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Isolation, Characterization and Anti-Diabetic Potentials of Oleanolic Acid from the Leaves of *Aspilia africana* (Pers) C.D Adams (Asteraceae)

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ABSTRACT

To isolate the anti-diabetic principle of the leaves of *Aspilia africana* through bioassay guided phytochemical investigation. The methanol extract previously screened for anti-diabetic activity was partitioned successively with n-Hexane, CH_2Cl_2, EtOAc and n-BuOH. The butanol fraction, the only fraction that responded positively to *in-vitro* anti-diabetic tests (like the crude methanol extract) was treated with diethyl ether to precipitate the saponins. The crude saponin was hydrolyzed with 4M HCl and the organic part subjected to silica gel chromatography which on further purification through gel filtration and prep TLC afforded a compound which was identified using melting point determination, extensive nuclear magnetic resonance spectroscopy (1D, 2D, COSY, HMBC, HQMC), Fourier Transform-Infra-red (FT-IR) spectroscopy and basic chemical reactions. The isolated compound was identified as 2-hydroxyolean-12-en-28-oic acid or oleanolic acid. The in vitro anti-diabetic assays showed inhibition of α-amylase and α-glucosidase with the following IC_{50}: in α-amylase assay: crude methanol 96.40±0.2 mg/mL, butanol fraction 72.50±0.65 mg/mL, isolated compound 25.45±0.45 µg/mL, compared to the reference drug (acarbose) 46.31±0.58 µg/mL; in the α-glucosidase assay crude methanol 95.10±0.20 mg/mL, butanol fraction 72.65±3.20 mg/mL, isolated compound 47.57±0.40 µg/mL, compared to the reference drug (acarbose), 48.10±0.13 µg/mL. Based on these findings the compound responsible for the anti-diabetic activity of *Aspilia africana* is 2-hydroxyolean-12-en-28-oic acid.

1. Introduction

Aspilia africana (Pers) C.D. Adams (Asteraceae) is an herb which can grow up to 2 m high and occurs throughout the savannah and forested zones of tropical Africa. It is found in all parts of Nigeria [1]. *Aspilia africana* is commonly known as wild sunflower or haemorrhage plant due to its ability to stop bleeding even from severed artery [2].

The previous phytochemical studies of the plant [3-5], (which were confirmed in this study) show that it contains saponins, tannins, terpenes and flavonoids. The detection of a number of terpenoids like cubebene, copaene, caryophyllene, germacrene B, patchoulene, elemene, cedrene, isocaryophyllene, guaiene and germacrene D from the leaves of *A. africana* has been reported [6]. Page et al. [3] has reported the isolation of diterpenes - kaurenoic and grandiflorenic acids from the leaves of the plant. Triterpeoids such as 3β-acetoxyolean-12-ene and 3β-O-(α-rhamnopyransyl)-(1→6)glucopyranosyl(1→3)ursan-12-ene have also been isolated from the leaves of the plant [7]. Biological studies of the extract of the leaves have highlighted anti-diabetic, hypolipidemic, antimicrobial, anti-coagulant, antiulcer and anti-plasmodial activities [5, 8 - 10]. Antibacterial activity of root extracts has also been reported [11]. The plant *A. africana* naturally grows luxuriantly without any pastoral care in all parts of Nigeria almost all-year-round and can be cheaply harnessed as a rich source of supply of drug materials to tackle the emerging problems of terminal ailments like diabetes (especially the Type 2) which has become a worrisome disease condition affecting over 422 million people worldwide [12]. Diabetes Mellitus is a metabolic disorder characterized by hyperglycemia caused by deficit or malfunction in insulin secretion and/or insulin action both of which cause the impaired metabolism of glucose, lipids and proteins [13].

Diabetes prevalence has been increased by the aging of population, socioeconomic disadvantages and lifestyles that trend toward physical inactivity and overweight/obesity [14]. Although many drugs with different modes of action are available, natural anti-diabetic agents with insulin-sensitizing effects and preventive actions are highly desirable [12]. It is on this basis that this study was carried out to isolate and characterize a compound with anti-diabetic potential from the leaf of *A. africana.* No previous study has linked any of the numerously reported biological activities to the isolated compounds from this plant. This is the first report of isolation of 3-hydroxyolean-12-en-28-oic acid (oleanolic acid) from *A. africana* as far our search can reach.

2. Experimental Methods

2.1 Reagents and Analytical Equipment

Melting point of the isolate was determined using Electrothermal melting point apparatus (IA9100, USA). UV was run on Spectro UV-Vis 2700 Dual beam (200 – 1100 nm) Labomed, Inc. USA. The silica gel for column chromatography, sephadex LH-20 and the analytical grade reagents used were obtained from Sigma Aldrich. Alpha amylase (from *Aspergillus oryzae*; CAS No 9001-19-8, Enz No 3-2-1.1) and alpha glucosidase (from Baker's yeast; CAS No 9001-42-47), 4-Nitrophenyl-α-D-glucopyranoside (CAS No 3767-28-0) were obtained from Sigma-Aldrich (USA). The preparative thin layer chromatography (TLC) plates were obtained from Merck, Germany. The Proton NMR (^{1}H NMR) and Carbon-13 NMR (^{13}C NMR) spectra were run on a JEOL AS 400 (400 MHz) spectrometer (Japan) using DMSO-d_6 and $CDCl_3$ as solvents and TMS as internal standard. Solvent peaks were at 7.26 ppm for $CDCl_3$ and 2.50 ppm for DMSO-d_6. The chemical shifts were reported as δ ppm relative to TMS. The samples were all run at temperature of 25 °C. FTIR (KBr) was recorded using 8400 S Shimadzu, Japan.

2.2 Collection and Identification of Plant Materials

The fresh leaf plant material was collected at Wusasa in Zaria L.G.A. of Kaduna State, Nigeria. The plant was identified and authenticated by

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Herbarium unit of the department of Biological Science, Faculty of Science, Ahmadu Bello University, Zaria, as *Aspilia africana* (pers.) C.D. Adams (Asteraceae). A specimen with voucher No. 1146 was deposited at the herbarium. The plant material was dried under the shed for several weeks then ground, sieved into fine powder and extracted with H_2O-MEOH (30:70).

2.3 Extraction and Purification Procedures

The powdered leaf of *Aspilia africana* (1 kg) was cold-macerated in H_2O-MEOH (30:70) for 72 h and then filtered. The filtrate was concentrated in vacuo to give the extract (150.8 g). The extract was partitioned successively with n-Hexane, CH_2Cl_2, EtOAc and n-BuOH. The fractions were concentrated to dryness in vacuo. The extracts and fractions were subjected to phytochemical screening using standard protocols. Crude saponin was precipitated from the n-BuOH fraction (6 g) by means of diethyl ether. The crude saponin was subjected to acid hydrolysis with 4M HCl. The resulting mixture was extracted with $CHCl_3$ to obtain the organic portion. The dried $CHCl_3$ extract was subjected to column chromatography (silica gel, $CHCl_3$: MEOH in order of increasing polarity). 100 eluates of 10 mL aliquot each were collected. Eluates with same R_f on TLC were pooled together to afford 12 fractions (HYD1 -12). Fraction HYD4 (eluates 17-26) on gel filtration with sephadex LH_{-20}, eluted with $CHCl_3$ and methanol afforded compound 1, when re-crystallized in methanol. The aqueous part was analyzed for the presence of sugars through TLC comparison with standard sugars [15] and chemical tests (Fehling's and Benedict's) for simple for sugars.

2.4 Determination of Anti-diabetic Activities

2.4.1 Determination of In-vitro α-Amylase Inhibition

This experimental procedure was carried out as reported by Sindhu et al. [16]. The assay mixture comprising of 200 µL of 0.02 M sodium phosphate buffer, 20 µL of enzyme and the crude methanol extract, prepared in the concentrations of 20, 40, 60, 80 and 100 µg/mL was incubated for 10 minutes at room temperature followed by addition of 200 µL of 1% starch (1.0 g of starch in 100 mL of sodium phosphate buffer (20 mM), pH, 6.8) in all the test tubes. The reaction was terminated with addition of 400 µL of 3, 5-dinitrosalicylic acid and placed in boiling water for 5 minutes, cooled and diluted with 15 mL of distilled water. The absorbance was measured at 540 nm. This procedure was repeated for the butanol fraction, the isolated compound and the reference drug.

2.4.2 Determination of In-vitro α-Glucosidase Inhibition

The assay was carried out using the method of Sindhu et al. [16] and Adefegha and Oboh [17]. The α-glucosidase was dissolved in 100 mM phosphate buffer (pH 6.8) and used as the enzyme extract. p-Nitrophenyl-α-D-glucopyranoside was used as the enzyme substrate. Different concentrations of test samples (as in 2.4.1 above) were mixed with 320 µL of 100 mM phosphate buffer pH 6.8 at 30 °C for 5 minutes. 3 mL of 50 mM sodium hydroxide was added to the mixture and the absorbance was read at 410 nm. The control samples were prepared without the plant materials. The % inhibition was calculated using the formula [18].

$$\% \text{ Inhibition} = \frac{\text{Abs}_{(w/l)\text{Control}} - \text{Abs}_{(w/l)\text{plant extract}} \times 100}{\text{Abs}_{(w/l)\text{Control}}}$$

The concentration of the compound required to inhibit 50% of the enzymes activities was defined by the IC_{50} value determined from the plot of percentage inhibition versus log of inhibitor concentration and were calculated by non-linear regression analysis from the mean of inhibitory values. Acarbose was used as the reference α-amylase and α-glucosidase inhibitor. All the tests were performed in triplicate.

3. Results and Discussion

The result of the phytochemical screening showed that the leaf methanol extract of the plant contained much of saponins, tannins, terpenes and poly phenols than alkaloids and flavonoids and the same result was replicated in butanol fraction. Compound 1 was obtained as a white amorphous solid (225 mg), m. p. 299-302 °C (Litt 301- 302 °C; [19]), mixed melting point 299-302 °C. TLC R_f value was 0.75 ($CHCl_3$–MeOH, 9:1). It was soluble in n-hexane, chloroform and methanol, and insoluble in water. It responded positively to the Liebermann-Burchard's test for pentacyclic triterpene and produced CO_2 with $NaHCO_3$. The ^{13}C-NMR and ^{1}H-NMR results are shown in Table 3. FTIR (V_{max}KBr): showed absorption at 3436 cm^{-1}, 2931 cm^{-1}, 2404 cm^{-1}, 1728 cm^{-1}, 1389 cm^{-1}, 1152 cm^{-1}, 103 cm^{-1}, 1016 cm^{-1}, 769 cm^{-1}, 695 cm^{-1} (Fig. 3).The analysis of the aqueous hydroxylate indicated that glucose was the major constituent.

3.1 Result of Anti-Diabetic Test

The results of the *in-vitro* anti-diabetic tests are shown on Tables 1 and 2. In α-amylase inhibition assay: the IC_{50} for crude methanol was 96.40±0.2 µg/mL, butanol fraction 72.50±0.65 µg/mL, isolated compound 25.45±0.45 µg/mL, compared to the reference drug (acarbose) 46.31±0.58 µg/mL; in the alpha glucosidase inhibition assay crude methanol IC_{50} was 95.10±0.20 µg/mL, butanol fraction 72.65±0.32 µg/mL, isolated compound 47.57±0.40 µg/mL, compared to the reference drug (acarbose), 48.10±0.13 µg/mL. The result showed that the compound inhibited the actions of alpha amylase and alpha glucosidase.

Table 1 % Inhibition of α-Amylase by products from *A. africana* and Acabose

Conc.	MeOH extract (%)	MeOH extract IC50	BuOH extract (%)	BuOH FR IC50	Compd 1 (%)	Compd 1 IC50	Acarb (%)	Acarb IC50
20	30	96.40±	30	72.50±	47	25.45±0.	32	46.31
40	38	0.20*	35	0.65*	58	45*	44	±0.58
60	40		43		72		63	*
80	44		47		73		69	
100	52		52		85		74	

*Conc. in mg/mL for MeOH and BuOH; µg/mL for ECJ17-26HYD and Acarbose; Data are given as mean ±SD (n=3; * p<0.05)*

Table 2 % Inhibition of α-Glucosidase by products from *A. africana* and Acabose

Conc.	MeOH extract (%)	MeOH extract IC50	BuOH extract (%)	BuOH FR IC50	Compd 1 (%)	Compd 1 IC50	Acarb (%)	Acarb IC50
20	33	95.10±	30	72.65±	34	47.57±0.	33	48.10
40	36	0.20*	42	3.20*	45	40*	44	±0.13
60	43		46		63		60	*
80	45		52		66		67	
100	49		56		74		71	

*Conc. in mg/mL for MeOH and BuOH; µg/mL for ECJ17-26HYD and Acarbose; Data are given as mean ±SD (n=3; * p<0.05)*

Table 3 ^{1}H and ^{13}C NMR chemical shifts of compound ECJ17-26HYD compared with ref compound

Position of Carbon	Compd 1 δC (ppm)	DEPT	Compd 1 δH ppm	Coupling constant (Hz)	HMBC correlations	* Ref. compd δC (ppm)
1	39.35	CH_2	1.53		25	39.0
2	27.89	CH_2	1.10			28.1
3	77.78	CH	3.38	dd, 3.5, 13.5	23, 24	78.2
4	38.51	C	-			39.4
5	55.39	CH	0.77		3, 5, 23, 24	55.9
6	18.33	CH_2	1.33			18.8
7	32.55	CH_2	1.57			33.4
8	38.69	C	-			39.8
9	47.71	CH	1.61			48.2
10	37.01	C	-			37.4
11	22.95	CH_2	1.86			23.8
12	122.24	CH	5.39	brs		122
13	144.12	C	-			144
14	41.70	C	-			42.2
15	27.29	CH_2	1.38			28.4
16	23.07	CH_2	1.54			23.8
17	46.05	C	-			46.7
18	41.43	CH	2.27	q, 14		42.1
19	45.99	CH_2	1.14		20, 21, 30	46.6
20	32.55	C	-		19, 29, 30	31.0
21	33.64	CH_2	1.21			34.3
22	25.45	CH_2	1.30			33.2
23	30.51	CH_3	0.99	s	3, 4, 5, 24	28.8
24	16.80	CH_3	0.99	s		16.5
25	15.50	CH_3	0.76	s	1, 5, 9, 10	15.6
26	14.98	CH_3	0.79	s	7, 8, 9, 14	17.5
27	27.63	CH_3	1.13	s		26.2
28	178.03	C	-			180
29	32.84	CH_3	0.91	s	19, 20, 21, 30	33.4
30	23.32	CH_3	0.89	s	19, 20, 21, 29	23.8

*400MHz ^{1}H, 100MHz ^{13}C $CDCl_3$; *Ref Compound [20]*

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3.2 Structural Elucidation of Compound 1

The ^{13}C-NMR and ^{1}H-NMR spectroscopic data in combination with analysis of DEPT and HMQC (Fig. 1b) showed the presence of thirty carbon atoms consisting of 8 quaternary, 5 tertiary, 10 secondary carbons and seven methyl groups. ^{1}H-NMR spectrum (Fig. 2) showed a downfield broad singlet signal at δ5.39 assigned to the olefinic proton on C-12. 1H-NMR spectral data also revealed seven methyl singlets at δ= 0.76, 0.79, 0.89, 0.91, 0.99, 0.99, and 1.13. The ^{13}C NMR chemical shifts at δ178.03, 144.12 and 122.24 were typical characteristics peaks of oleanane type of triterpene skeleton assigned to C-28, C-13 and C-12 respectively. The de-shielding chemical shift at δ77.78 was assigned to C-3. In the HMBC experiment the following correlations were observed: Me-25→C-1 → C-5 → C-9 → C-10; Me-26 →C-7→ C-8→ C-9→ C-14; Me-23→C-3→ C-4→ C-5→ C-24; Me-29→C-19→ C-20→ C-21→ C-30; Me-30→C-19 → C-20→ C-21→ C-29. The 1H-NMR signals at δ 0.99 (s, 3H, Me-23), δ 0.99 (s, 3H, Me-24), 0.76 (s, 3H, Me-25), δ 0.79(s, 3H, Me-26), δ1.13 (s, 3H, Me-27), δ 0.91 (s, 3H, Me-29), δ 0.89 (s, 3H, Me-30), and a broad signal at δ 5.39 (H-12) together with the 13C-NMR suggests the presence of olean-12-ene skeleton [21]. The HQMC has also helped in assigning the proton resonances to the respective carbon atoms. The signals at δC 77.78 ppm (C-3) corresponds to δH 3.38 (H-3); δC 55.39 ppm (C-5)/ δH 0.77 ppm (H-5); δC18.33 ppm (C-6)/ δH 1.33 ppm (H-6); δC 47.7 ppm (C-9)/ δH 1.41 ppm; (H-9); δC 122.20 ppm (C-12)/ δH 5.39 ppm (H-12); δC 41.7 ppm (C-18)/ δH 2.27 ppm (H-18); δC 15.50 ppm (C-25)/ δH 0.76 ppm (3H, H-25) ppm.

The COSY experiment (Fig. 1b) has shown the following couplings: δH1.53 ppm (H-1)/ δH 3.38 (H-3); δH 2.27 ppm (H-18)/ δH 1.14 ppm (H-19); δH 0.99 (H-23)/ δH 0.99 (H-24); δH 0.79 ppm (H-26)/ δH 1.13 ppm (H-27). A triplet observed at 2.27 ppm with a J value of 14.0 Hz indicated coupling between a single proton at C-18 and two protons at C-19. This triplet only appears if the compound is of the β-type triterpene (oleanane) (where only two protons are attached to C-19) [22].

The FTIR spectrum (Fig. 3) confirmed the presence of hydroxyl group (3436 cm^{-1}), C-H str (2931cm^{-1}), carbonyl group (1728 cm^{-1}), olefinic group, C=C (695, 769 cm^{-1}) and C-O (acid) functional group (1152, 1039 and 1016 cm^{-1}). The FTIR spectrum also showed absorption peaks at 1458 cm^{-1} (CH_2 and CH_3), 1389 cm^{-1} (gem-dimethyl – at C-4), 1218 cm^{-1} (two methyls on qua-ternary carbon atom- indicating the 2 methyl groups on C-20 which distinguishes it from the isomer (ursolic acid), which has one methyl group at this position [23].

On the basis of above spectral and chemical evidences compound 1 was identified as 3β-hydroxyolean-12-en-28-oic acid also known as oleanolic acid (Fig. 1a).The identity of the compound was finally determined by CO-TLC and MMP with an authentic sample and by comparison of ^{13}C-chemical shifts with the reported data [20].

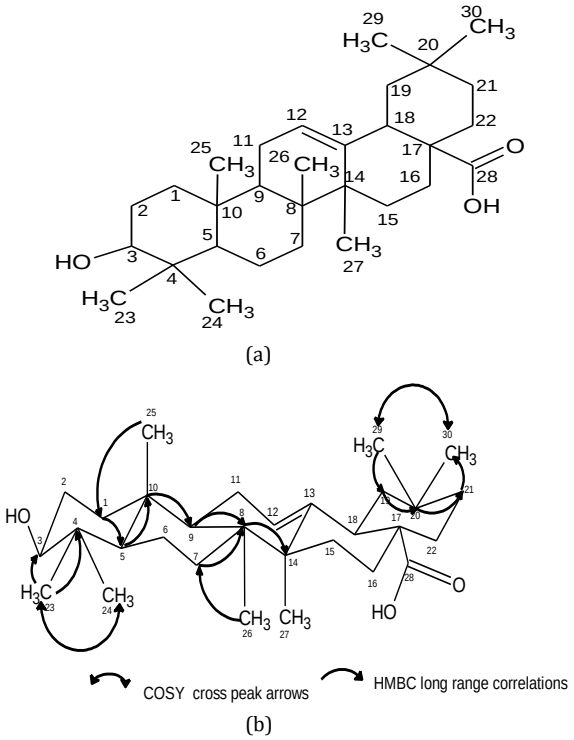


Fig. 1 (a) Structure of compound 1 (b) 2D NMR Correlations of compound 1

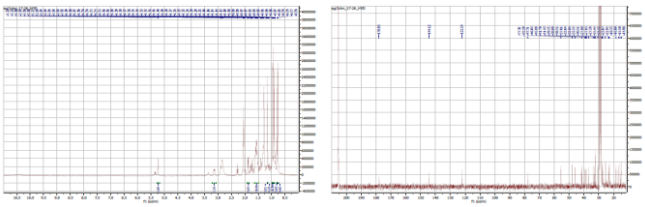
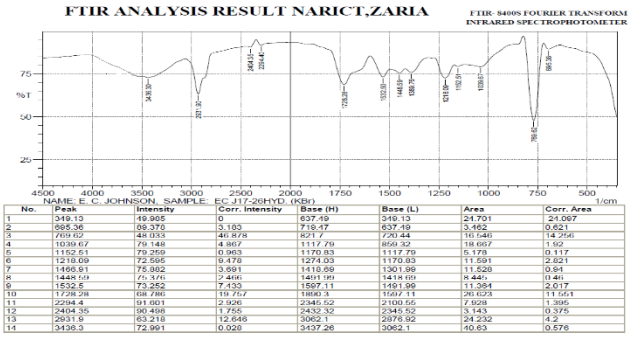


Fig. 2 NMR spectra of the isolated compound (a) ^{1}H NMR, (b) ^{13}C NMR



No.	Peak	Intensity	Corr. Intensity	Base (H)	Base (L)	Area	Corr. Area
1	349.13	49.905	0	657.49	349.13	24.701	24.067
2	695.36	89.376	3.183	718.47	637.49	3.462	0.621
3	769.12	48.033	46.878	821.7	720.44	16.546	14.298
4	1039.67	79.148	4.867	1117.79	859.32	18.667	1.59
5	1152.51	79.259	0.963	1170.83	1117.79	5.178	0.117
6	1218.09	72.595	9.478	1274.03	1170.83	11.591	2.821
7	1466.91	75.862	3.891	1518.69	1301.99	11.528	0.94
8	1448.69	75.376	2.498	1491.99	1414.69	6.447	0.46
9	1532.5	73.252	7.433	1597.11	1491.99	11.384	2.017
10	1728.28	68.786	19.767	1868.3	1697.11	26.628	11.551
11	2294.4	91.601	2.926	2345.52	2100.55	7.928	1.395
12	2404.35	90.498	1.755	2432.32	2345.52	3.143	0.375
13	2931.5	63.218	12.646	3062.1	2876.92	24.232	4.2
14	3436.3	72.991	0.928	3437.26	3062.1	40.83	0.578

Fig. 3 FTIR Spectrum of the isolated compound

3.3 Anti-Diabetic Activity

The bioassay guided phytochemical investigation of *Aspilia africana* has led to the isolation of an anti-diabetic agent that has dose-dependently inhibited the actions of two important enzymes, α-amylase and α-glucosidase implicated in the metabolism of carbohydrate in human body. Alpha amylase catalyses the breaking down of starch to disaccharides and oligosaccharides and the intestinal α-glucosidase catalyses the breakdown of disaccharides to release glucose which is later absorbed from small intestine into the blood circulation [24].This inhibition in digestion and absorption of carbohydrate which in turn decreases rise in postprandial hyperglycemia is a very simple but highly effective therapeutic approach to check the prevalence of hyperglycemia [25].The isolated compound has been identified as oleanolic acid, a very well-known anti-hyperglycemic agent employing this therapeutic approach [12]. Isolation of oleanolic acid from a very common and abundantly available herb like *Aspilia africana* is a great gain to the fight against diabetes mellitus.

4. Conclusion

The results showed that the methanol extract, the butanol fraction and the isolated compound inhibited the actions of alpha amylase and α-glucosidase in a dose dependent and uncompetitive manner. The results also confirmed the anti-diabetic activity of *Aspilia africana* and established the fact that oleanolic acid is probably the bioactive agent responsible for the activity in the extract of the leaves of plant previously reported. It is however suggested that further studies are undertaken to establish the *in-vivo* activity.

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We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. E. Johnson conceived and designed the research work as well carried out phytochemical studies and analyzed the results. M. Ilyas supervised the phytochemical studies and interpreted the spectroscopic data. O. Eseyin, E. Etim, A. Udobre, A. Udoh and E. Edem conducted the anti-diabetic studies.

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