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Anti-*Bacillus* and nitric oxide inhibitory activities of Malaysian *Garcinia mangostana* extracts and their major constituents

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Abstract

Four types of crude extracts were obtained from the stem bark of *Garcinia mangostana* from which six xanthone derivatives: α-mangostin, β-mangostin, garcinone D, mangostenol, fuscaxanthone C and dulcisxanthone F were isolated. The structures of these compounds were elucidated and determined using spectroscopic techniques such as MS, 1D and 2D NMR. The *in vitro* anti-*Bacillus* assay was performed using the crude extracts as well as α-mangostin and β-mangostin against four *Bacillus* species; *B. subtilis* ATCC 6633, *B. cereus* ATCC 33019, *B. megaterium* ATCC 14581 and *B. pumilus* ATCC 14884. The ethyl acetate extract showed strong inhibitory activity against B. subtilis, B. cereus, B. megaterium and B. pumilus in disc diffusion assay with 10.33 ± 0.44 mm, 10.33 ± 0.44 mm, 9.00 ± 0.00 mm and 11.33 ± 0.17 mm inhibition zones, respectively. Nitric oxide inhibition activities indicated that two major compounds (α-mangostin, β-mangostin) exhibited very significant activity in the inhibition of LPS/IFN-γ stimulated RAW 264.7 macrophages with IC_{50} values of 29.81 ± 0.77 and 11.72 ± 1.16 μM, respectively. The chloroform and ethyl acetate extract of *G. mangostana* showed very potent activities.

Introduction

Garcinia mangostana is a well-known species with a lot of ethnomedicinal properties, and an important crop cultivated in Asian continent especially in Southeast Asia. *Garcinia mangostana* is one of the famous species been studied from the genus *Garcinia* and the family Clusiaceae. Different plant parts of *G. mangostana* especially the leaf and the fruit hull have been used in the Southeast Asia region for hundreds of years in traditional medicine. In Thailand and India, as well as in other parts of the Asian region, *G. mangostana* fruit hull has been used as anti-microbial and anti-parasitic treatments (Saralamp *et al.*, 1996; Moongkarndi *et al.*, 2004b; and Obolskiy *et al.*, 2009). Moreover, *G. mangostana* is rich in polyphenolic compounds such as benzophenones, flavonoids and xanthones which possess very good bioactivities such as cytotoxicity, anti-inflammatory and anti-microbial. A lot of pharmaceutical and nutraceutical products have been developed based on *G. mangostana* extracts especially from the fruit hull.

It has also been reported that crude extracts of various parts of *G. mangostana* as well as the compounds isolated from it have significant anti-inflammatory and antifungal activities (Obolskiy *et al.*, 2009). The α-mangostin, which is a major compound in *G. mangostana*, has been reported to have a very potent activity against vancomycin resistant *Enterococci* (VRE) and methicillin resistant *Staphylococcus aureus* (MRSA) (Sakagami *et al.*, 2005). Moreover, α-mangostin has also been proven to be a very good anti-inflammatory candidate when tested *in vitro* and *in vivo* (Obolskiy *et al.*, 2009; Karunakaran *et al.*, 2017).

Bacillus is a type of foodborne pathogen which cause various types of diseases such as arthritis, anthrax, cutaneous infection and gastroenteritis. *Bacillus* species such as *B. cereus*, *B. pumilus* and *B. anthracis* are among the primary causes of the mentioned diseases (Ahmed *et al.*, 1995; Chan *et*

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al., 2003; Tena *et al.*, 2007; Kimouli *et al.*, 2012; Shivamurthy *et al.*, 2016; Karunakaran *et al.*, 2018). The present work thus reports the inhibition of the *G. mangostana* crude extracts as well as α-mangostin and β-mangostin against four *Bacillus* species (*B. subtilis* ATCC 6633, *B. cereus* ATCC 33019, *B. megaterium* ATCC 14581, *B. pumilus* ATCC 14884), and cell based cytotoxicity and nitric oxide inhibition against RAW 264.7 macrophages and LPS/IFN-γ stimulated RAW 264.7 macrophages, respectively. To the best of our knowledge, the present work is the first attempt at demonstrating anti-*Bacillus* activities of *B. megaterium* and *B. pumilus* from *G. mangostana*.

Materials and methods

Chemistry

General experimental procedures

Ultraviolet spectra were recorded in ethanol on a Shimadzu UV-160A, UV-Visible Recording Spectrophotometer. Infrared spectra were obtained using the universal attenuated total reflection (UATR) technique on a Perkin Elmer 100 Series FT-IR spectrophotometer (Perkin Elmer, Waltham, MA, USA). EIMS were recorded on a Shidmadzu GCMS-QP5050A spectrophotometer. ^{1}H NMR spectra were recorded on JEOL JNM-ECX500 (500 MHz) spectrophotometer (Jeol, Tachikawa, Japan). ^{13}C NMR spectra were obtained using the above instrument operating at 125 MHz. Melting points were obtained on a Leica Galen III instrument (Leica Micro-systems, Redwood City, CA, USA). Tetramethylsilane (TMS) was used as an internal standard, and deuterated solvents were used in the analysis.

Plant material

The stem barks of *G. mangostana* were collected from Parit Buntar, Perak, Malaysia, and were identified by Prof. Dr. Rusea Go from the Department of Biology, Faculty of Science, Universiti Putra Malaysia after which the voucher specimen (RG5033) was deposited at the Department's herbarium.

Extraction and isolation

The stem bark of *Garcinia mangostana* (3 kg) was air dried and ground to powder. The crude extracts were concentrated under reduced pressure by using a rotary evaporator to yield n-hexane (10.2 g), chloroform (68.0 g), ethyl acetate (15.50 g) and acetone (28.4 g) extracts. The chloroform extract of *Garcinia mangostana* yielded two xanthone derivatives which are *α-mangostin* (1) (50.0 mg), *β-mangostin* (2) (250.0 mg) and dulcisxanthone F (6) (6.0 mg). The ethyl acetate extract gave *α-mangostin* (1) (150.0 mg), *β-mangostin* (2) (50.0 mg), garcinone D (3) (8.5 mg), mangostenol (4) (5.5 mg) and fuscaxanthone C (5) (5.0 mg).

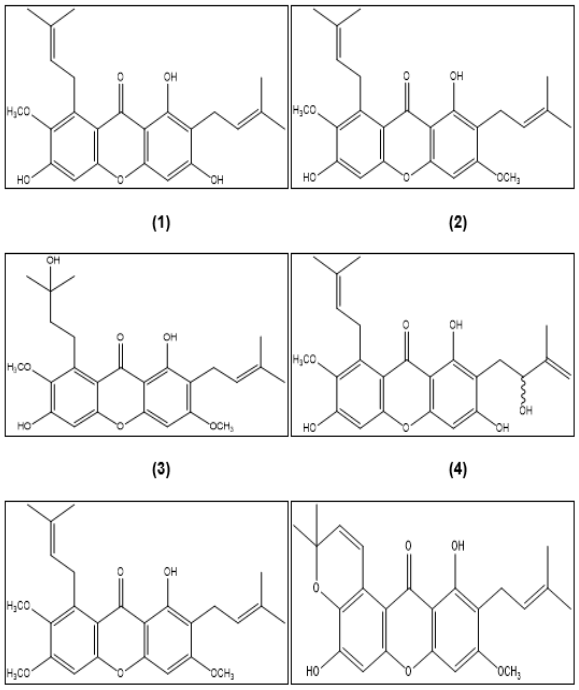

Figure 1. *α-mangostin* (1), *β-mangostin* (2), garcinone D (3), mangostenol (4), fuscaxanthone C (5) and dulcisxanthone F (6) isolated from *Garcinia mangostana*.

Biological assay

General experimental procedures

Curcumin (97% purity), lipopolysaccharide (LPS), phosphate-buffered saline (PBS) and recombinant murine interferon-gamma (IFN-γ) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). The cell culture medium, Dulbecco's Modified Eagle's Medium (DMEM), contained both HEPES and L-glutamine with phenol red and that without phenol red, penicillin-streptomycin antibiotic solutions, foetal bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) and triple Express enzyme, which were acquired from Gibco/BRL Life Technologies Inc. (Eggenstein, Germany). Müeller-Hinton broth (MHB; Difco, Becton Dickinson, Sparks, Md.) was used for the aerobic culture of *B. subtilis*, *B. cereus*, *B. megaterium* and *B. pumilus*.

Sample preparation

The samples were prepared separately for MTT, NO and antimicrobial assays. For the cell assays, 1.0 mg samples (crude extracts, α-mangostin, β-mangostin) was dissolved in 7 µL dimethyl sulfoxide (DMSO), sonicated, and added to 993 µL DMEM (no phenol red) to give a concentration of 1,000 µg/mL (1 mg/mL). For antimicrobial assay, 993 µL sterile distilled water was used instead.

Cell culture

The RAW 264.7 murine macrophage purchased from American Type Culture Collection (ATCC, Rockville, MD, USA) was grown in DMEM containing 10% foetal bovine serum (FBS) and 1% penicillin/streptomycin in a 95% air and 5% CO2 atmosphere at 37°C.

Cell Cytotoxic Test (MTT)

Cell cytotoxic assay of crude extracts, α-mangostin and β-mangostin were determined against RAW 264.7 macrophages. Raw 264.7 macrophages (1×10^5 cells/mL) with DMEM (100 µL) were seeded in a 96-well plate, and incubated for 24 h. Next, the cells were separately treated with 100 µL crude extracts, α-mangostin and β-mangostin (1 mg/mL) and made up to a final volume of 200 µL, and further incubated for 20 h. Following incubation, 20 µL MTT reagent (5 mg/mL) was added in all the wells, and the plate was further incubated in 95% air and 5% CO2 atmosphere at 37°C for 4 h. The mixture of culture media and MTT in all wells were removed and the purplish formazan crystals formed were dissolved in dimethyl sulfoxide (DMSO) and further incubated for 15 min at room temperature in the dark. The colour intensity was then measured at 570 nm at room temperature with cells treated with fresh culture medium only used as control (Karunakaran *et al.*, 2018).

Nitrite determination

Raw 264.7 macrophages (1×10^5 cells/mL) were seeded in a 96-well plate, and incubated for 24 h. These cells were stimulated with 5 mg/mL lipopolysaccharide and 1 ng/mL IFN-γ in the presence of (100 µL) (1 mg/mL) plant crude extracts or pure compounds (α-mangostin, β-mangostin) and made up to a final volume of 200 µL, and further incubated for 20 h. Nitrite concentration was then determined by Griess assay (1% sulphanilamide and 0.1% *N*-(1-naphthyl)ethylene diamine dihydrochloride in 2.5% phosphoric acid) through the reaction of 100 µL Griess reagent with 100 µL of cell culture supernatant at room temperature. The optical density was measured at 550 nm after 15 min of incubation at room temperature with a microplate reader. The supernatant from the cells treated with fresh culture medium was used as control, and curcumin [IC_{50}: 7.36 µg/mL or 20 µM] served as positive control (Karunakaran *et al.*, 2018).

Preparation of bacterial strains and inoculum

Four *Bacillus* type strains were obtained from American Type Culture Collection namely *B. subtilis* ATCC 6633, *B. cereus* ATCC 33019, *B. megaterium* ATCC 14581 and *B. pumilus* ATCC 14884 (Rockville, MD, U.S.A.). The strains were grown in TSB (tryptic soybean broth) at 37°C overnight. A loop of each strain was taken from MHA plates and diluted in 1 mL MHB, and the serial dilution was carried out to get approximately 10^7 CFU/mL.

Disc diffusion assay

The plant crude extracts and pure compounds (α-mangostin, β-mangostin) were tested for antimicrobial activity against *B. subtilis*, *B. cereus*, *B. megaterium* and *B. pumilus* using the disc diffusion method as described by Clinical and Laboratory Standards Institute (CLSI, 2012). Firstly, all bacterial strains were streaked onto MHA plates with a sterile cotton swab. Sterile filter paper discs with 6 mm diameter were placed on top of the agar and 10 µL of 1 mg/mL (w/v) plant extracts and pure compounds were loaded on the paper discs. 1 mg/mL of chlorhexidine (CHX) was used as positive control and 10% of DMSO as negative control. The plates were then incubated at 30°C for 24 h. Clear zone indicated bacterial growth inhibition and the zone diameters were measured in mm. This assay was carried out three times in three replicates ($n = 3 \times 3$) (Karunakaran *et al.*, 2018).

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The determination of MIC and MBC were carried out according to the methods recommended by CLSI (2003). The MICs and MBCs of plant extracts and pure compounds against all bacterial strains were performed in a 96-well microtiter plate using two-fold standard broth microdilution method with an inoculum of approximately 10^7 CFU/mL. Plant extracts and pure compounds stock solutions were mixed with the test organism in MHB. The final concentration of samples ranged from 0.5 – 0.0098 mg/mL. Column 12 of the microtiter plate contained the highest concentration of samples, column 3 the lowest concentration of samples, column 2 served as

the positive control for all the samples (only medium and inoculum or antimicrobial agent-free wells). Column 1 was the negative control (only medium, no inoculum, and no antimicrobial agent). The microtiter plates were incubated at 37°C for 24 h. The MIC is defined as the lowest concentration of antimicrobial agent that resulted in the complete inhibition of visible growth, whereas MBC is the lowest concentration of antimicrobial agent at which no growth occur in the MHA plate. MBC showed no bacterial growth after wells was removed onto MHA medium (included the control). The plates were then incubated at 37°C for 24 h (the experiment was repeated three times with duplicate/experiment) as the corresponding concentration required to completely kill the bacterial strains. The MIC and MBC tests were repeated thrice with three replicates each (Karunakaran *et al.*, 2018).

Results

Extraction and isolation

The crude extracts *G. mangostana* stem barks yielded n-hexane (10.2 g), chloroform (68.0 g), ethyl acetate (15.50 g) and acetone (28.4 g) extracts. The chloroform extract of *G. mangostana* yielded two xanthone derivatives; α-mangostin (50.0 mg), β-mangostin (250.0 mg) and dulcisxanthone F (6.0 mg). The ethyl acetate extract, chromatographed using a silica gel column under gravity, yielded α-mangostin (150.0 mg), β-mangostin (50.0 mg), garcinone D (8.5 mg), mangostenol (5.5 mg) and fuscaxanthone C (5.0 mg). Similarly, the chloroform extract, also chromatographed using a silica gel column under gravity, yielded α-mangostin (50.0 mg), β-mangostin (250.0 mg) and dulcisxanthone F (6.0 mg). The spectral data of these compounds are shown in Figure 1.

Disc diffusion assay

The disc diffusion assay showed that the ethyl acetate extract yielded the most significant inhibition against *B. subtilis* (10.33 ± 0.44 mm), *B. cereus* (10.33 ± 0.44 mm), *B. megaterium* (9.00 ± 0.00 mm) and *B. pumilus* (11.33 ± 0.17 mm) inhibition zone as compared to the positive control which showed the inhibition zone of 11.17 ± 0.17 mm, 10.83 ± 0.17 mm, 10.50 ± 0.29 mm and 10.50 ± 0.29 mm, respectively. The inhibition activities of the other extracts of *G. mangostana* are shown in Table 1.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Table 2 shows that the ethyl acetate and chloroform extracts showed excellent bioactivities in the MIC and MBC tests. Hexane extract also showed good MIC activity, but not on MBC test. Acetone extract did not exhibit MIC and MBC activity.

Cytotoxicity Test (MTT)

The cytotoxicity assay (MTT) provided substantial information on the toxicity profile of the screened compounds, based on the cell viability of the evaluated RAW 264.7 macrophage cells. Moreover, the toxicity assay aids to determine the concentration of drug/sample that would exhibit null or minimal toxicity, thereby the dose fixation can be achieved for further analysis. Four crude plant extracts in different solvents from *G. mangostana* as well as two pure compounds, *α-mangostin* and *β-mangostin* were subjected to the cytotoxicity analysis. The percentage of cell viability was plotted against extract concentration and is shown in Figure 2. The crude plant extracts of *G. mangostana* especially the chloroform and ethyl acetate extracts exhibited toxicity at high concentrations of 125 µg/mL to 500 µg/mL, whereas below 62.5 µg/mL they did exhibit substantial toxicity towards the cells. The *n*-hexane

Table 1. Inhibition activity of plant crude extracts and pure compounds against *Bacillus* spp.

Plant crude extracts / pure compounds (1 mg/mL)	Diameter of inhibition zone (mm)			
	B. subtilis	*B. cereus*	*B. megaterium*	*B. pumilus*
GMH	6.33 ± 0.44	6.33 ± 0.17	6.83 ± 0.17	6.33 ± 0.17
GMC	10.00 ± 0.00	9.33 ± 0.33	8.33 ± 0.33	8.33 ± 0.33
GME	10.33 ± 0.44	10.33 ± 0.44	9.00 ± 0.00	11.33 ± 0.17
GMA	–	–	–	–
α-mangostin	9.17 ± 0.17	9.17 ± 0.17	8.50 ± 0.29	8.33 ± 0.17
β-mangostin	9.33 ± 0.33	9.33 ± 0.33	8.50 ± 0.29	9.33 ± 0.33
Positive control (CHX)	11.17 ± 0.17	10.83 ± 0.17	10.50 ± 0.29	10.50 ± 0.29

Data are mean ± S.E.M of three independent experiments. GMH: n-hexane extract of *G. mangostana*; GMC: chloroform extract of *G. mangostana*; GME: ethyl acetate extract of *G. mangostana*; GMA: acetone extract of *G. mangostana*.

Table 2. MIC and MBC of selected plant crude extracts against Bacillus spp.

Plant crude extract	*B. subtilis*		*B. cereus*		*B. megatarium*		*B.pumilus*	
	MIC (µg/mL)	MBC (µg/mL)	MIC (µg/mL)	MBC (µg/mL)	MIC (µg/mL)	MBC (µg/mL)	MIC (µg/mL)	MBC (µg/mL)
GMC	15.625	500	1.9531	500	15.625	–	15.625	–
GME	15.625	500	7.8125	125	15.625	500	62.5	500

and acetone extracts of G. magostana showed high cell viability of more than 80% from as low as 7.81 µg/mL to 500 µg/mL. α-mangostin and β-mangostin exhibited toxicity at concentrations between 125 µg/mL to 500 µg/mL, and did not exhibit substantial toxicity at concentrations below 62.5 µg/mL.

Nitrite Determination (NO)

The concentrations of the samples used in the evaluation of NO inhibition were based on the non-toxic concentration ranges acquired from the MTT results. The concentration of LPS used to stimulate the RAW 264.7 macrophage cells was 5 mg/mL which was higher than the concentration used by most researchers which is 10 µg/mL to 1 mg/mL. The purpose of using higher LPS concentration was to inspect the sensitivity of induced cells under high stimulation of LPS towards the samples tested. The ethyl acetate plant extract of *G. mangostana* showed potential inhibitory activity towards NO release from the IFN-γ/LPS stimulated RAW 264.7 macrophage cells, with an IC_{50} value of 8.14 ± 1.76 µg/mL. The optimal concentration to inhibit the NO production was found to be 31.25 µg/mL. The bioactive prenylated xanthones, α-mangostin and β-mangostin with IC_{50} values of 12.43 ± 0.32 µg/mL (29.81 ± 0.77 µM) and 4.97 ± 0.92 µg/mL (11.72 ± 1.16 µM) could contribute towards the activity of the ethyl acetate extract. The IC_{50} values of the respective samples are shown in Table 3.

Table 3. Inhibitory activities of plant extracts and pure compounds on IFN-γ/LPS induced NO production in RAW 264.7 macrophages.

Plant extract / pure compound	IC_{50} (µg/mL)	IC_{50} (µM)
GMH	>100	–
GMC	52.97 ± 2.76	–
GME	8.14 ± 1.76	–
GMA	>100	–
α-mangostin	12.43 ± 0.32	29.81 ± 0.77
β-mangostin	4.97 ± 0.92	11.72 ± 1.16
Curcumin[a]	7.36 ± 0.00	20 ± 0.00

Data are mean ± S.E.M of three independent experiments. GMH: n-hexane extract of *G. mangostana*; GMC: chloroform extract of *G. mangostana*; GME: ethyl acetate extract of *G. mangostana*; GMA: acetone extract of *G. mangostana*. [a]positive control

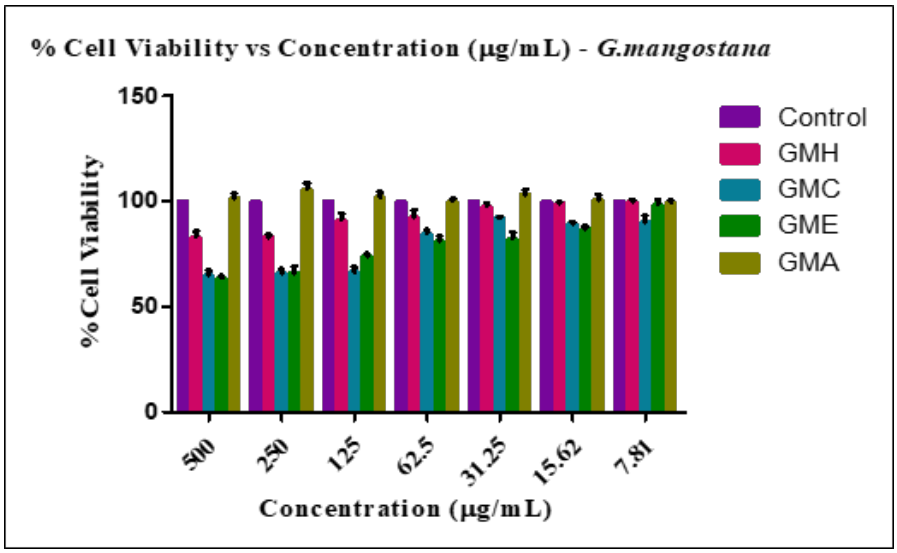

Figure 2. Cytotoxicity of crude extracts of G. mangostana against RAW 264.7 macrophages. Data are mean ± S.E.M.

GMH: n-hexane extract of *G. mangostana*; GMC: chloroform extract of *G. mangostana*; GME: ethyl acetate extract of *G. mangostana*; GMA: acetone extract of *G. mangostana*

Discussion

The significant activities exhibited by the chloroform and ethyl acetate extracts on anti-*Bacillus* and anti-inflammatory tests might be due to the synergism activity of the secondary metabolites (α-mangostin, β-mangostin, garcinone D, mangostenol) present in the extracts. These compounds have two to three hydroxyl groups in their structure and might be one of the core reasons on the activity showed by the ethyl acetate extract. Moreover, these compounds also have prenyl moiety attached to the parent structure which could be responsible for the significant activity of the ethyl acetate extract activity (Pinto *et al.*, 2005). The chloroform extract also showed good activity towards the nitric oxide inhibitory activities as well as to the four tested *Bacillus* species in the disc diffusion, MIC and MBC tests. α-mangostin, β-mangostin, and dulcisxanthone F were isolated from chloroform extract. Since α-mangostin and β-mangostin are the major compounds of *G. mangostana*, the disc diffusion test was performed on them but these two compound only showed moderate activity towards the tested bacteria. The anti-inflammatory activities exhibited by α-mangostin and β-mangostin were well reported and studied by previous researchers (Obolskiy *et al.*, 2009; Karunakaran *et al.*, 2017). Perhaps these compounds work better in a group which provides excellent synergistic activity towards the anti-*Bacillus* and nitric oxide inhibitory activities.

Conclusion

In conclusion, ethyl acetate extract of *G. mangostana* exhibited highly significant NO inhibitory activity on LPS/IFN-γ induced RAW 264.7 macrophages as well as potent anti-*Bacillus* activity against the tested *Bacillus* species. Chloroform extract of *G. mangostana* also displayed significant effects on the anti-*Bacillus* and NO inhibitory activities. Since the chloroform and ethyl acetate plant extracts of the stem bark of *G. mangostana* showed very significant activities, it is thus recommended that these extracts be used in potential herbal formulations of pharmaceutical and nutraceutical products.

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Disclosure Statement

No potential conflict of interest was reported by the authors.

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