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Typhonium flagelliforme decreases tyrosine kinase and Ki67 expression in mice

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ABSTRACT

BACKGROUND
Worldwide, breast cancer is the most frequent cancer in women after lung cancer. Treatments include surgery, radiation, immunotherapy and chemotherapy, but are not effective. Tyrosine kinase and Ki67 protein are markers of proliferation. *Typhonium flagelliforme* ethanol extract (TFEE) has been shown to inhibit proliferation of Michigan Cancer Foundation-7 (MCF7) cells in culture. The aim of the present study was to examine the effect of administration of TFEE on tyrosine kinase and Ki67 expression in mice.

METHODS
This experimental study using post test randomized design with control group was conducted in 24 tumor-bearing CH3 mice. They were randomly divided into 4 groups, consisting of one control and 3 treatment groups (TI, T2, T3) treated daily for 30 days with 0.2 ml TFEE at dosages of 200, 400, and 800 mg/kgBW, respectively. On day 31 the tumor tissues were collected and their tyrosine kinase and Ki67 expression were levels assessed using ELISA and immunohistochemical staining, respectively. Tyrosine kinase and Ki67 expression levels were analyzed, respectively using Kruskal Wallis test and one-way Anova followed by Bonferroni post hoc test.

RESULTS
Mean tyrosine kinase level was highest in the control group, followed by T3, T2 and T1 (p=0.019). Mean level of Ki 67 expression was highest in the control group, followed by T2, T3 and T1 (p=0.000).

CONCLUSSIONS
Oral administration of TFEE at a dose of 200 mg/kgBW decreases tyrosine kinase levels and Ki 67 expression.

Key words: *Typhonium flagelliforme*, tyrosine kinase, Ki67 expression, mammary adenocarcinoma**,** mice

Umbi keladi tikus menurunkan kadar enzim tirosin kinase dan protein Ki67 pada mencit

ABSTRAK

LATAR BELAKANG

Kanker payudara merupakan jenis kanker terbanyak pada wanita di seluruh dunia setelah kanker paru. Pengobatan kanker payudara dengan obat, radioterapi, immunoterapi dan kemoterapi masih belum memuaskan. Enzim tirosin kinase dan protein Ki67 merupakan parameter proliferasi sel kanker. Umbi keladi tikus (Typhonium flagelliforme) diketahui dapat menghambat proliferasi kultur sel Michigan Cancer Foundation-*7 (MCF7/*adenokarsinoma mamma*). Tujuan penelitian ini adalah untuk menilai efek ekstrak etanol umbi keladi tikus terhadap kadar enzim tirosin kinase dan ekspresi protein Ki67 pada mencit.*

METODE

Studi eksperimen dengan post test randomized design with control group *digunakan pada 24 ekor mencit C3H bertumor adenokarsinoma mamma. Secara random mencit dikelompokkan menjadi kelompok kontrol (K), kelompok perlakuan 1 (P1), kelompok perlakuan 2 (P2), dan kelompok perlakuan 3 (P3) masing masing diberi 0,2 cc ekstrak umbi keladi tikus dosis 200, 400 dan 800 mg/kgBB peroral perhari selama 30 hari. Hari ke 31, jaringan tumor mencit diambil, sebagian diperiksa kadar enzim tirosin kinase dengan metode ELISA, sebagian dibuat preparat blok parafin serta dilakukan pengecatan imunohistokimia. Data kadar enzim tirosin kinase di uji dengan uji* Kruskal Wallis. *Data ekspresi protein Ki67 diuji menggunakan* One Way Anova *dilanjutkan dengan uji* Benferoni.

HASIL

Terdapat perbedaan penurunan kadar enzim tirosin kinase yang bermakna antara kelompok kontrol dengan T1, T2 dan T3 (p=0,019). Rerata ekspresi Ki67 tertinggi pada kelompok kontrol, diikuti T2, T3 dan yang paling rendah kelompok T1 (p=0,000).

KESIMPULAN

Pemberian ekstrak umbi keladi tikus dosis 200 mg/kgBB dapat menurunkan kadar enzim tirosin kinase dan ekspresi protein Ki67.

Kata kunci: *Typhonium flagelliforme, tirosin kinase, Ki67, adenokarsinoma mamma, mencit*

INTRODUCTION

Breast cancer is the most frequently found cancer in women worldwide after lung cancer. The number of new cancer cases in the US was 1,638,910 in 2012, with a mortality of 577,190 persons.[1] In Indonesia, breast cancer is the most commonly found cancer after cervical malignancies.[2] The mortality rate of patients with breast cancer worldwide is from 6 to 9 per 100,000 women. In Europe, in the period of 1998 to 2002 there were 420,800 cases in the age range of 50 to 60 years, with a mortality rate of 129,300 persons. In Europe, in the year 2008, among 238.8 million inhabitants 7.5% had breast cancer.[3]

Radiotherapy following mastectomy for treatment of breast cancer carries the risk of esophageal cancer after 5-10 years.[4] Chemotherapy administered after mastectomy serves mainly as adjuvant therapy to reduce recurrence. The mode of action of chemotherapeutic drugs is to decrease the enzyme tyrosine kinase in cellular proliferation

pathways, e.g. Irresa/gefitinib (tyrosine kinase receptor inhibitor).[5] The side effects of chemotherapeutic drugs include depression of the immune system, nausea, vomiting, loss of hair, changes in the menstrual cycle, infections of the oral cavity, and fatigue.[6] Immune and cytokine therapy are aimed at strengthening the immune system against cancer, but the results are still unsatisfactory.

Tumor cell proliferation is marked by increases in tyrosine kinase and vascular endothelial growth factor (VEGF) levels, which are produced by the tumor cells and may be measured by means of ELISA. Histopathologically, the levels of tumor cell proliferation markers may be seen from the expression of the Ki67 protein found in the nucleus. The Ki67 protein in normal tissues is expressed at very low levels, but is increased in breast cancer and can be measured by immunohistochemical methods.[7,8]

Bioflavonoids, alkaloids, and other natural plant substances are known to be able to inhibit cell proliferation, thus constituting potential anticancer agents. For example, flavonoids are reportedly capable of inhibiting tyrosine kinase activity and tumor growth in vivo.[9] An extract of the tubers of *Typhonium flagelliforme (*rodent tuber, *umbi keladi tikus)* is known to inhibit cancer cell proliferation.[10,11] An ethanol extract of *Typhonium flagelliforme* added to Michigan Cancer Foundation-7 (MCF7) mammary cell cultures at a concentration of 89.15 µg/nL can inhibit cell proliferation by 50% within 24 hours.[9] The hexane and dichloromethane fractions of *Typhonium flagelliforme* tubers are known to inhibit the growth of NCL-H23 cells.[11,12] An extract of the dichloromethane (DCM) fraction of *Typhonium flagelliforme* administered orally at dosages of 200, 400 and 800 mg/KgBW to leukemic BALB/c rats for 28 days resulted in decreased numbers of peripheral blood immature granulocytes.[12] To date, there have been few invitro and invivo studies on ethanol extracts of *Typhonium flagelliforme*. Therefore, there is a need to study the invivo effect of *Typhonium flagelliforme* ethanol extracts on cancer cell proliferation. The aim of the present study was to evaluate the effect of a *Typhonium flagelliforme* ethanol extract (TFEE) on tyrosine kinase levels and Ki67 protein expression levels in C3H mice bearing mammary adenocarcinoma tumors.

METHODS

Study design

An experimental study using post test randomized design with control was conducted from October 2012 up to April 2013 at the Laboratory for Experimental Pathologic Anatomy and the Biomolecular and Biochemistry Laboratories, Faculty of Medicine, University of Indonesia, Jakarta, and at the Prof dr Sardjito Hospital, Yogyakarta.

Experimental animals

The subjects of this study were 25 ten-week old C3H mice weighing 19-20 grams kept at the Laboratory for Experimental Pathologic Anatomy, Faculty of Medicine, University of Indonesia. The animals were adapted for one week, then inoculated with mammary adenocarcinoma cells. Tumors were already palpable at day 10, and one mouse was sacrificed for preparation of tumor tissue paraffin blocks with Hematoxyline-Eosin staining.

Preparation of *Typhonium flagelliforme* ethanol extract

One-year old *Typhonium flagelliforme* plants were obtained from the area around Jalan Sri Rejeki, West Semarang, for utilization of their tubers. TFEE was prepared at the Center for Drug Development from Natural Substances, Diponegoro University Research Institute, Semarang.

Fresh tubers weighing 1 kg were washed in running water, cut into small pieces and left in a drying chamber at 40^0C for 48 hours. The

dried material was ground to powder in a mill grinder. The powder was extracted in a Soxhlet apparatus with ethanol as the solvent, then evaporated to a concentrate in a rotary vacuum evaporator at 70^{0}C. The concentrate was diluted with water to yield 20 mg/mL, 40 mg/mL and 80 mg/mL, respectively.

Intervention

The prepared dosages were administered at the Laboratory for Experimental Pathologic Anatomy, University of Indonesia. A total of 24 tumor-bearing mice were assigned randomly into 4 groups, each consisting of 6 animals. The control group was left untreated. Experimental group T1 was given orally 0.2 ml of TFEE at a daily dosage of 200 mg/kgBW for 30 days. Experimental groups T2 and T3 also received oral 0.2 ml TFEE for 30 days, at daily dosages of 400 mg/kgBW and 800 mg/kgBW, respectively.

Laboratory analysis

At day 31, tumor tissue was taken for determination of tyrosine kinase levels by the ELISA method and Ki67 protein expression using immunohistochemically stained paraffin sections. Ki67 protein expression was assessed under a Nikon Eclipse E400 light microscope by the hot spot method, which uses 5 high power fields containing 1000 adenocarcinoma cells, so that each high power field contains 200 cells. Assessment of tyrosine kinase levels was carried out at the Departments of Molecular Biochemistry and Biology, Faculty of Medicine, University of Indonesia. Immunohistochemical assessment of Ki57 protein expression was done at Sardjito Hospital, Yogyakarta.

Preparation of tumor tissue samples for homogenization

From each tissue tumor sample, 50 mg was weighed, lysed and dissolved in 250 µl extraction buffer in a test tube. Each sample was mixed at 4^{0}C for 3 minutes in a homogenizer at 1000 rpm to yield soluble material (the test tubes with the tissue samples were put in a jar containing ice cubes). The insoluble tissue remnants at the bottom of the tube were transferred to a different test tube. All samples were centrifuged at 1000 g for 10 minutes, then stored at – 80^0 C until required for determination of tyrosine kinase levels.

ELISA assay for tyrosine kinase

The prepared tissue sample solution was diluted more than five-fold with kinase reacting solution (GenWay Biotech, San Diego, CA). A total volume of 7200 mL kinase reacting solution was prepared, with the addition of 10 mM mercapto-ethanol just before use (5 µl mercapto-ethanol + 7195 µl kinase reacting buffer). One vial of lyophilized protein tyrosine kinase (PTK) control was dissolved in 100 µl twice-distilled water, then 400 µl kinase reacting buffer solution (at a concentration of 108 x 10 units per microliter) was added, resulting in a five-fold dilution of PTK control, and subjected to serial dilution. Phosphorylation of tyrosine was carried out in duplicate by micropipetting 40 µl of PTK control or tissue sample into each microplate well (containing immobilized PTK substrate). Then 10 µl of 40 nM ATP-2Na solution was added to each well and well-mixed, after which the microplate was incubated for 30 minutes at 37^0 C. The remaining sample solution was removed and the plate washed 4 times in washing buffer (PBS 0.1 M + 0.05 % Tween). To each well of the 7 pairs of duplicate wells (in total 14 wells) 100 µl blocking solution was added, then the wells were again incubated for 30 minutes at 37^0 C. The blocking solution was discarded, 50 µl antiphosphotyrosine-horse radish peroxidase (GenWay Biotech) was added, then incubated for 30 minutes at 37^0 C. The remaining antibody solution was discarded and washed four times, then HRP substrate was added, consisting of 100 µl tetramethyl-benzidine dihydrochloride (TMBZ [2HCl]). After the plate was covered with aluminum foil, it was incubated for 15 minutes at 37^0 C. After the addition of 100 µl

stop solution, the assay result was read at 400 nm wavelength using a plate reader.

Immunohistochemical Ki67 protein assay (direct method)

In this method, the monoclonal antibody used to detect Ki67 protein is directly labelled with an enzyme. The following chemicals were used: H_2O_2 3% for inactivation of endogenous peroxidase; trypsin 0.025% in PBS for clearing protein debris presumably covering epitopes of the substance to be detected); DAB working solution, consisting of 50 drops of H_2O_2 substrate buffer and 1mL of distilled water, as color indicator of the enzymatic reaction. Briefly, the assay procedure was as follows: Deparaffinizing of tissue sections in xylol (2 minutes) and graded alcohols (absolute, 95%, 80%, 70%) for 1 minute each. The deparaffinzed sections were then immersed in H_2O_2 3% (30 minutes at room temperature), trypsin 0.025% (6 minutes at 37 ^{0}C), enzyme-labelled anti-Ki67 monoclonal antibody (60 minutes), with a rinse in PBS (3 x 2 minutes) after each step. After the last PBS rinse and addition of chromogen substrate (5 minutes), the sections were rinsed in PBS (3x 2 minutes), then in distilled water, immersed in Mayer's haematoxylin (6 minutes), rinsed in running water until free of excess stain, dehydrated, cleared, and mounted.

Data analysis

Tyrosine kinase concentrations and Ki67 protein expression levels were tested for normality using the Kolmogorov Smirnov test and for homogeneity using the Levene test. These tests indicated that the tyrosine kinase levels were non-normally distributed and non-homogenous, therefore the data were subjected to the non-parametric Kruskal Wallis test. Since the Ki67 protein expression levels were normally distributed and homogenous, they were analyzed by one-way Anova, followed by the Bonferroni post hoc test.

Ethical clearance

The study protocol was approved by the Commission on Health Research Ethics, Faculty of Medicine, Diponegoro University and dr Kariadi Central General Hospital, Semarang.

RESULTS

During the whole intervention period until the completion of the study, there were no deaths among the test animals. Mean tyrosine kinase levels by experimental group are presented in Table 1. The results showed that the highest mean tyrosine kinase level was found in the control group, followed by treatment group T3 (receiving TFEE 800 mg/kgBB), T2 (400mg/kgBB), while the lowest mean level was in T1 (200 mg/kg BW). The Kruskal Wallis test results on tyrosine kinase levels showed that tyrosine kinase levels were significantly different between the four groups (p=0.019). On the basis of these results, it can be stated that the greatest reduction in tyrosine kinase levels in tumor-bearing C3H mice was found at a dose of 200 mg/kgBB TFEE. One-way Anova results on Ki67 expression were

Table1. Comparison of tyrosine kinase dan Ki67 expression by experimental group

Parameter	Control group (n=6)	Group T1 (n=6)	Group T2 (n=6)	Group T3 (n=6)	p
Tyrosine kinase (x 10^{-5} unit/ug)	22.13 ± 10.09	9.50 ± 1.08	17.46 ± 6.03	19.15 ± 5.65	0.019*
Ki 67 (%)	90.83 ± 4.49	21.66 ± 3.14	73.33 ± 3.01	66.00 ± 4.19	0.000**

T1 = TFEE at a daily dosage of 200 mg/kgBW; T2 = TFEE at a daily dosage of 400 mg/kgBW; T3 = TFEE at a daily dosage of 800 mg/kgBW; Values are mean ± SD; *Kruskal Wallis; **One-way ANOVA

Table 2. Post hoc Benferroni test results on mean between-group Ki67 expression

Group	Control	T1	T2	T3
Control	-	0.000	0.000	0.000
T1	-	-	0.000	0.000
T2	-	-	-	0.018
T3	-	-	-	-

T1 = TFEE at a daily dosage of 200 mg/kgBW; T2 = TFEE at a daily dosage of 400 mg/kgBW; T3 = TFEE at a daily dosage of 800 mg/kgBW

significantly different between the four experimental groups (p=0.000). The greatest reduction in Ki67 expression was found in group T1 (receiving 200 mg/kgBB TFEE) (Table 1).

The results of the Bonferroni post hoc test showed that administration of TFEE at dosages of 200 mg/kgBB, 400mg/kg BW and 800 mg/kg BW, respectively, to tumor-bearing C3H mice for 30 days, was able to decrease the expression of Ki67, in comparison with the control group ($p<0.05$) (Table 2).

DISCUSSION

The highest tyrosine kinase level was found in the control group, followed by T3 (receiving TFEE at a dose of 800mg/kgBB) and T2 (400 mg/kg BW), while the lowest level was found in T1 (200 mg/kgBB). TFEE at dosages of 200, 400 and 800 mg/kg BW, respectively, was able to decrease tyrosine kinase levels, indicating that abovementioned dosages still contain the flavonoids, alkaloids and other substances capable of decreasing tyrosine kinase levels invivo. *Typhonium flagelliforme* was reported to be capable of inhibiting tyrosine kinase activity invitro.[13] A study on the flavonoid- and alkaloid-containing tubers of *Curcuma zedori (temu putih)* also showed that these substances were capable of inhibiting tyrosine kinase activity invitro.[14] Tyrosine kinase belongs to the protein kinases that function in signal transduction pathways of cell proliferation. Inhibition of tyrosine kinase prevents transduced signals from entering the nucleus, leading to inhibition of tumor cell proliferation, which presumably also occurred in the present study. There are many pathways for signal transduction in cell proliferation, such as the pathway in which VEGF binds to its receptor (VEGFR). This will activate receptor-associated tyrosine kinases, resulting in autophosphorylation and activation of the tyrosine domain, leading to activation of the Ras transduction factor. Then the growth factor receptor - bound protein-2 (Grb-2) and son of sevenless (SOS) proteins effect the conversion of guanosine diphosphate (GDP) in the Ras protein into guanosine triphosphate (GTP). Binding of GTP to the Ras protein results in Ras activation, leading to phosphorylation of the MAPKKK-MAPKK-MAPK cascade, after which these activated kinases in turn activate transcription factors.[16,17] At the dosages of 400 mg/kgBW and 800 mg/kg BW, the decrease in tyrosine kinase levels was minimal and almost equal to that in the control group. This may be due to saturation of the tumor cell receptors at

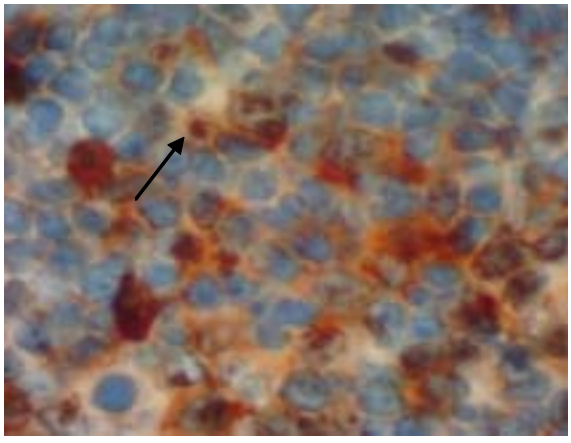

Figure 1. Ki67 expression in the control group (brown-colored nuclei [arrow]) Magnification 1000x

the dosages of 400 and 800 mg/kgBW, preventing maximal inhibition of proliferation. This was presumably also caused by the anti-apoptotic protein Hsp 70, a chaperone molecule capable of inhibiting apoptosis. The role of Hsp 70 in malignancies is to effect tumor cell survival and is involved in stabilization of the Akt kinase, a cytoplasmic protein regulating various control pathways in cell metabolism, growth, death and mobility. Akt kinase dysfunction leads to increased cell growth, metabolism and mobility, while inhibiting apoptosis.[22] The fact that the inhibition of cell proliferation was not maximal in the present study, is presumably due to the effect of a number of proteins. The cooperation between stem cells and cancer cells in the regulation of cell proliferation may become the target of cancer treatment.

The EphB receptor contributes to epithelial cell proliferation, but also functions as a tumor suppressor in the development of colonic carcinoma. How one protein can play the different roles of cell proliferation and tumor suppression of the same tissue is unclear.[15]

The results of the Ki67 assay showed that Ki67 had the highest level of expression in the control group (indicating increased tumor cell proliferation), followed by T2 (400 mg/kgBB), T3 (800 mg/kgBB) and T1 (200 mg/kg BW). Statistically there were significant differences in Ki67 expression between groups T1, T2, and T3 on the one hand and the control group on the other. This indicates that TFEE at dosages of 200 mg/kgBB, 400 mg/kgBB and 800 mg/kg BW was capable of decreasing tumor cell proliferation, as shown by the expression levels of Ki67 as a proliferation marker. The greatest decrease in cell proliferation (shown by increased Ki67) was in T1 at a dosage of 200 mg/kgBW. Quercetin, a ubiquitous flavonoid, is also known to decrease Ki67 protein expression in cancer, e.g. nasopharyngeal carcinoma invitro.[21]

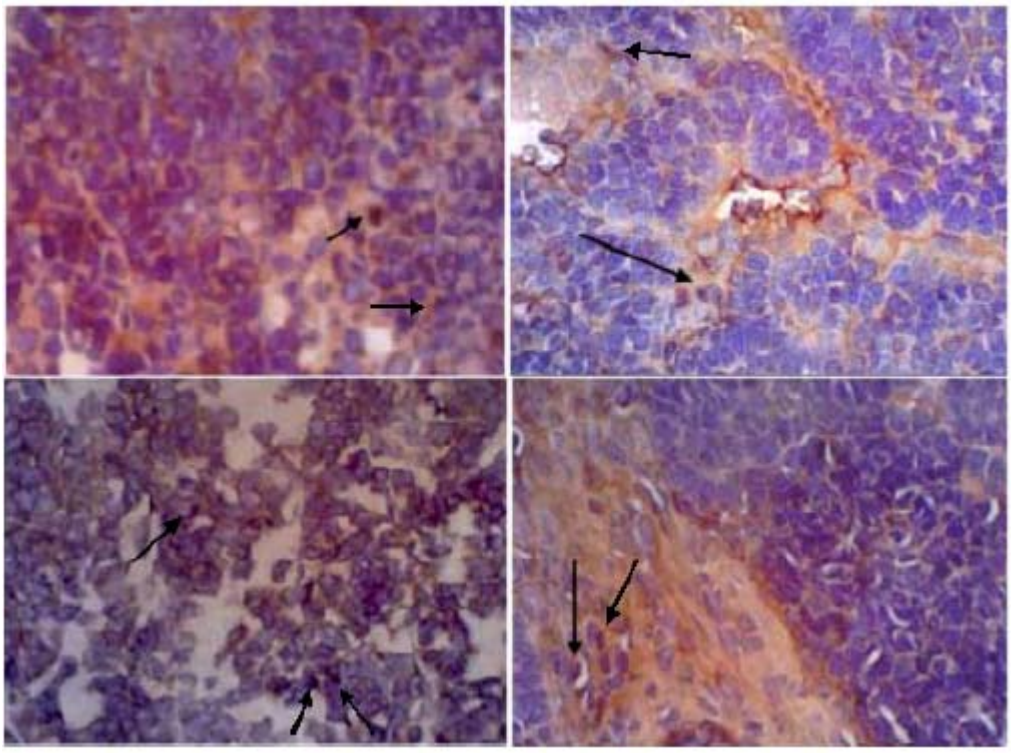

Figure 2. Ki67 expression (brown-colored nuclei [arrow]). Magnification 400x.
A, control group. B, group T1. C, group T2. D, group T3

The Ki67 protein is a proliferation marker in malignancies, where inhibition of proliferation leads to decreased Ki67 expression. The gene for the Ki 67 proliferation marker protein is located at the long arm of chromosome 10 (10q25). Ki67 expression varies in intensity during the cell cycle and is very low (<3%) in normal mammary or epithelial tissues.[18] Ki67 is used for the evaluation of mammary cancer proliferation, although its prognostic role in mammary cancer is unclear. According to a meta-analysis, Ki67 is positively associated with early recurrence of mammary cancer.[20] BCl2 is an anti-apoptotic protein that is correlated to Ki67. Ki67 expression is associated with invasive in situ ductal mammary carcinoma.[20]

A limitation of this study was that it did not assess other proliferation parameters, such as cyclin-dependent kinase (CDK), mitogen-activated protein kinases (MAPKs) and others. Hopefully this study may be extended to a clinical trial, so that it may be of benefit for the prevention of malignancies in the community.

CONCLUSIONS

Oral administration of *Typhonium flagelliforme* ethanol extract is capable of decreasing tyrosine kinase levels and Ki57 expression in C3H mice with mammary adenocarcinoma. It is recommended that further studies be conducted on the effect of *Typhonium flagelliforme* ethanol extract on CDK and MAPK levels.

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