MODIFICATION AND EVALUATION OF THE POTATO DISC ASSAY AND ANTITUMOR SCREENING OF EUPHORBIACEAE SEEDS

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ABSTRACT.—Galsky *et al.* (10, 11) have reported that the inhibition and growth of crown gall tumors, initiated on potato discs by *Agrobacterium tumefaciens*, apparently has good agreement with 3PS *in vivo* antileukemic activity in mice. We have now modified and evaluated this assay for its potential use as a prescreen and fractionation monitor of plant extracts for 3PS activity. The modified assay was performed on a series of natural compounds and plant extracts (known to have 3PS activity) and on extracts of seeds of 41 Euphorbiaceae species. The results suggest that the potato disc assay is a safe, simple, rapid, in-house, low cost, prescreen for 3PS antitumor activity; it detects some false positives, but few false negatives; it is statistically much more predictive ($p=0.002$) of 3PS activity than either the 9KB ($p=0.140$) or the 9PS ($p=0.114$) cytotoxicity assays. Its extended use could easily obviate the expense and extensive need for animals in the initial stages of antitumor screening and plant fractionation.

Crown gall is a neoplastic disease of plants induced by specific strains of the Gram negative bacterium, *Agrobacterium tumefaciens* (1,2). The bacteria contain large Ti (tumor-inducing) plasmids which carry genetic information (T-DNA) that transforms normal plant cells into autonomous tumor cells (3–5). Thus, certain tumorigenesis mechanisms, in both plants and animals, have in common the intracellular incorporation of extraneous nucleic acids (6,7), and it could be anticipated that some antitumor drugs might inhibit tumor initiation and growth in both plant and animal systems. The development of a simple antitumor prescreen, using convenient and inexpensive plant tumor systems, could, thus, offer numerous advantages as alternatives to extensive animal testing in the search for new anticancer drugs.

In 1977, crown gall tumorigenesis on discs of potato tubers (*Solanum tuberosum* L.) was proposed as an ideal system for investigating the transformation process (8). In 1979, crown gall tumors on pea seedlings (*Pisum sativum* L.) showed promise as predictors of the cancer/anticancer dual action of certain drugs (9). In 1980, Galsky *et al.* (10) combined these ideas and demonstrated that inhibition of crown gall tumor initiation on potato discs showed apparent agreement with compounds and plant extracts known to be active in the 3PS (*in vivo*, mouse leukemia) antitumor assay. In 1981 these workers expanded their study to show that inhibition of the growth of the tumors, in addition to the inhibition of tumor initiation, agreed well with 3PS activity (11). The action of the antitumor compounds is neither via antibiosis nor through inhibition of bacterial attachment to the tumor-binding sites (10).

The present investigation was initiated to modify Galsky's potato disc assay for the routine assay of plant fractionation extracts and to test the effectiveness of the modified assay as an antitumor prescreen for crude plant extracts. Tumors were initiated on potato discs (usually Pontiac red or red Russett varieties), essentially as previously described (8,10). Two note-worthy modifications were made: 1. the use of dimethylsulfoxide (DMSO) as a universal solvent for the plant extracts; and 2. the use of iodine/potassium iodide solution to stain the

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TABLE 1. Determination of best solvent for plant extracts: viable bacteria counts after dilution and plating.[a]

Solvent	0 min. contact	30 min. contact
water	5.6×10^8 bact/ml	5.9×10^8 bact/ml
ethanol	0.0 bact/ml	0.0 bact/ml
acetone	0.0 bact/ml	0.0 bact/ml
DMSO	6.0×10^8 bact/ml	5.9×10^8 bact/ml

[a]2 ml solvent + 2 ml culture (6.5×10^8 bact/ml) and dilutions in water to about 200 bact/agar plate.

TABLE 2. Inhibition of tumor development by DMSO.[a]

	Mean No. tumors/disc	% inhibition
water	36	0
12.5% DMSO	31	−14
25% DMSO	27	−25
50% DMSO	16	−56

[a]Each tested on 5 plates with 5 discs/plate.

background, starch-containing, nontumorous (normal) cells to facilitate tumor counts. DMSO did not affect bacteria viability (table 1), but the DMSO did inhibit tumor initiation (table 2); however, inoculation of control discs with identical final concentrations (12.5% is used routinely) of DMSO compensated for this inhibition. Scheme 1 summarizes the steps involved in the modified bioassay. With these procedures, we routinely administer 25 μg of sample/potato disc while Galsky *et al.* administered 10 μg/disc (10,11). The use of

SCHEME 1. The potato disc bioassay modified for plant extracts and compounds.

1. Fresh, disease-free potato tubers (preferably red) are obtained from local markets and are kept under refrigeration until used.
2. Tubers of moderate size are surface sterilized by immersion in sodium hypochlorite (Clorox) for 20 minutes. The ends are removed and the potatoes are soaked for 10 minutes more in Clorox.
3. A core of the tissue is extracted from each tuber with a surface-sterilized (ethanol and flame) 1.5 cm cork borer.
4. 2 cm pieces are removed from each end and discarded, and the remainder of the cylinder is cut into 0.5 cm discs with a surface-sterilized scalpel, or a special cutter. This is done in the laminar flow hood.
5. The discs are then transferred to 1.5% agar plates (1.5 g of Difco agar is dissolved in 100 ml of distilled water, autoclaved, and 20 ml poured into each sterile petri dish). Each plate contains 5 discs, and 3–5 petri dishes are used for each experimental sample. This is done in the laminar flow hood.
6. A total of 8 mg of extracts or compounds is dissolved in 2 ml of dimethylsulfoxide; this solution is filtered through Millipore filters (0.22 μm) into a sterile tube; 0.5 ml is added to 1.5 ml of sterile water; 2 ml of a broth culture of *A. tumefaciens* strain B_6 (a 48 hour culture containing 5×10^9 cell/ml) are added aseptically.
7. Controls are made in this way: 0.5 ml of dimethylsulfoxide is filtered through Millipores (0.22 μm) into 1.5 ml of sterile, distilled water and added to tubes containing 2 ml of *A. tumefaciens* strain B_6 (from the same 48 hour culture containing 5×10^9 cell/ml).
8. Using a sterile disposable pipette, 1 drop (0.05 ml) from these tubes is used to inoculate each potato disc, spreading it over the disc surface. No more than 30 minutes should elapse between cutting the potatoes and incubation (8).
9. The plates are incubated at room temperature. Under dry conditions, the lids may be taped down, after the first two days, to minimize moisture loss.
10. Twelve (12) days after inoculation, the tumors are counted with the aid of a dissecting scope, after staining with Lugol's solution (I_2·KI). The tumor cells lack starch. Controls with 12.5% DMSO average about 30 tumors/disc (Table 2).
11. All material (petri dishes and potato discs used) should be sterilized before clean-up or discarding.
12. The results are expressed as + or − percentages versus the number of tumors on the control discs; inhibition is expressed as a negative percentage and stimulation is expressed as a positive percentage. Significant activity is indicated when two or more independent assays give consistent negative values of *ca.* 20% or greater inhibition.

DMSO, rather than water, as a solvent circumvents most solubility problems and permits routine testing with higher concentrations of samples.

To determine preliminary dose-response effects, four known 3PS active natural compounds were used. The results (table 3) for bouvardin and vincristine show

TABLE 3. Potato disc dose-response results (% Tumor Inhibitions) with known 3PS active natural compounds.[a]

Conc/Disc	Homoharringtonine	Bouvardin	Vincristine	Trewiasine
10 μg	−79,−85,[−83,−58][b]	−40,−43,[−48][b]	−45,−44	−100
5 μg	−70,−64	−15,−18	−36,−37	−100
2.5 μg	−72,−77	−12,+14	−17,−20	−100
1.25 μg	NT	NT	NT	−100
3PS activity	(% T/C)[c] 238(10,11)	188(10,11)	242(12)	168(13)

[a]Mean values from 5 plates with 5 discs/plate.
[b]Values reported by Galsky *et al.* at 10 μg/disc (10, 11).
[c]% T/C represents increased life span for treated leukemic mice vs. controls; T/C values of *ca.* 120% or more are considered significantly active.
NT=not tested.

a dose-response relationship. With homoharringtonine and trewiasine, the concentrations tested were maximal; a dilution of trewiasine to 1.25 μg/disc still inhibited 100% of the tumors. Our values at 10 μg/disc corresponded quite well with those obtained in Galsky's laboratory (10,11).

The modified assay was tested with a series of twelve plant extracts which had been previously evaluated in the 3PS system (table 4). Significant activity

Table 4. Potato disc results with known 3PS active plant extracts.

NCI Plant identification No.	Species and extract description	Best 3PS activity[a] (% T/C at mg/kg)	Potato disc activity[b] (% Tumor Inhibition)	Pertinent reference
B-811242	*Chione allenii* (1973) Costa Rica; sap dried at 100°	158% at 200	−44/−61	—
B-839610	*Chione allenii* (1978) Costa Rica; aq. ext. of shavings, freeze dried	inactive	−40/−55	—
B-696788	*Pogonopus speciosus* (1973) Costa Rica; sap dried at 100°	181% at 25 (tox. 200)	−20/−34	—
B-850371	*Pogonopus speciosus* (1979) Costa Rica; aq. ext. of shavings, freeze dried	142% at 25 (tox.>50)	−25/−20	—
B-669674	*Pouteria* sp. LJP-316 (1972) Costa Rica; sap dried at 100°	150% at 200 (tox.>400)	−21/−23	—
B-839502	*Pouteria* sp. LFP-316 (1978) Costa Rica; aq. ext. of shavings, freeze dried	inactive (tox.>400)	−25/−20	—
B-696794	*Quassia amara* (1973) Costa Rica; sap dried at 100°	181% at 12.5 (tox.>50)	−45/−60	20
B-839420	*Quassia amara* (1979) Costa Rica; aq. ext. of shavings, freeze dried	126% at 200 (tox.>400)	−40/−50	20
B-811412	*Rondeletia amoena* (1973) Costa Rica; sap dried at 100°	155% at 100	−20/−35	—
B-839338	*Rondeletia amoena* (1977) Costa Rica; aq. ext. of shavings, freeze dried	inactive	−25/−28	—
B-811157	*Tabernaemontana arborea* (1973) Costa Rica; sap dried at 100°	157% at 400	−36/−29	21
B-839508	*Tabernaemontana arborea* (1978) Costa Rica; aq. ext. of shavings, freeze dried	119% at 400	−30/−36	21

[a]3PS activity is assumed when plant extracts increase the life span of leukemic mice by ca. 20% or more, (T/C≧120%).
[b]Potato disc activity is assumed when the crown gall tumor inhibition is *ca.* 20% or more in two successive determinations.

in the potato disc assay was found in all twelve extracts, while nine of the twelve were 3PS positive. Thus, three false positives (and no false negatives) were obtained. In each case with the false positives, however, previous similar extracts of the same species had tested positive in 3PS; consequently, the reliability of the 3PS tests themselves seem in question.

The Euphorbiaceae family was selected for a demonstration screening project. This family has a past history of yielding diverse 3PS active compounds, *e.g.*, phorbol esters, jatrophone, trewiasine and other maytansanoids, etc. (13–15). Seeds were available from the antitumor screening program involving the USDA seed collection in Peoria (16). Hexane and ethanol extracts were made of the pulverized seeds of 41 species and were subjected to the modified potato disc assay to test for the inhibition of crown gall tumor initiation. Concurrently, the ethanol extracts were subjected to the 9KB (*in vitro* human nasopharynx carcinoma) and the 9PS (*in vitro* mouse leukemia) cytotoxicity assays (17). Potato disc actives were defined as those which inhibited *ca.* 20% or more of the tumors in two subsequent assays; this decision was made when considering the activity (−18%/−21%) of *Trewia nudiflora*, a known 3PS active, as a model. The eleven potato disc active extracts (plant nos. 5, 25, 31, 33–35, and 37–42) and eleven random potato disc inactives (nos. 2, 8–11, 14, 18, 21, 23, 32, and 36) were submitted for 3PS testing. Additional inactive samples were not submitted because of the high cost of the 3PS assay.

Seven of the eleven potato disc positives (ethanol extracts, table 5) were

TABLE 5. Summary of antitumor cytotoxicity screening results with extracts of Euphorbiaceae seeds.

Identification	Species	Ethanol extract				Hexane extract potato disc	Pertinent References
		9PS[a]	9KB[a]	3PS[b]	Potato disc[c]		
NF-1 NU 43554..	*Euphorbia paralias* L.	2.0×10^{1}	inact.	NT	−14/−7	−20/−8/−10	(22)
NF-2 NU 62372..	*Antidesma nigricans* Tul.	inact.	inact.	inact.	−11/−5	−15	—
NF-3 NU 46143..	*Euphorbia eriophora* Boiss.	3.0×10^{1}	inact.	NT	−12/+14	−5	—
NF-4 NU 48849..	*Daphniphyllum humile* Maxin.	inact.	inact.	NT	−24/+31/−12/−14	−5	—
NF-5 NU 50787..	*Euphorbia lagascae* Spreng.	8.0×10^{-1}	inact.	active (137% at 200 mg/kg)	−30/−39	+12	—
NF-6 NU40293..	*Manihot rubricaulis* I.M. Johnston	inact.	inact.	NT	−23/+1/−5/−7	+7	—
NF-7 NU 40250..	*Reverchonia arenaria* A. Gray	inact.	inact.	NT	+3/+15	+0	—
NF-8 NU 41307..	*Chrozophora tinctora* (L.) A. Juss.	inact.	inact.	inact.	−7/−11	+8	—
NF-9 NU 62046..	*Mallotus phillipensis* (Lam.) Muell and Arg.	inact.	inact.	inact.	−6/−2	−7	—
NF-10 NU 26094..	*Euphorbia marginata* Pursh.	7.7×10^{0}	inact.	inact.	+0/−17	−8	—
NF-11 NU 44425..	*Sapium japonicum* Pax. and K. Hoffm.	inact.	inact.	inact.	−5/−23	−15	—
NF-12 NU 43330..	*Sapium haematosperum* Muell. and arg.	inact.	inact.	NT	−15/−2	−18	—

TABLE 5. Continued.

Identification	Species	Ethanol extract				Hexane extract potato disc	Pertinent references
		9PS[a]	9KB[a]	3PS[b]	Potato disc[c]		
NF-13 NU 61780..	*Jatropha gossypifolia* L.	inact.	inact.	NT	−4/−16	+10	(23,24)
NF-14 NU 62049..	*Croton tiglium* L.	$<10^{-5}$	3.2×10^{-6}	inact.	−6/−10	+20	(25,26)
NF-15 NU 32935..	*Euphorbia heterophylla* L.	inact.	inact.	NT	+21/−9/−10/−7	−18	—
NF-16 NU 43947..	*Euphorbia falcata* L.	inact.	inact.	NT	−7/−19	+4	—
NF-17 NU 41970..	*Cnidosculus tepiquensis* (Cst. and Gall.) McVaugh	7.1×10^{0}	inact.	NT	−11/−10	+11	—
NF-18 NU 61153..	*Excoecaria bussei* Pax and Pax.	1.3×10^{1}	inact.	inact.	−2/−8	+20	—
NF-19 NU 62869..	*Daphniphyllum himalaense* (Benth.) Muell. and Arg.	inact.	inact.	NT	−9/−3	+10	—
NF-20 NU 49124..	*Bishofia javanica* Blume.	inact.	inact.	NT	+11/−8	+15	—
NF-21 NU 63208..	*Jatropha curcas* L.	9.0×10^{0}	inact.	inact.	+11	+11	—
NF-22 NU 54763..	*Aleurites fordii* Hemsl.	1.8×10^{1}	inact.	NT	+69/−13/−12/−8	+14	—
NF-23 NU 44741..	*Putranjiva roxubrghii* Wall.	inact.	inact.	inact.	+52	+5	—
NF-24 NU 62587..	*Macaranga perakensis* Hookf.	7.9×10^{0}	inact.	NT	−27/−12	−10	—
NF-25 B 820915...	*Trewia nudiflora* L.	3.9×10^{-2}	$<10^{-5}$	active (178% at 200 mg/kg)	−18/−21	−5	(10,13)
NF-26 NU 40193..	*Manihot isoloba* Standley	4.0×10^{0}	3.4×10^{0}	NT	−6/−14	−2	—
NF-27 NU 41968..	*Cnidosculus elasticus* Lumdell	inact.	inact.	NT	+2/−4	−13	—
NF-28 NU 43962..	*Euphorbia medicaginea* Boiss.	4.1×10^{0}	inact.	NT	+5/−19	−11	—
NF-29 NU 41175..	*Euphorbia myrsinites* L.	inact.	inact.	NT	+19/−7	−12	—
NF-30 NU 43115..	*Chrozophora hierosolynitana* Spreng.	inact.	inact.	NT	+1/−25/−4/−8	−10	—
NF-31 NU 43364..	*Sapium montevidense* Klotzsch.	4.3×10^{0}	inact.	active (133% at 100 mg/kg)	−38/−30	−14	—
NF-32 NU 49151..	*Euphorbia lathris* L.	1.2×10^{0}	inact.	H_2O ex. inact.	−13/−8	−9	—
NF-33 NU 45077..	*Euphorbia cyparissias* L.	2.5×10^{-2}	3.9×10^{0}	active (133% at 25 mg/kg)	−38/−29	−11	(27)
NF-34 NU 43815..	*Manihot tweediana* Muell. and Arg.	5.2×10^{0}	inact.	inact.	−21/−27	−8	—

TABLE 5—Continued.

Identification	Species	Ethanol extract				Hexane extract potato disc	Pertinent references
		9PS[a]	9KB[a]	3PS[b]	Potato disc[c]		
NF-35 NU 42075..	*Euphorbia cybirensis* Boiss.	$6.9x10^{0}$	$1.5x10^{1}$	inact.	−24/−30	+10	—
NF-36 NU 61156..	*Bridelia retusae* (L.) Spreng.	$7.8x10^{0}$	inact.	inact.	−29/−6	+15	—
NF-37 NU 40168..	*Eremocarpus setigerus* (Hook.) Benth.	$1.8x10^{-2}$	inact.	active (144% at 100 mg/kg)	−20/−53	+8	—
NF-38 NU 42374..	*Euphorbia amygdaloides* L.	$6.8x10^{0}$	inact.	inact.	−18/−23	+12	—
NF-39 NU 40033..	*Jatropha spathulata* Muell. and Arg.	$2.7x10^{-1}$	inact.	active (135% at 100 mg/kg)	−29/−19	+10	—
NF-40 NU 44013..	*Sapium sebiferum* (L.) Roxb.	$8.2x10^{-1}$	inact.	inact.	−18/−33	+12	—
NF-41 NU 19955..	*Aleurites moluccana* (L.) Willd.	$5.4x10^{0}$	$2.6x10^{0}$	active (146% at 400 mg/kg)	−23/−31	−12	—

[a]9PS and 9KB activity is assumed when plant extracts exhibit ED_{50} values $\leq 30\ \mu g/ml$.

[b]3PS activity is assumed when plant extracts increase the life span of leukemic mice by *ca.* 20% or more, ($T/C \geq 120\%$).

[c]Potato disc activity is assumed when the crown gall tumor inhibition is *ca.* 20% or more in two successive determinations.

NT=not tested.

also active in the 3PS assay, every 3PS active was also active in the potato disc assay, and four of the eleven were false positives. None of the eleven random potato disc inactives were active in 3PS; thus, no false negatives were obtained. Two of the potato disc actives (nos. 31 and 33) were initially toxic to the 3PS test mice and were reported as inactive, but when retested at adjusted doses both were found to be 3PS active. Only one of the hexane extracts (no. 1) exhibited activity in the initial potato disc screen, so these were not tested further.

Six of the 41 Euphorbiaceae species were active in the 9KB cytotoxicity assay, and three of these (nos. 24, 33, and 41) were 3PS active; one of the 9KB actives (no. 26) was not tested in 3PS, but the other two (nos. 14 and 35) were false positives when compared with 3PS as a standard. Furthermore, the four additional 3PS actives (nos. 5, 31, 37, and 39) were not detected by 9KB and are, thus, false negatives. As Galsky *et al.* indicated (10,11), the potato disc assay seems more reliable than 9KB in detecting 3PS activity.

The seven 3PS actives were all included among the twenty-four species which demonstrated 9PS cytotoxicities. However, the 9PS assay produced a very high number (ten) of false positives when correlated with 3PS. While false positives in a prescreen are more tolerable than false negatives, such a high number of false positives necessitates excessive follow-up and is quite undesirable. It must be added that the high number of 9PS actives in this selection of plants may be indicative of phorbol esters, a group of compounds to which the 9PS cells are especially sensitive (14).

Statistical relationships between 3PS and the other assays were assessed by 1. four-fold tables (scheme 2); 2. the Fisher-Irwin test for significance of association between 3PS and the other assays (as displayed in scheme 2); and 3. calculation of kappa values to indicate the degree of data agreement between 3PS and the

SCHEME 2. Four-fold tables for assessment of data agreement (18) between 3PS, potato disc, 9PS, and 9KB screening results with 22 ethanol extracts of Euphorbiaceae seeds.

Potato Disc Results				
3PS Results	positive	negative	total	
positive	7	0	7	
negative	4	11	15	
total	11	11	22	p=0.002, kappa=0.64

9PS Results				
3PS Results	positive	negative	total	
positive	7	0	7	
negative	10	5	15	
total	17	5	22	p=0.114, kappa=0.24

9KB Results				
3PS Results	positive	negative	total	
positive	3	4	7	
negative	2	13	15	
total	5	17	22	p=0.140, kappa=0.40

other assays (18). The results of the potato disc assay were strongly associated with the 3PS results (p=0.002), while the cytotoxicity assays, 9PS (p=0.114) and 9KB (p=0.140), were much less reliable as predictors of 3PS activity. The calculated kappa values for potato disc, 9PS, and 9KB were 0.64, 0.24, and 0.40, respectively. Kappa values greater than 0.75, or so, represent excellent agreement beyond chance; values less than 0.40, or so, represent poor agreement beyond chance; and values between 0.40 and 0.75 represent fair to good agreement beyond chance (19). Thus, the kappa values substantiate the favorable agreement between the potato disc and 3PS results.

We conclude that with these modified procedures crown gall tumors on potato discs could routinely be employed as comparatively rapid, inexpensive, safe, and statistically reliable prescreens for 3PS antitumor activity. The methodology (scheme 1) is simple, and the assay can be performed in-house, with minimal technical training and equipment, to detect potentially useful compounds and active extracts. The assay also circumvents mouse toxicity, which is an inherent disadvantage of the 3PS assay; and it offers a reliable alternative to extensive antitumor screening with small animals for models.

EXPERIMENTAL[2]

PLANT MATERIAL.—Authenticated dried seeds of 40 species of Euphorbiaceae were selected from the USDA, NRRC, seed collection in Peoria; collection numbers are listed in table 1. Seeds of *Trewia nudiflora* (NCI B820915) were obtained from India as previously described (13). The samples were pulverized through a 2-mm screen in a small Wiley mill.

EXTRACTION.—Powdered seed samples, weighing 100 g each, were exhaustively extracted by four or more macerations with 250 ml portions of hexane in 500 ml Erlenmeyer flasks on a reciprocal shaker. After the marc was dried, the extractions were repeated with ethanol. The extracts were concentrated under rotary vacuum at 40°, weighed, and stored under refrigeration.

POTATO DISC ASSAY.—*A. tumefaciens* strain B_6 was maintained on solid slants under refrigeration. Subcultures were grown in 0.8% nutrient broth (Difco) supplemented with 0.5% sucrose and 0.1% yeast extract. The medium was solidfied as required with 1.5% agar (Difco). For inoculation of the potato discs, 48 hour broth cultures containing *ca.* $5x10^9$ cells/ml were used. Samples were dissolved in DMSO, filter sterilized, diluted, and mixed with the bacterial culture for inoculation; the potato discs were aseptically prepared, rapidly inoculated,

[2]3PS tests were performed at Illinois Institute of Technology under a contract from the National Cancer Institute. 9KB and 9PS tests were performed at the Purdue Cell Culture Laboratory following protocols set by the NCI (17). Homoharringtonine, bouvardin, and vincristine were provided by Dr. M. Suffness from the NCI; trewiasine was isolated from *Trewia nudiflora* (13). *A. tumefaciens* strain B_6 was obtained from Dr. A. G. Galsky, Bradley University. Known 3PS active extracts reported in table 4 were provided by Hudson Consulting Service, Box 2451, Spartenburg, S.C. 29302.

and incubated for 12 days, at room temperature, after which I_2·KI solution was added, the tumor counts were made and compared with controls. These procedures are outlined step-by-step in scheme **1**.

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