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Anti-Acetylcholinesterase Compounds Isolated from the Leaves of *Kigelia africana* (LAM) Benth (Bignoniaceae)

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Authors' contributions

This work was carried out in collaboration among all authors. Authors JAF, OOC, ACA and MTO designed the stud. Author JAF, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors OOC and SOF wrote the final draft of the manuscript. Author SOF carried out the structural elucidation of the compounds and processed the manuscript to submission. Authors CAE supervised the isolation of the compounds. Author IVO generated the NMR and MS data of the compounds. Authors OOC, ACA, MTO and AAA supervised the research work. All authors read and approved the final manuscript.

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ABSTRACT

Acetylcholinesterase (AChE) is an enzyme that is involved in the breakdown of some neurotransmitters. Its inhibition is one of the treatment strategies employed in the management Alzheimer diseases. Flavonoids isolated from the leaves of *Kigelia africana* were investigated for their comparative AChE inhibition.

The extract of the leaves was subjected to vacuum liquid chromatography (VLC) to obtain four fractions using n-hexane (n-hex, 100%), n-hexane/dichloromethane (hex/DCM, 1:1), dichloromethane/ethyl acetate (DCM/EtOAc, 1:1) and ethyl acetate/methanol (EtOAc/MeOH, 1:1). The four fractions were subjected to AChE inhibitory study with DCM/EtOAc (1:1) fraction showing the highest inhibitory activity. Three flavonoids were isolated from this fraction and their structures were elucidated and characterised using 1D- and 2D-nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) techniques. Their spectroscopic data compared well with literature.

The compounds demonstrated considerable inhibition of AChE activity with luteolin (1), rutin (2) and quercetin (3) that showed IC_{50} of 945.0, 282.1, 254.8 µg/ml respectively as against the IC_{50} of 38.93 µg/ml for rivastigmine, a well-known cholinesterase inhibitor. Compound 3 showed 17.89 ± 0.57 and 7.70 ± 0.64 µ/l/mg protein at 200 and 400 µg/ml respectively, for AChE activity as against 10.37 ± 0.99 and 6.24 ± 1.24 µ/l/mg protein showed by rivastigmine at 200 and 400 µg/ml respectively.

This study showed that the constituents responsible for the AChE inhibition in the crude extract as reported by Falode *et al*., 2017 resided in the DCM/EtOAc (1:1) fraction. The structure-activity relationship of the flavonoids revolves around substitution in position 3 of the compounds.

Keywords: Kigelia africana; flavonoids; structural elucidation; dementia; acetylcholinesterase inhibition.

1. INTRODUCTION

Alzheimer disease is the main cause of dementia accounting for about 75% of all dementia cases. Dementia refers to a very large group of brain diseases that bring about a long term and frequently gradual decline in the ability to think and remember things which in turn affect the individual's daily performance, but oftentimes, consciousness is not affected [1]. Dementia is one of the most common causes of disability among the old [2], which has been estimated to result in economic costs of 604 billion United States dollar (USD) a year (WHO, 2014). People with dementia are often physically or chemically restrained and undemonstrative. Globally, dementia affected about 46 million people in 2015 [3]. About 10% of people develop the disorder at some point in their lives [4]; it becomes more common with age [5]. A dementia diagnosis requires a change from a person's usual mental functioning and a greater decline than one would expect due to ageing [1]. The most common type of dementia is Alzheimer's disease, which makes up 50% to 70% of cases; the most common symptoms of Alzheimer's disease are short-term memory loss and word-finding difficulties. The part of the brain most affected by Alzheimer's is the hippocampus; other parts of the brain that show shrinking (atrophy) include the temporal and parietal lobes [6]. Other frequent types include vascular dementia (25%), Lewy body dementia (15%), and front temporal dementia [1,2]. More than one type of dementia may exist in the same person [1]. Cholinesterase inhibitors such as rivastigmine, donepezil and galantamine are often used and may be beneficial in mild to moderate disorder [7,8,9]; overall benefit, nevertheless, may be minor [9], this necessitated the need for alternative medicines.

Despite the evolvement of various pharmacotherapy used in the management of dementia, galanthamine is the only naturally occurring drug in the treatment of dementia. The use of alternative medicine has been encouraged in the management of disease due to the multi-target potentials they have *Kigelia aficana* is used in Southwestern Nigeria in the management of dementia. It is even one of the ingredients used in the treatment of mentally retarded patients. It is referred to in Yoruba as 'ewe isoye'. *Kigelia africana* (Lam) Benth (family Bignoniaceae) is commonly called sausage tree in English and "Pandoro" in South-Western Nigeria [10]. The *Bignoniaceae* family is noted for the occurrence of iridoids, naphthoquinones, flavonoids, terpenes, tannins, steroids, saponins and caffeic acid in the fruits, stem, leaves and roots [11,12,13,14,15]. Several pharmacological properties have been attributed to the fruits of *K*.

africana but there is a relative paucity of data on the leaves [16]. The aqueous extract of the leaves was reported to possess central nervous system (CNS) stimulatory effect [17]. In addition, the antioxidant, antiulcerogenic, arginase inhibitory, hepatoprotective and antibacterial properties of the aqueous leaf extract have been reported [18,19,20,21]. Previous studies have shown that among compounds isolated from the Bignoniaceae, naphthoquinones in general and furanonaphthoquinones in particular, exhibit a broad spectrum of biological activities [22-25,26]. [27] evaluated and compared the antioxidant activity of the fruit and leaf extracts of the plant. The sausage tree flavonoid extract has been reported to be neuroprotective in $AlCl_3$-induced Alzheimer's disease model [28]. Previous phytochemical studies on *Kigelia africana* extract showed central nervous system stimulant properties [16]. However, there has been no report about neuropharmacological studies on its isolated compounds.

The present study assessed the acetylcholinesterase inhibitory capacity of some flavonoids (quercetin, luteolin and rutin) isolated from the leaf extract of *Kigelia africana*.

2. MATERIALS AND METHODS

2.1 Chemicals

Aluminium chloride ($AlCl_3$), rivastigmine, acetylcholine iodide, 5′, 5′-dithiobis-2-nitrobenzoate (DTNB), thiobarbituric acid (TBA) and sucrose were obtained from Sigma-Aldrich Co. (Munich, Germany). n-Hexane, Dichloromethane (DCM), Ethylacetate (EtOAc), Methanol (MeOH), Vanillin/Sulphuric acid (H_2SO_4) was used as TLC spray reagent. Visualization was done by observing under UV at 254 and 366 nm prior to detection with a chemical spray. Other chemicals and reagents used were of analytical grade and obtained from standard suppliers.

2.2 Materials

Spatula, Masking tape, aluminium foil, sample vials, ruler, pencil, silica gel (100-200 mesh), volumetric flasks of various volumes, TLC Silica gel 60 F_{254} (Aluminium sheet 20 x 20 cm), rotary evaporator (Buchi), Vacuum-Liquid Chromatography (VLC)-Setup, glass column (48x4 cm, 50x2.3 cm).

2.3 Plant Material and Extraction

Leaves of *K. africana* were obtained from Ikere-Ekiti, South-Western Nigeria and authenticated at the Botany Department, University of Ibadan, Ibadan, Nigeria (voucher number KA06). The leaves were cleaned with distilled water, air-dried and powdered. The pulverized sample (1.2 kg) was extracted by maceration in 80% methanol for 72 h and then filtered. The filtrate was concentrated in a rotary evaporator and freeze-dried to obtain the crude extract (55 g, KAE).

2.4 Bioassays on the Fractions Obtained and the Compounds Isolated

Acetylcholinesterase activity was carried out on the four fractions obtained and the isolated compounds according to standard procedure [29]. Precisely 1000 µl of sodium phosphate buffer (100 mM, pH 7.5) containing 10 mM DTNB, 100 µl of the substrate at 25, 50, 100, 200 and 400 µg/ml concentrations and 100 µl of whole-brain homogenate were added in a cuvette and incubated for 2 min at 37ºC. The reaction was then initiated with the addition of 200 µl of acetylthiocholine iodide (8 mM). Hydrolysis of acetylthiocholine iodide was monitored (by the formation of the yellow 5-thio-2-nitrobenzoate anion as a result of the reaction of DTNB with thiocholine catalysed by enzymes) at 412 nm. Absorbance was read at 30 seconds intervals for 5 mins. The absorbance reading was started immediately after adding the substrate. The absorbance usually increases with time.

The inhibition of acetylcholinesterase was calculated with the following formula:

$$\text{Activity (U/l)} = 1.4\ (\text{ml}) \times 10^9 \times \Delta\text{Abs/min} / 100\ (\mu\text{l}) \times \text{Extinction coeefficient } (\varepsilon)$$

Where (ε)= 13.6×10^3 =molar extinction coefficient of DTNB at 412 nm and pH 8.0.

2.5 Isolation and Characterization

The crude extract (50 g) was dissolved in the minimum volume of methanol and adsorbed on silica gel (150 g). The adsorbed silica gel was air-dried and then packed into a sintered glass funnel. This was eluted gradient by using n-Hex., DCM, EtOAc and MeOH. Four fractions eluting with n-hex (600 ml), n-hex: DCM (1:1, 1000 ml), DCM: EtOAc (1:1, 2600 ml), EtOAc: MeOH (1:1, 1800 ml) were collected that yielded 1.12 g, 0.17 g, 6.00 g and 38.25 g of their respective fractions.

Fraction, DCM: EtOAc(1:1) was subjected to column chromatography based on the results of the bio-assay. About 5.7 g of the fraction was

dissolved in 5 ml MeOH and adsorbed on silica gel. The adsorbed silica gel was air-dried and then packed into a column (4:48 cm) that contained plain silica gel in ratio 1:5. Then gradient elution followed from n-hex. through EtOAc to MeOH and 157 (20 ml each) fractions were collected which was bulked into 10 fractions according to their TLC profile: K1 (0.10 g), K2 (0.08 g), K3 (0.93 g), K4 (0.81 g), K5 (0.68 g), K6 (0.32 g), K7 (0.12 g), K8 (0.42 g), K9 (0.23 g) and K10 (0.09 g).

Bulked fraction K5 (0.68 g) was further subjected to repeated column chromatography based on the colour (yellow) and fewer number of spots on the TLC plate. The fraction K5 was dissolved in methanol (2 ml) and the solution was adsorbed on silica gel. The adsorbed silica gel was allowed to air-dry before packing into the column. The column was eluted gradient using n-hex through EtOAc to MeOH mixtures. Eluants were collected in 15 ml test tubes and a total of 88 fractions were collected which were bulked into 6 fractions according to their TLC profile: K5a (10 mg), K5b (200 mg), K5c (100 mg), K5d (300 mg), K5e (5 mg) and K5f (10 mg). K5c (light yellow) was eluted with 40% n-hex. in EtOAc and it gave a single spot on analytical TLC plate. This was labelled compound 3 (100 mg).

K5b (200 mg) gave two spots on TLC which were coded K5b1 and K5b2 from the top of the plate and were separated by PTLC. K5b2 (59 mg) gave a light yellow single spot on TLC which was labelled compound 2 (59 mg) while K5b1 (125 mg), an impure solid, was further purified on column chromatography by dry packing followed with gradient elution of n-hex/EtAOc/MeOH mixture. At 40% n-hex.: EtOAc mixture K5b1 yielded a light yellow single on TLC that was labelled compound 1 (20 mg).

2.5.1 2-(3,4-Dihydroxyphenyl)-5,7-dihydroxychromen-4-one (compound 1)

Yellow powder, ^{1}H and ^{13}C NMR: Table 1. HRMS-ESI (negative mode): m/z $[M-H]^+$ calcd for $C_{15}H_9O_6$, 285.0399; found 285.0393.

2.5.2 2-(3,4-Dihydroxyphenyl)-3-O-glucosyl-6-O-rhamnosyl-5,7-dihydroxychromen-4-one (compound 2)

Yellow powder, ^{1}H and ^{13}C NMR: Table 1. HRMS-ESI (negative mode): m/z $[M-H]^+$ calcd for $C_{27}H_{29}O_{16}$, 609.1456; found 609.1455.

2.5.3 2-(3.4-dihydroxy phenyl)-3,5,7-trihydroxychromen-4-one (compound 3)

Yellow powder, ^{1}H and ^{13}C NMR: Table 1. HRMS-ESI (negative mode): m/z $[M-H]^+$ calcd for $C_{15}H_9O_7$, 301.0348; found 301.0339.

3. RESULTS AND DISCUSSION

3.1 Effect of Fractions on AChE Activity

The effect of n-Hex (100 %), Hex:DCM (1:1), DCM:EtOAc (1:1) and EtOAc:MeOH (1:1) on cerebral activity of AChE is illustrated in Fig. 1. The result showed that fraction DCM:EtOAc (1:1) had the highest inhibitory effect on the activity of acetylcholinesterase. This result also showed that the inhibitory activity on the AChE by the fraction is significantly comparable to the reference sample, rivastigmine and this suggested that the phytochemical(s) responsible

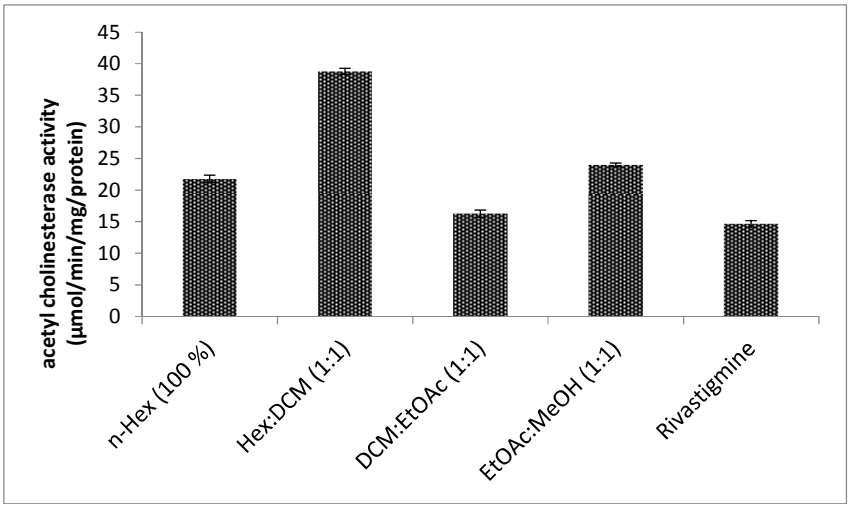


Fig. 1. Effects of various solvent fractions on AChE activity

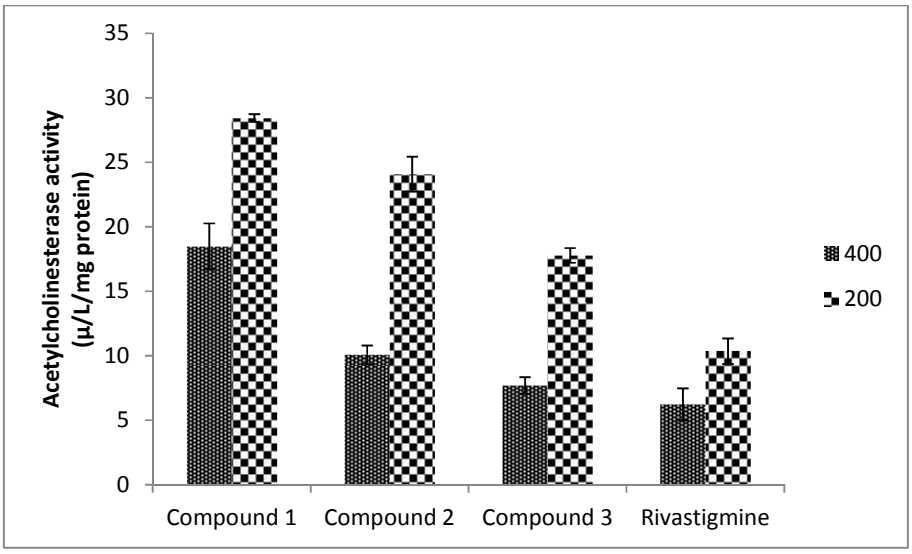


Fig. 2. Effect of isolated compounds on AChE activity

for the inhibitory activities observed in the crude extract as reported by Falode et al., 2017 reside in this fraction.

3.2 Effect of Isolated Compounds on AChE Activity

The effect of the isolated compounds from the DCM:EtOAc (1:1) fraction on the AChE activity were shown in Fig. 2. The result showed dose-dependent activity with compound 3, quercetin, demonstrating highest inhibitory activity in the assay and the inhibitory activity of compound 3 was significantly comparable at 400 µg/ml dose to the reference sample, rivastigmine. This suggested that the compound responsible for the inhibitory activity of AChE was compound 3. The structural-activity relationship of the compounds was noted to revolve around position 3. It was noted that the more the electronegative the atom or group of atoms in position 3 of the compounds are the more the inhibitory activity of the compound.

3.3 Structural Elucidation of the Isolated Compounds

3.3.1 Compound 1

The HRESIMS (negative mode) of the compound showed a pseudo-molecular ion peak at m/z 285.0393 of the molecular ion peak calculated to be 286.0477 which is consistent with a molecular formula $C_{15}H_{10}O_6$. The ^{1}H NMR of the compound showed resonances for aromatic protons at δ 6.20 (d, J = 3 Hz), δ 6.43(d, J = 3 Hz), δ 6.56 (s), δ 6.79 (d, J = 3 Hz), 6.81 (d, J = 3 Hz) and δ 6.93 (dd, J = 3, 8 Hz) each integrated to be one proton suggesting two protons on ring A and three protons on ring B at positions 6 and 8, and 2', 5' and 6' respectively of a flavonoid compound. The ^{13}C NMR showed very distinct signals for fifteen carbon atoms at δ 93.6, 98.7, 101.9, 102.4, 112.7, 114.5, 121.8, 125.9, 144.9, 145.0, 158.0, 161.3, 161.8, 166.8 and 182.5. The NMR data coincided well with Luteolin in literature [30]. Hence the compound was identified as Luteolin (Fig. 3).

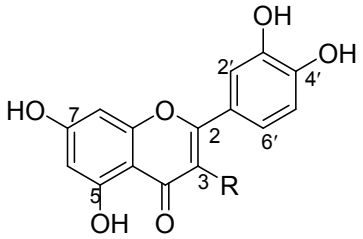
3.3.2 Compound 2

The HRESIMS (negative mode) of the compound showed a pseudo-molecular ion peak at m/z 609.1455 of the molecular ion peak calculated to be 610.1534 which is consistent with a molecular formula $C_{27}H_{30}O_{16}$. The ^{1}H NMR of the compound showed resonances for aromatic protons at δ 6.19 (s), δ 6.37(s), δ 6.88 (d, J = 8 Hz), δ 7.64 (d, J = 8 Hz) and δ 7.67 (s) each integrated to be one proton suggesting two protons on ring A and three protons on ring B at positions 6 and 8, and 2', 5' and 6' respectively Of a flavonoid compound. The ^{13}C NMR showed very distinct signals for twenty seven carbon atoms at δ 178.0, 164.7, 161.5, 157.9, 157.1, 148.4, 144.4, 134.3, 122.2, 121.7, 116.3, 114.7, 104.2, 103.4, 101.0, 98.6, 93.5, 76.8, 75.8, 74.3, 72.6, 70.9, 70.7, 70.0, 68.3, 67.2 and 16.5. The ^{1}H and ^{13}C data indicated a glycoside with two sugar moiety and aglycone to be C6-C3-C6 compound which was easily identified as rutin. The HMBC spectrum showed correlations between the anomeric proton of glucose at δ_H

Table 1. NMR data for compounds 1, 2 and 3 in CD_3OD (Multiplicities and J values are given in Hz in parenthesis)

Position	1		2		3	
	δ_H (600 MHz)	δ_C (150 MHz)	δ_H (600 MHz)	δ_C (150 MHz)	δ_H (600 MHz)	δ_C (150 MHz)
2	-	168.8	-	157.9	-	144.9
3	6.93	101.9	-	134.3	-	135.8
4	-	182.5	-	178.0	-	175.6
4a	-	102.4	-	104.2	-	103.2
5	-	161.3	-	161.5	-	162.2
6	6.20 (s)	93.6	6.19	98.6	6.28 (s)	98.2
7	-	161.8	-	164.7	-	164.1
8	6.43 (s)	98.7	6.37	93.5	6.54 (s)	93.6
8a	-	158.0	-	157.1	-	156.9
1'	-	121.8	-	122.2	-	122.9
2'	6.79 (s)	112.7	7.67 (s)	116.3	7.83 (s)	114.8
3'	-	145.0	-	148.4	-	146.0
4'	-	144.9	-	144.4	-	147.4
5'	6.56 (d)	114.5	6.88 (d)	114.7	7.01 (d)	115.3
6'	6.81 (d)	125.9	7.64 (d)	121.7	7.72 (d)	120.6
1"	-	-	5.11	103.4	-	-
2"	-	-	-	74.3	-	-
3"	-	-	-	75.8	-	-
4"	-	-	-	70.9	-	-
5"	-	-	-	76.8	-	-
6"	-	-	3.83, 3.38	67.2	-	-
1"'	-	-	4.53	101.0	-	-
2"'	-	-	-	70.7	-	-
3"'	-	-	-	70.0	-	-
4"'	-	-	-	72.6	-	-
5"'	-	-	-	68.3	-	-
6"'	-	-	1.12	16.5	-	-

5.11 (1H, d) with a carbon atom at δ_c 134.3 which indicated point of attachment of one of the sugars at position 3 of the aglycone. The protons on C-6 of the glucose at δ_H 3.83 and 3.38 (2H) showed HMBC correlation with the anomeric carbon atom of rhamnose at 101.0 which indicated the attachment of the second sugar, rhamnose, at position 6 of the first sugar, glucose. The NMR data coincided well with Rutin in literature [31]. Hence the compound was identified as Rutin (Fig. 3).



Compound	R
1	H
2	O-D-glucose-L-rhamnose
3	OH

Fig. 3. Structures of flavonoids isolated from the leaves of *Kigelia africana*

3.3.3 Compound 3

The HRESIMS (negative mode) of the compound showed a pseudo-molecular ion peak at m/z 301.0339 of the molecular ion peak calculated to be 302.0427 which is consistent with a molecular formula $C_{15}H_{10}O_7$. The ^{1}H NMR of the compound showed resonances for aromatic protons at δ 6.28 (d, J = 4 Hz), δ 6.54 (d, J = 4 Hz), δ 7.01 (d, J = 8 Hz), δ 7.72 (dd, J = 0, 8 Hz) and δ 7.83 (d, J = 0 Hz) each integrated to be one proton suggesting two protons on ring A and three protons on ring B at positions 6 and 8, and 2', 5' and 6' respectively Of a flavonoid compound. The ^{13}C NMR showed very distinct signals for fifteen carbon atoms at δ 175.6, 164.1, 162.2, 156.9, 147.4, 146.0, 144.9, 135.8, 122.9, 120.6, 115.3, 114.8, 103.2, 98.2 and 93.6, The only correlation in the COSY spectrum is between protons at δ 7.72 (dd, J = 0, 8 Hz) and δ 7.01 (d, J = 8 Hz) which agreed with the coupling constant from the ^{1}H NMR spectrum. The HMQC spectrum showed correlations between these carbon atoms at δ_c 120.6, 115.3, 114.8, 98.2 and 93.6 with protons at δ_H 7.72, 7.01, 7.83, 6.28 and 6.54 respectively. The HMBC spectrum showed correlations between proton at δ_H 7.01 with carbon atoms at δ_c 147.4, 146.0 and 122.9. A correlation was also observed with a proton at 6.54 and a carbon atom at 156.9. The NMR data coincided well with Quercetin in literature [31]. Hence the compound was identified as Quercetin (Fig. 3).

4. CONCLUSION

This work showed the isolation of luteolin, rutin and quercetin from the leaves of *Kigelia africana* and their comparative cholinesterase inhibitory capacities on AChE. It showed quercetin as the major inhibitor of acetylcholinesterase in the leaves of the plant. Though the cholinesterase inhibitory capacity of luteolin, rutin and quercetin have been reported their activity had not been correlated in this manner.

Conclusively, this research has established the phytoconstituents responsible for potential neuroprotection in the extract; it has also elucidated the mechanism of action of the extract. This report could be built upon in further researches leading to drug discovery.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTEREST

The authors declare that they have no conflict of interest.

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