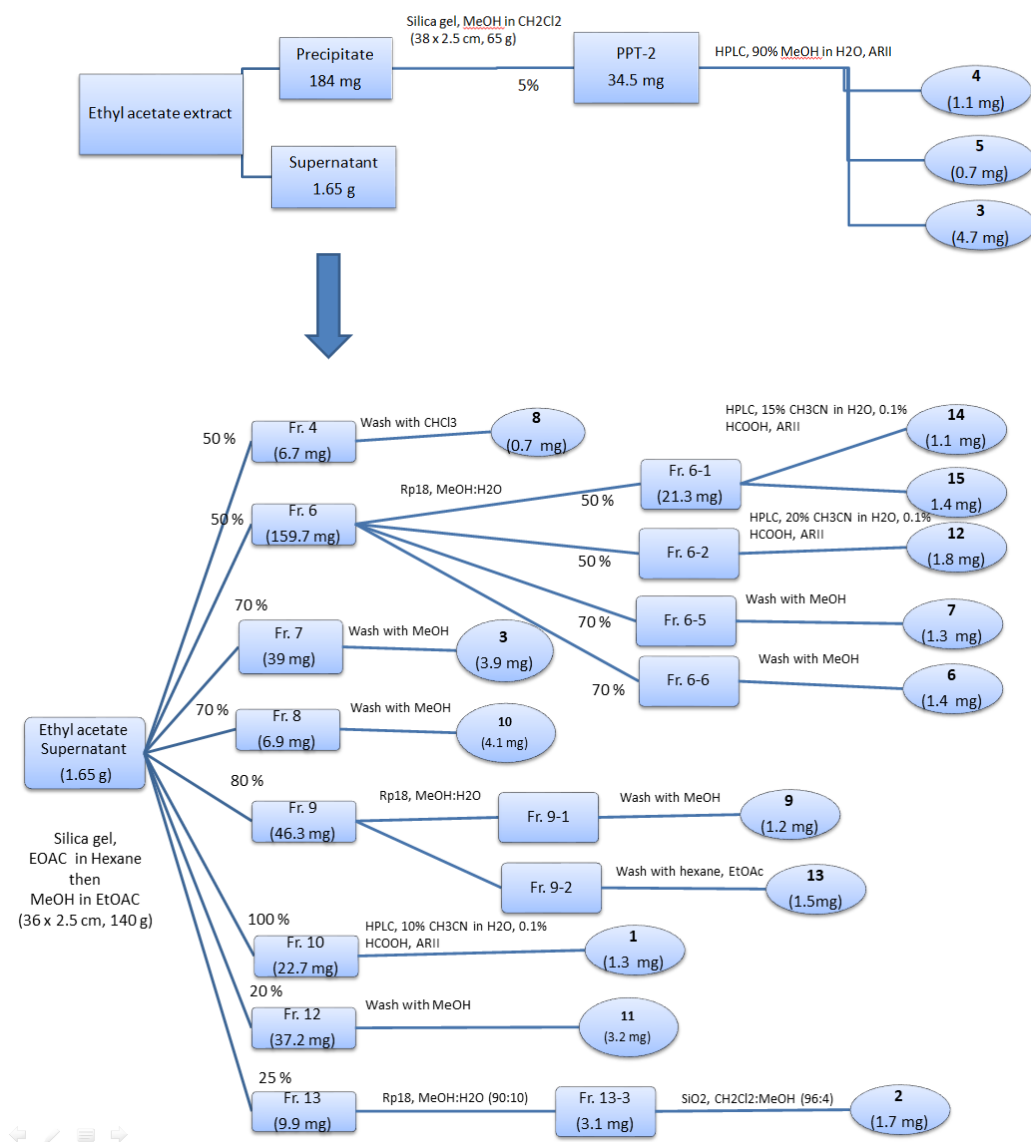


Figure S19: Detailed isolation procedure for the fungal metabolites.

References:

1. Gumnicka O, Oleszek W. *Acta societatis botanicorum Poloniae* 1998; 67: 65-68.
2. Bandara BR, Jayasinghe L, Karunaratne V, Wannigama GP, Kraus W, Bokel M, Sotheeswaran S. *Phytochemistry* 1989; 28: 2783-2785.
3. Pitt JI, Hocking AD (2009) *Fungi and food spoilage*, Vol. 519, Springer.
4. White T, Bruns T, Lee S, Taylor J. *PCR protocols: a guide to methods and applications* 1990: 315-322.