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PLANTS AS SOURCES OF ANTIMALARIAL DRUGS, PART 4[1]: ACTIVITY OF *BRUCEA JAVANICA* FRUITS AGAINST CHLOROQUINE-RESISTANT *PLASMODIUM FALCIPARUM* IN VITRO AND AGAINST *PLASMODIUM BERGHEI* IN VIVO[2]

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ABSTRACT.—Extracts of *Brucea javanica* fruit have been prepared and monitored for their in vitro and in vivo antiplasmodial activities. The antimalarial activity of the fruit was found to be attributable to its quassinoid constituents. Nine of the quassinoids possessed in vitro IC_{50} values between 0.046-0.0008 μg/ml against the chloroquine resistant *Plasmodium falciparum* strain (Kl) tested. The two quassinoid glycosides tested were considerably less active in vitro than the aglycones. Four quassinoids were found to possess activity in vivo against *Plasmodium berghei* infections in mice after oral dosing. All five quassinoids tested in vivo showed some toxicity.

In our continuing search for natural products with antimalarial activities that could provide alternatives to chloroquine, we have investigated the fruits of *Brucea javanica* (L.) Merr. (Simaroubaceae). This species of shrub is widely distributed throughout Asia, where the bitter fruits (known as "ya dan zi," "ya tan tzu," "kho-sam," "Macassar kernals," "Makassaarse pitjes") are used in traditional medicine for various ailments, including cancer (1), amoebic dysentery (2), and malaria (3). In common with other Simaroubaceae species, the bitter principles of this plant are quassinoids, and some of these have been investigated extensively as antitumor agents (4). These investigations have included recent clinical trials in the United States of one of the most potent of the *B. javanica* quassinoids, bruceantin [**1**] (4-7). Bruceantin has also been shown to possess high activity in vitro against *Entamoeba histolytica* (8,9), although three closely related quassinoids, bruceantinol [**2**], bruceine B [**4**], and brusatol [**7**], from the same plant were inactive at 2 μg/ml, the highest dose tested.

Certain quassinoids, including bruceantin (10-12), have been found to exhibit antimalarial activity in vitro against *Plasmodium falciparum* at concentrations somewhat lower than those needed to inhibit tumor cells in vitro. Undoubtedly, some quassinoids are highly toxic to mammalian cells (13), but our investigations (12) indicate that in vitro antimalarial activity of quassinoids does not always parallel their in vitro mammalian cytotoxicity. Recently, we reported briefly (14) on the activities of some *B. javanica* fruit extracts and of six isolated quassinoids, viz. bruceantin [**1**], bruceantinol [**2**], bruceines A [**3**], B [**4**], and C [**5**], and dehydrobruceine A [**6**], against a chloroquine-resistant strain of *P. falciparum* in vitro (15). Our results for bruceines A, B, and C have since been largely corroborated by other investigators (16) against a series of different *P.*

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[1]For Part 3 see Chan *et al.* (32).

[2]This article commemorates the 50th year of publication of the *Journal of Natural Products* (formerly *Lloydia*).

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falciparum strains. In the present communication, we describe the extraction and fractionation of *B. javanica* fruits, the isolation of 12 quassinoids, and the assessment of their in vitro and in vivo antiplasmodial activities.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—^{1}H-nmr spectra were determined in $CDCl_3$ or C_5D_5N on a Bruker WM-250 MHz instrument. Fab/ms were obtained using 8 Kev argon atoms with ion currents of 0.5 mA on a Va analytical ZAB-IF mass spectrometer. Tlc used Kieselgel GF_{254} (Fluka) 0.5×200×200 mm plates. Hplc was performed on an Altex model 334 gradient liquid chromatograph.

PLANT MATERIAL.—*B. javanica* fruits from Thailand were supplied and authenticated (17) by Professor P. Tantivatana, Chulalungkorn University, Bangkok and Dr. N. Supavita, Prince Song-Khla University, Song-Khla.

INITIAL EXTRACTION AND FRACTIONATION.—The fruits (780 g) were coarsely powdered and extracted sequentially with petroleum ether, MeOH, and H_2O. The concentrated methanolic extract was further partitioned between $CHCl_3$, *n*-BuOH, and H_2O as described previously (15) and shown in Figure 1. Extracts were concentrated and were monitored for in vitro antimalarial activity against a multi-drug resistant strain of *P. falciparum* as described earlier (15). Results are given in Table 1.

TABLE 1. Inhibition of Uptake of ^{3}H-hypoxanthine into *Plasmodium falciparum* in vitro by Extracts from *Brucea javanica* Fruits

Extract	IC_{50} μg/ml[a]
Petroleum ether	50
MeOH	0.5
aqueous 1	0.5
$CHCl_3$	0.5
n-BuOH	0.5
aqueous 2	50

[a]Based upon tenfold dilutions in duplicate.

SEPARATION AND ISOLATION, $CHCl_3$ EXTRACT (4).—The syrupy $CHCl_3$ soluble extract (9 g) was chromatographed over a column of polyamide SCMN (600 g) eluted with $CHCl_3$ followed by a $CHCl_3$/MeOH gradient and finally MeOH. Fractions of 25 ml were collected, and fractions containing similar tlc profiles were pooled so that ultimately 15 fractions were obtained. Each fraction was monitored for in vitro antimalarial activity, and the results are given in Table 2. Column fractions 2-6 were further purified by

TABLE 2. Inhibition of Uptake of ^{3}H-hypoxanthine into *Plasmodium falciparum* in vitro by Fractions from a Column Chromatogram of the $CHCl_3$ Extract of *Brucea javanica* Fruits

Column fraction	ca. IC_{50}[a] (μg/ml)	Column fraction	ca. IC_{50}[a] (μg/ml)
1	0.5	9	0.5
2	0.05	10	0.5
3	0.05	11	5
4	0.05	12	5
5	0.05	13	5
6	0.05	14	5
7	0.5	15	5
8	0.5		

[a]Based upon tenfold dilutions in duplicate.

successive tlc over Silica gel GF_{254} developed in $CHCl_3$-MeOH (9:1) and $CHCl_3$-iPrOH (9:1). Final purification was achieved by semi-preparative hplc using an Ultrasphere ODS 5 μm column (10×250 mm) fitted with an Ultrasphere ODS 5 μm precolumn (46×45 mm). Gradient program: solvent A=20% MeOH in H_2O, solvent B=80% MeOH in H_2O; flow rate 2.5 ml/min; 0↦2 min % B=50, 2↦27 min %B increases to 90, 27↦32 min %B decreases to 50; detection by uv spectrophotometer at 280 nm. The following quassinoids were obtained: bruceantin [**1**] (Rt 23.8 min) (0.0015%), bruceantinol [**2**] (Rt 20.4 min) (0.0019%) bruceine A [**3**] (Rt 19.6 min) (0.0004%), brusatol [**7**] (Rt 13.0 min) (0.0002%), bruceine C [**5**] (Rt 9.8 min) (0.0038%), bruceine B [**4**] (Rt 7.5 min) (0.0020%), and dehydrobruceine A [**6**] (Rt 17.0 min) (0.0002%).

BUTANOL EXTRACT (5).—The *n*-BuOH extract was concentrated to dryness (2.5 g) and partitioned between $CHCl_3$ and H_2O. The aqueous phase was purified initially by semi-preparative hplc using an Ultrasphere ODS 5 μm column (10×250 mm) fitted with an Ultrasphere ODS 5 μm pre-column (46×45 mm). Gradient program: solvent A=20% MeOH in H_2O, solvent B=80% MeOH in H_2O; flow rate 2.5 ml/min; 0↦12 min %B=40, 12↦27 min, %B increases to 70, 27↦37 min, %B held at 70, 37↦42 min, %B decreases to 40; detection by uv spectrophotometer at 254 nm. Twelve fractions were collected and monitored for in vitro antimalarial activity, and the results are given in Table 3. Column fractions 2-6

TABLE 3. Inhibition of Uptake of ^{3}H-hypoxanthine into *Plasmodium falciparum* in vitro by Fractions from an hplc Separation of the Aqueous Phase from the *n*-BuOH Extract of *Brucea javanica* Fruits

Column fraction	ca. IC_{50}[a] (μg/ml)	Column fraction	ca. IC_{50}[a] (μg/ml)
1	50	7	50
2	0.05	8	5
3	0.05	9	50
4	5	10	50
5	5	11	5
6	5	12	5

[a]Based upon tenfold dilutions in duplicate.

were further purified by tlc over silica gel GF_{254} developed in $CHCl_3$-MeOH-H_2O (65:35:10) (lower phase), followed by hplc using the column described above, eluted with 30% MeOH in H_2O at 2 ml/min. A series of quassinoids was isolated in very small quantities. The following compounds were characterized: bruceine D [**8**] (Rt 16.6 min) (0.002%), yadanziolide A [**9**] (Rt. 14.6 min) (0.001%), yadanzioside C [**10**] (Rt 5.0 min) (0.0003%), yadanzioside F [**11**] (Rt 20.6 min) (0.002%), yadanzioside I [**12**] (Rt 16.0 min) (0.0006%). The 12 quassinoids listed in Table 4 were characterized by their spectral properties (uv, ^{1}H nmr, ms), which were consistent with literature values (18-25).

IN VITRO ANTIMALARIAL ACTIVITY.—Plant extracts and isolated quassinoids were assessed for activity in vitro in human blood against *P. falciparum* strain K-1, which originated in Thailand and which is multidrug resistant (26). The technique relies on the inhibition of incorporation of ^{3}H-hypoxanthine into the parasite (27,28), and precise details of the protocol used are given in references (12,15). Extracts were tested in duplicate each at 6 concentrations in tenfold dilutions. Quassinoids were tested in duplicate at 12 concentrations in 2-fold dilutions. Two series of controls were performed: one with infected red blood cells in the absence of drug and the other with uninfected red blood cells. Chloroquine diphosphate was tested simultaneously to monitor the sensitivity of the *P. falciparum* strain.

IN VIVO ANTIMALARIAL ACTIVITY.—Plant extracts and quassinoids were monitored for activity in vivo in a 4-day suppressive test against *P. berghei* infections in mice (29). Mice (TSW, male, 15-20 g) were inoculated (iv) with *P. berghei* (strain N, 10^7 infected red blood cells) randomized into groups of 5 on day 0 and dosed orally with quassinoid or plant extract (micronized in 0.2 ml H_2O containing not more than 0.1% EtOH) 3 h after inoculation. Further doses of drug were given on days 1, 2, and 3. The animals received a total of four doses of drug. On day 4, tail blood smears were taken, parasitaemia (% red blood cells infected) was determined by counting at least 20 parasitized cells of 1,000 erythrocytes where parasites were few, and the animals were sacrificed. Quassinoids and crude extracts were tested at four concentrations using five mice for each concentration. Five infected, H_2O-dosed mice were used as a negative control.

TABLE 4. Inhibition of Uptake of ^{3}H-hypoxanthine into *Plasmodium falciparum* in vitro by Quassinoids from *Brucea javanica* Fruits

Quassinoid	IC_{50}[a] µg/ml	confidence interval (95%)
bruceantin [1]	0.0008	0.0004- 0.002
bruceantinol [2]	0.002	0.001 - 0.003
bruceine A [3]	0.011	0.007 - 0.014
bruceine B [4]	0.011	0.008 - 0.013
bruceine C [5]	0.005	0.004 - 0.007
dehydrobruceine A [6]	0.046	0.043 - 0.050
brusatol [7]	0.003	0.002 - 0.005
bruceine D [8]	0.015	0.008 - 0.039
yadanziolide A [9]	0.031	0.013 - 0.072
yadanzioside C [10]	N.T.[b]	
yadanzioside F [11]	5.00[c]	
yadanzioside I [12]	22.04	11.95 -40.65
chloroquine diphosphate	0.210	0.190 - 0.240

[a]Based upon twofold dilutions in duplicate.
[b]N.T. = not tested.
[c]Based upon tenfold dilutions in duplicate.

Death occurring before sacrificing on the 5th day of the test was considered as toxic death. ED_{50} and ED_{90} values were determined for each compound/extract.

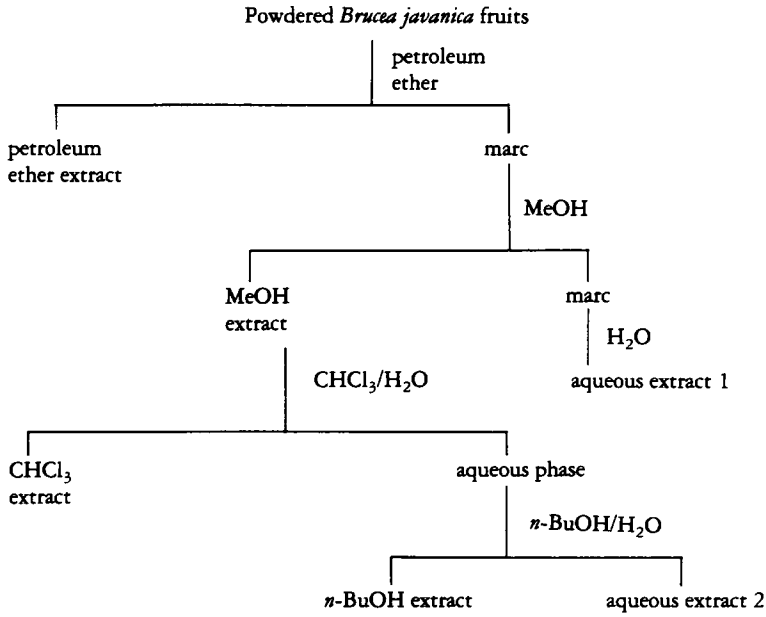


FIGURE 1. Extraction of *Brucea javanica* fruits

RESULTS AND DISCUSSION

IN VITRO ACTIVITIES OF CRUDE EXTRACTS.—Results in Table 1 indicate that of the crude extracts, the methanolic, aqueous 1, chloroformic, and butanolic were relatively highly active in vitro having IC_{50} values of ca. 0.5 µg/ml, whereas the petroleum ether and the aqueous 2 extracts showed only low activity (IC_{50} ca. 50 µg/ml).

CHLOROFORM EXTRACT.—The $CHCl_3$ extract upon column chromatography yielded 15 fractions, all of which showed some in vitro antimalarial activity at 5 μg/ml (Table 2). The most active of these fractions, 2-6, contained the greatest quantities of quassinoids, seven of which were identified as bruceantin [**1**], bruceantinol [**2**], bruceine A [**3**], B [**4**], and C [**5**], dehydrobruceine A [**6**], and brusatol [**7**]. It is almost certain that the activities of the remaining column fractions reflect the presence of other minor quassinoids (30).

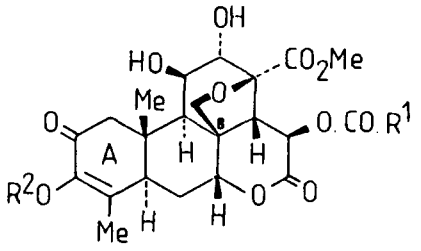
BUTANOL EXTRACTS.—Considering that in traditional medicine, aqueous teas are made from *B. javanica* fruits, we were particularly interested in the in vitro antimalarial activities of the polar extracts (aqueous 1) and (*n*-BuOH). Both of these extracts were devoid of the lipophilic quassinoids **1-7**. Purification of the *n*-BuOH extract by hplc resulted in 12 fractions having in vitro IC_{50} values between 0.05-50 μg/ml (Table 3). Acid hydrolysis of some of these fractions yielded a series of lipophilic quassinoids including bruceantin and bruceine A, B, and C and suggested that possibly glycosidic forms of these compounds were present in the *n*-BuOH extract from the fruits. During the course of the study, other workers (24,25,31) described the isolation and structural characterization of some 13 glycosidic quassinoids, yadanziosides A-J,L,N, and O, from *B. javanica* fruits.

Our purification of active fractions from the *n*-BuOH extract yielded a series of quassinoids, two of which, bruceine D [**8**] and yadanziolide A [**9**], are highly polar, non-glycosidic quassinoids and three of which, yadanziosides C [**10**], F [**11**], and I [**12**], are glycosidic forms of bruceines B and C.

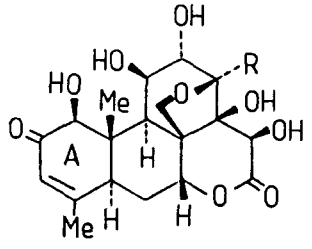
IN VITRO ACTIVITIES OF ISOLATED QUASSINOIDS.—Table 4 indicates that nine of the isolated quassinoids had IC_{50} values <0.05 μg/ml against chloroquine-resistant *P. falciparum* in vitro. The lipophilic, $CHCl_3$-soluble quassinoids **1-7** were generally more active than the *n*-BuOH soluble compounds. Although the non-glycosidic quassinoids, bruceine D [**8**] and yadanziolide A [**9**], exhibited a similar order of activity to some of the lipophilic quassinoids [**3**, **4**, **6**], the quassinoid glycosides, yadanziosides F [**11**] and I [**12**] were much less active, having IC_{50} values of 5.0 μg/ml and 22.04 μg/ml, respectively. In fact the 3-*O*-glycoside **12** is over 2000 times less active than its aglycone, bruceine B [**4**]. The in vitro activity of the *n*-BuOH extract from *B. javanica* fruits is almost certainly attributable to highly active non-glycosidic quassinoids diluted with relatively inactive glycosides.

It is obvious from our results, that relatively small molecular changes produce quite large differences in the in vitro activities of the quassinoids tested. We have noted earlier (12,14) the important contribution to in vitro antimalarial activity of the C-15 ester function of the quassinoids. Compounds **1-5** and **7** differ only in the nature of the ester moiety at C-15 but have IC_{50} values ranging from 0.0008 μg/ml for bruceantin, the most potent compound, to 0.011 μg/ml for bruceines A [**3**] and B [**4**], the least potent compounds. In particular, brusatol [**7**] and bruceine A [**3**] are identical except for the presence (as in **7**) or absence (as in **3**) of an unsaturated C-2′/C-3′ bond, and yet brusatol is more than three times more active in vitro than bruceine A. Bruceine D [**8**] is about twice as active as yadanziolide A [**9**] in the in vitro test, and the two compounds differ only in the presence of a methyl [**8**] or an hydroxymethyl [**9**] function at C-13. Also noteworthy is the alteration in in vitro activity produced as a result of modifications of the A-ring substitution pattern. Bruceine A [**3**] is about 4 times more active than its ring A dehydro derivative, dehydrobruceine A [**6**].

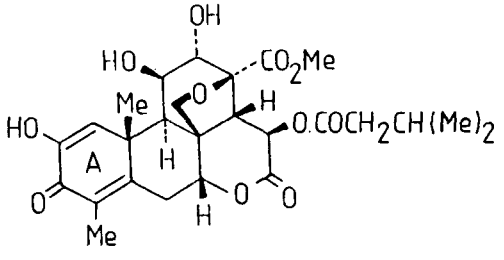
IN VIVO ANTIMALARIAL ACTIVITIES OF *B. JAVANICA* EXTRACTS AND QUASSINOIDS.—*Crude extracts.*—Results presented in Table 5 indicate that the $CHCl_3$ and *n*-BuOH extracts were both highly active and highly toxic in vivo. The ED_{90} of the



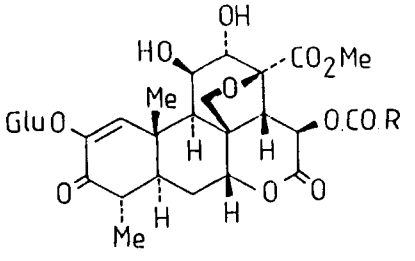
		R^1	R^2
1	bruceantin	$CH=C(Me)CH(Me)_2$	H
2	bruceantinol	$CH=C(Me)C(OAc)(Me)_2$	H
3	bruceine A	$CH_2CH(Me)_2$	H
4	bruceine B	Me	H
5	bruceine C	$CH=C(Me)C(OH)(Me)_2$	H
7	brusatol	$CH=C(Me)_2$	H
12	yadanzioside I	Me	D-glucose



8 bruceine D R=Me
9 yadanziolide A R=CH_2OH



6 dehydrobruceine A



10 yadanzioside C R=CH=C(Me)C(OH)$(Me)_2$
11 yadanzioside F R=Me

FIGURE 2. Structures of quassinoids isolated from *Brucea javanica* fruits

$CHCl_3$ extract was 80.42 mg/kg/day, but at a dose of 100 mg/kg/day three out of five animals showed toxic death, and at 300 mg/kg/day, all five animals died. The ED_{90} of the *n*-BuOH extract was 265.6 mg/kg/day. However, at 100 mg/kg/day four out of five mice died, and at 300 mg/kg/day, the highest dose tested all five mice died. The aqueous extracts 1 and 2 from the fruits were considerably less active in vivo. The extracts produced inhibitions of parasitaemia of 29% (aqueous extract 1) and 19% (aqueous extract 2) at doses of 300 mg/kg/day, the highest doses tested for each extract. Neither extract showed any toxicity at this dose. The activities of these aqueous extracts lend some support to the drinking of teas made from *B. javanica* fruits in traditional medicine for the treatment of malaria.

TABLE 5. Suppression of Parasitaemia in *Plasmodium berghei* (strain N) Infected Mice by Crude Extracts and Some Quassinoids from *Brucea javanica* Fruits

	Oral dose[a] mg/kg/day			Highest dose tested mg/kg/day	Inhibition at highest dose at which animals survived (%)	Toxic deaths out of 5 mice at each dose
	ED_{50}	Confidence interval (95%)	ED_{90}			
Crude extracts						
aqueous extract 1	—	—	—	300	29% at 300	Nil
$CHCl_3$ extract	17.89	13.06- 24.51	80.42	900	95% at 100	5 at 300 3 at 100
n-BuOH extract	73.77	49.31-110.36	265.60	300	95% at 100	5 at 300 4 at 100
aqueous extract 2	—	—	—	300	19% at 300	Nil
Quassinoids						
bruceine A [**3**]	3.36	2.25- 6.03	26.72[b]	9	72% at 9	1 at 9
bruceine B [**4**]	0.90	0.70- 1.17	2.82	9	95% at 3	5 at 9
bruceine C [**5**]	—	—	—	9	40% at 3	5 at 9
brusatol [**7**]	1.27	1.05- 1.54	3.03	3	98% at 3	4 at 3
bruceine D [**8**]	2.79	1.51- 6.75	8.19[b]	3	50% at 3	Nil

[a]Based upon four oral doses given daily for four days.
[b]Extrapolated result.

Isolated quassinoids.—The five quassinoids tested in vivo **3**, **4**, **5**, **7**, and **8**, all showed some activity. Bruceine B [**4**] and brusatol [**7**] were particularly active having ED_{90} values of 2.82 and 3.03 mg/kg/day, respectively. Of these two compounds, bruceine B was 100% lethal at 9 mg/kg/day, the highest concentration tested, but it produced no toxic deaths at 3 mg/kg/day. Brusatol killed four out of five animals at 3 mg/kg/day, the highest dose tested. Thus, bruceine B appears to be the less toxic of these compounds. Bruceine C [**5**] was the least active compound tested. It produced 40% inhibition at 3 mg/kg/day. At 9 mg/kg/day, the highest concentration tested, all animals died. Bruceine D [**8**] has several structural differences compared with the other quassinoids tested. Its oral potency lies between bruceines A [**3**] and B [**4**].

Not surprisingly, the in vitro anti-*P. falciparum* activities of the quassinoids do not parallel their in vivo activities against *P. berghei* after oral dosing. For instance, **3** and **4** show virtually identical activities in vitro whereas the former compound has an estimated in vivo ED_{90} value greater than ten times that of the latter compound. The pharmacokinetics of the two compounds following oral dosing are clearly quite different, and this requires investigation.

Quassinoids from simaroubaceous plants are known to possess a range of biological activities. Most of the studies reported in the literature have used parenteral dosage forms (iv, ip, subcu.). Our study is perhaps the first to demonstrate that quassinoids are orally active antiplasmodials. Further studies, when more compounds have been isolated, will enable a fuller determination of relaxtive toxicity in vivo. To date, more than 30 quassinoids have been reported to occur in *B. javanica*; however, there are accounts in the literature of the biological activities for only very few of these compounds. In the light of our findings that two out of five *B. javanica* quassinoids tested exhibit oral antiplasmodial activities, the remaining minor quassinoids from the plant should be evaluated for antimalarial activity.

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