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In vitro and *In vivo* Evaluation of *Sida Acuta burm.f. (Malvaceae)* for its Anti-oxidant and Anti-Cancer Activity

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ABSTRACT

Sida acuta Burm.f. (Malvaceae) is known for its high flavonoid content and potent antioxidant activity.The present study was designated to evaluate the various extracts of Sida acuta burm.f. (Malvaceae) in vitro for its radical scavenging activity,cytotoxicity,short term and long term in vivo anticancer activity.The extracts were evaluated for their anti-cancer activity through DPPH assay, Cytotoxicity by MTT and SRB assays in Vero, HEP-2, A-549 and HeLa cell lines followed by short term toxicity studies on Dalton's Lymphoma Ascites (DLA) and Ehrlich's Ascites Carcinoma (EAC) cells.The results showed the significant decrease of the viability of the cells in a concentration-dependent manner. According to the IC_{50} obtained, the chloroform and toluene extracts of S. acuta showed significant antiproliferative activitywhich was further evaluated in vivo. The results revealed a dose dependent increase in lifespan and mean survival time in the animals treated with chloroform and toulene extracts. However, animals treated with 200 mg/kg dose level showed significant improvement.In conclusion, Sida Acuta burm.f. possesses significant anti cancer potential and needs to be further evaluated by isolating key phytochemicals and investigating the proteins that play a major role in this activity.

Keywords: Cytotoxicity, DLA, EAC, DPPH, Free Radical Scavenging, Cancer

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INTRODUCTION

Cancer, a disease in which a cell or a group of cells display uncontrolled proliferation, invasion and sometimes metastasis contributes to a multitude of abnormalities in the normal functioning of the body. It affects people of all ages with the risk for most types increasing with age. Majority of its occurrence is due to lifestyle and environmental factors while 5-10% is attributed to genetics. Genetic abnormalities found in cancer typically affect two general classes of genes. Cancer-promoting oncogenes are typically activated in cancer cells, giving those cells new properties, such as hyperactive growth and division, protection against programmed cell death, loss of respect for normal tissue boundaries, and the ability to become established in diverse tissue environments. Tumor controlled genes are then inactivated in cancer cells, resulting in the loss of normal functions in them, such as accurate DNA replication, control over the cell cycle, orientation and adhesion within tissues, and interaction with protective cells of the immune system.

Over the past two decades, herbal medicines have become a topic of global importance, making an impact on both world health and international trade. This is particularly true in developing countries, where herbal medicine has a long and uninterrupted history of use. In addition, herbal medicines are more acceptable in these countries from their cultural and spiritual standpoints. Plants have long history of use in the treatment of cancer; several studies have been conducted on herbs under a multitude of ethno botanical grounds. Plant derived components have played an important role in the development of several clinically useful anticancer agents. These include Vinblastine, Vincristine, the Camptothecin derivatives, Topotecan and Irinotecan, Etoposide, derived from Epipodophyllotoxin and Paclitaxel (taxol). Several promising new agents are in clinical development based on selective activity against cancer related molecular targets, including Flavopiridol and Combretastin A4 phosphate, and some other agents which failed in earlier clinical studies are stimulating renewed interest. Sixty percent of currently used anti-cancer agents are derived in one way or another from natural source [1]. *Sida* is an ethnomedically important plant genus consisting of important species like *S. acuta, S. cordifolia, S. rhombifolia, S. spinosa, S. carpenifolia, S. humilis* and *S. veronicaefolia* which were used extensively in Ayurveda [2]. *Sida Acuta burm.f. (Malvaceae)* also known as *S. Carpinifolia* mast and commonly called as broom weed belongs to the family Malvaceae. Earlier studies indicated presence of alkaloids in the aerial parts of the plant while seeds contain 0.26% and roots contain 0.066% of total alkaloids while ephedrine is present in higher concentration[3]. *Sida Acuta burm.f. (Malvaceae)* has significant anti-proliferative activity and is also known for its potent free radical scavenging activity[4]. Based on these facts, an attempt has been made to screen the anti-cancer activity of *Sida. Acuta. Burm. F in vitro* to evaluate the cytotoxicity effect on normal cell line comaprtively on different cancer cell line in order to evaluate significant effect on cancer cell lines , further identifying the same in primary cell lines contuined further to determine the cytotoxicity effect *in vivo.*was performed and 200mg/kg dose showd significant results.

MATERIALS AND METHODS

Methods:
Vero, HEp-2, A-549 and HeLa cell lines were obtained from National Centre for Cell Science (NCCS Pune, India. Dalton's Lymphoma Ascites (DLA) Ehrlich's Ascites Carcinoma (EAC) cells were obtained from Amala Cancer Research Center, Kerala. The chemicals used in all studies were of tissue culture/laboratory grade, all the cell lines are maintined with 5% CO_2 at 37^0C

Plant material
Sida Acuta burm.f. (Malvaceae) was authenticated and collected from Mr. V. Chelladurai, Research Officer-Pharmacognosy, Central Council for Research in Ayurveda and Siddha (AYUSH; Government of India), T.Nagar, Tirunelveli, Chennai.

The aerial part of the plant was dried under shade, chopped and coarsely powdered. The powdered parts were then subjected to extraction by hot continuous maceration. Based on previous studies petroleum ether, toluene, chloroform, acetone, ethyl acetate and hydroalcoholic extracts of whole plant of *Sida Acuta burm.f. (Malvaceae)* were prepared for investigation[5]. The tests for carbohydrates (Molisch, Fehling's, Benedict's and Barfoed's), alkaloids (Dragendroff's, Wagner's, Mayer's and Hager's), glycosides (Legal, Baljet, Borntrager and Keller Killani), flavonoids (Shinoda's), proteins and amino acids (Biuret's test, Ninhydrin test and Xanthoprotein) were performed as per the standard procedures[6].

***In Vitro* Diphenyl Picryl Hydrazine (DPPH) Free Radical Scavenging Assay [7]**
The DPPH radical scavenging assay was performed for extracts of whole plant extracts of *Sida Acuta burm.f. (Malvaceae).* The extract or standard solution was added to DPPH in methanol solution (200 µL) in a 96 well plate. After incubation at 37^0 C for 30 min, the absorbance of the each solution was determined at 490 nm using an ELISA Microtiter plate reader (Model 550, BioRAD USA).

The corresponding blank readings were taken and the remaining DPPH was calculated using the below formula

$$\% \ Growth \ inhibition = \frac{Control - Sample \ X \ 100}{\text{Control}}$$

***In Vitro* Cytotoxicity Studies**
The monolayer cell culture was trypsinized and cell count was adjusted to 1.0 x 10^5 cells /mL using Dulbecco's Modified Eagle Medium(DMEM) with 10% Fetal Calf Serum (FCS). To each well in a 96 well microtiter plate, 0.1 mL of the diluted cell suspension containing approximately 10,000 cells was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, the monolayer was washed once with the medium and 100 mL of different sample concentrations were added to the cells in the microtiter plate. The plates were then incubated

at 37 ^{0}C for 3 days in 5% CO_2 atmosphere, and microscopic examination was carried out and observation was recorded every 24 h. After 72 h, the extract solution in the wells were discarded and the cellular viability was determined by MTT and SRB assays [8] & [9].

$$\% \ Growth \ Inhibition \ for \ MTT \ Assay = \frac{100 - Mean \ O.D. \ of \ Individual \ Test \ Group}{Mean \ O.D. \ of \ Control \ Group} \ X \ 100$$

$$\% \ Growth \ Inhibition \ for \ SRB \ Assay = \frac{100 - Mean \ O.D. \ of \ Test \ Compound}{Mean \ O.D. \ of \ Control \ Cells} \ X \ 100$$

Short Term Toxicity Studies using Dalton's Lymphoma Ascites (DLA) cells And Ehrlich's Ascites Carcinoma (EAC) [10]

Cells were cultured in the peritoneal cavity of mice (Adult healthy Swiss albino mice weighing 60 g) by injecting the cell suspension by intra peritoneal administration ($1.0x10^5$ cells/mL).The cells were withdrawn from the peritoneal cavity of the mice after 10-15 days (significant increase in peritoneal cavity size) with the help of a sterile syringe. The cells were washed with HBSS and centrifuged for 5 min at 1000g. The procedure was repeated thrice after which the cells were suspended in a known quantity of HBSS and the cell count was adjusted to 2 x 10^6 cells/mL.The diluted cell suspension was distributed into eppendorf tubes (0.1 mL containing $2x10^6$cells). The cells were exposed to drug dilutions and incubated at 37 ^{o}C for 3 h. After 3 h, dye exclusion test was performed, i.e. equal quantity of the drug treated cells and trypan blue (0.4%) were mixed and left for a minute. It was then loaded in a haemocytometer and viable and non-viable count was recorded within two minutes.

The percentage growth inhibition was calculated by using the following formula:

$$\% \ Growth \ inhibiton = 100 - \left(\frac{\text{Total Cells—Dead Cells}}{\text{Total Cells}}\right) X100$$

Induction of DLA & EAC in Albino Mice

Male Swiss albino mice (20-28 g) were obtained from the centralized animal house facility of JSS College of Pharmacy, Udhagamandalam and maintained under standard environmental conditions (22±3 ^{o}C, 60±5 % relative humidity, and 12/12 light and dark cycle). Animals had access to standard laboratory feed (M/s Hindustan Lever Ltd., Bangalore, India) and water *ad libitum*. The experimental protocol was approved by Committee for the Purpose of Controlled Supervision of Experimentation on Animals (CPCSEA) approved Institutional Animal Ethics Committee (IAEC) of JSS college of Pharmacy, Udhgamandalam (IAEC Approval Number JSSCP/IAEC/ M.PHARM/PH.BIOTECH/01/2010-11). The mice were randomly divided into six groups (n = 6). Group A served as negative control administered with DLA cells i.p.; Group B served as positive control administered with DLA cells followed by 5-Fluorouracil (20 mg/kg i.p.) treatment for 11 days. Groups C-H served as treatment groups administered with *Sida Acuta burm.f. (Malvaceae)* chloroform and toluene extracts at 50, 100, and 200 mg/kg respectively by oral gavage.

Mean Survival Time (MST): The average life span of animals of all the groups were determined and noted.

Percentage increase in life span (% ILS): The effect of toluene and chloroform extracts of *Sida Acuta burm.f. (Malvaceae)* on tumour growth was monitored by recording the occurrence of mortalityon a daily basis for a period of 6 weeks and percentage increase in life span was calculated.

$$\% \ Increase \ in \ life \ span = \frac{MST \ of \ treated \ group - MST \ of \ control \ group}{\text{MST of control group}}$$

Where,

$$MST = \frac{day \ of \ first \ death \ observed + day \ of \ last \ death \ observed}{2}$$

Body weight analysis

Body weights of the experimental mice were recorded both in the treated and control groups at the beginning of the experiment (day 0) and sequentially on every day during the treatment period. Percentage increase in body weight was calculated on day 15 of the experiment using the following formula.

$$\%\ Increase\ in\ Body\ Wt. = \frac{Body\ Wt.\ of\ Animal\ on\ 15th\ day - Body\ Wt.\ of\ animal\ on\ 0\ day}{Body\ Wt.\ of\ animal\ on\ 0\ day} X\ 100$$

Statistical Analysis

Statistical significance of the *in vivo* data was analysed by One-Way Analysis of Variance (ANOVA) followed by Dunnett's multiple comparison test. $p < 0.05$ was considered as statistically significant. Graph Pad Prism (Ver. 5.01, GraphPad USA) was used for statistical analysis.

RESULTS AND DISCUSSION

Phytochemical Studies

Qualitative photochemical analysis of the extracts of *Sida acuta burm.f.* showed the presence of alkaloids, carbohydrates, flavonoids, tannins, saponins, aminoacids, proteins and glycosides in various extracts (Table 1).

Table 1: The Phytochemical analysis of *Sida acuta*

Tests	Pet.Ether	Toluene	Chloroform	Ethyl acetate	Acetone	Hydro-Alcoholic
Alkaloids	+	+	+	+	+	+
Carbohydrates	+	+	+	+	+	+
Flavonoids	+	+	+	+	+	+
Tannin	-	-	-	-	-	-
Saponin	-	-	-	-	-	-
Amino acids	-	-	-	-	-	+
Proteins	-	-	-	-	-	+
Cardiac glycosides	+	+	+	+	+	+

(+)- PRESENT, (-)- ABSENT

***In vitro* Diphenyl Picryl Hydrazine (DPPH) scavenging assay**

The toluene (TL), chloroform (CL) and ethyl acetate extracts of *Sida Acuta burm.f. (Malvaceae)* showed significant IC_{50} values (78±2, 142.33±2.51 and 113±3.60 µg/mL) compared to standard Rutin (21.66±2.08 µg/mL).

Table 2: Antioxidant activity of *Sida Acuta burm.f. (Malvaceae)* plant extracts by DPPH assay. Values expressed as Mean ± SEM. One way ANOVA followd by Dunnet's post hoc test.

S.No	Plant extracts	*SIDA ACUTA BURM.F. (MALVACEAE)* IC_{50}(µg/mL)
1.	Pet ether(PE)	321 ± 4.00
2.	Toluene(TU)	78 ± 2.00
3.	Chloroform(CL)	142.33 ± 2.51
4.	Ethyl acetate(EA)	113 ± 3.60
5.	Acetone(AC)	194.33 ± 4.04
6.	Hydro alcohol(HA)	402 ± 2.51
7.	Rutin	21.66 ± 2.08

***In vitro* Cytotoxicity Screening:**

The results of the effect of extracts of plants on the Vero, HEp-2, HeLa, A-549 - cells at different concentrations (15.675–1000 g/mL) demonstrated the significant ($p < 0.05$) decrease of the viability of cells in a concentration-dependent manner after 72 h incubation. The increasing of the concentration of extracts almost completely blocked the growth of cells. The antiproliferative activity of each extract was expressed as CC50 (50 cytotoxic concentration values) with a lower CC_{50} value indicating a higher antiproliferative activity. The cytotoxic concentration (CC_{50}) values varied from one extract to another. According to the results of CC50, The chloroform extract of *Sida Acuta burm.f. (Malvaceae)* showed highest antiproliferate activity with CTC_{50} values of 38.33, 38.00, 40.33 and 43.67µg/mL and 43.33, 33.33, 33.67and 35µg/mL respectively for Vero, HEp-2, HeLa and A-549 cell cultures against MTT and SRB assay. The the rest of the plant extracts demonstrated moderate CTC_{50} values by MTT assay.

Table 3: CTC_{50} values of *Sida Acuta burm.f. (Malvaceae)* extracts in MTT assay. Values expressed as Mean ± SEM. One way ANOVA followd by Dunnett's post hoc test.

Extract	Vero	HEp-2	HeLa	A549
PE	378.3±12.58	182.0±7.21	191.7±12.58	360±17.90
TU	214.6±6.429	48.67±16.80	44.67±8.963	43.33±5.859
CL	268.3±10.41	38.00±6.083	40.33±4.509	43.67±3.215
EA	125.0±27.84	52.33±10.02	58.33±2.082	49.42±5.811
AC	211.0±37.24	171.7±7.638	193.3±12.58	105±13.00
HA	178.0±2.00	95.33±12.74	118.7±12.06	108±2.646

Table 4: CTC_{50} values of *Sida Acuta burm.f. (Malvaceae)* extracts in SRB assay. Values expressed as mean ± SEM. One way ANOVA followd by dunnet's post comparision test

Extract	Vero	HEp-2	HeLa	A549
PE	378±12.58	181.0±7.000	200.0±26.46	371.7±7.638
TU	27.33±6.429	34.00±10.39	40.67±3.786	23.00±9.849
CL	43.33±5.774	33.33±11.55	33.67±2.082	35.00±5.000
EA	100.0±26.46	46.67±7.638	55.33±3.055	51.33±5.132
AC	207.7±23.46	166.7±57.64	181.7±12.58	116.7±15.28
HA	176.7±15.28	97.00±32.05	108.7±10.97	121.7±22.55

In vitro short term toxicity studies

All the extracts exhibited moderate toxicity against DLA and EAC cells. The *Sida Acuta burm.f. (Malvaceae)* toluene and chloroform extracts showed maximum activity with CTC_{50} of 170±.1.50 and 140±0.98 µg/mL in DLA; 190±2.45 and 150±1.85 µg/mL in EAC cells respectively. Other extracts have shown CTC_{50} values ranging from 200 µg/mL to 420 µg/mL for both DLA and EAC cells (Table 5).

Table 5: CTC50 values of *Sida Acuta burm.f. (Malvaceae)* extracts in DLA and EAC cells. Values expressed as Mean ± SEM. One way ANOVA followd by Dunnett's post hoc test

Extracts	Mean (DLA) CTC_{50} (µg/mL)	Mean (EAC)CTC_{50} (µg/mL)
Sidaacuta (Pet.ether)(PE)	350±2.56	370±4.79
Sidaacuta(Toluene)(TU)	170±1.50	190±2.45
Sidaacuta(chloroform)(CL)	140±0.98	150±1.85
Sidaacuta(Ethyl acetate)(EA)	375±4.96	420±3.12
Sidaacuta(Acetone)©	210±1.98	230±4.20
Sidaacuta(Hydro alcoholic)(HA)	350±2.96	340±3.10

In vivo Anti-cancer Studies:

The treatment with *Sida Acuta burm.f. (Malvaceae)* chloroform extract (CL) and toluene extract (TL) at 200 mg/kg dose level increased the average life span (ALS) of DLA bearing mice from 16.67±1.36 to 24.00±1.67 and 25.17±1.72 days ($p<0.05$) respectively compared to DLA tumor control group. *Sida Acuta burm.f. (Malvaceae)* (CL) and (TL) at 100 mg/kg dose level have shown moderate increase in ALS to 20.33±1.80 and 20.50±1.57 days ($p<0.05$) respectively. The standard 5-FU at 20 mg/kg, significantly increased the life span to 28.50±1.37 days ($p<0.05$). The significant and dose dependent reduction in percentage increase in body weight when compared to DLA control ($p<0.05$) reveal the potent anti-cancer nature of *Sida acuta*.

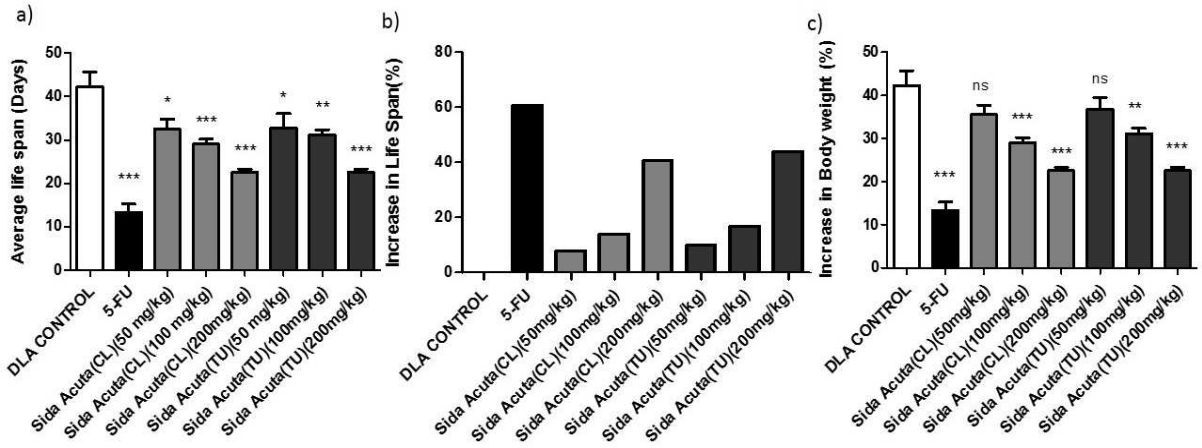

Figure 1: a) Effect of *Sida acuta* Chloroform and Toulene extracts on average life span of animals induced with Daltons Lymphoma Ascites (DLA) cells. b)Effect of *Sidaacuta* Chloroform and Toulene extracts on percentage increase in life span of animals induced with DLA cells. c) Effect of *Sidaacuta* Chloroform and Toulene extracts on body weights of animals induced with DLA cells. Values expressed as Mean ± SEM, One way ANOVA followed by Dunnett's post hoc test. *p < 0.05 is considered significant

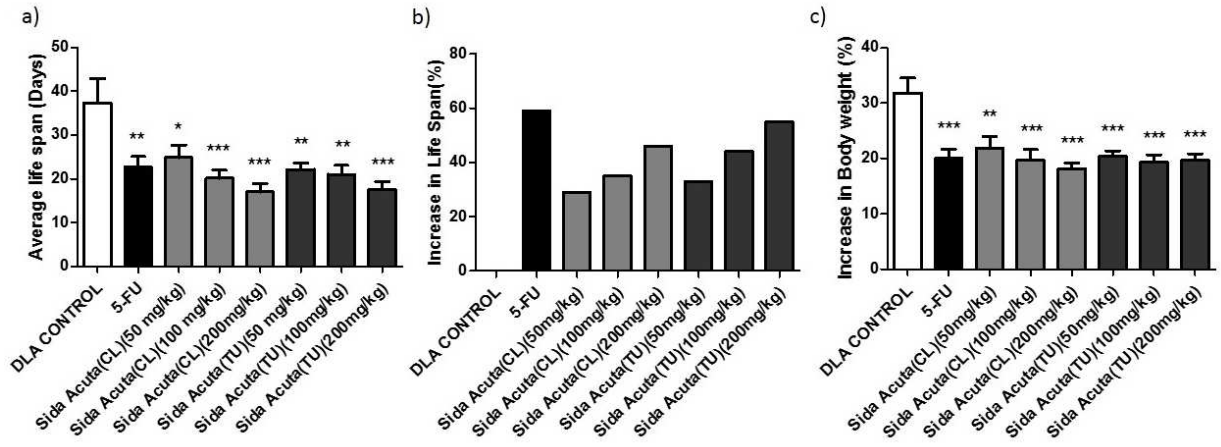

Figure 2: a) Effect of *Sidaacuta* Chloroform and Toulene extracts on average life span of animals induced with Ehrlich Ascites Carcinoma (EAC) cells.b)Effect of *Sidaacuta* Chloroform and Toulene extracts on percentage increase in life span of animals induced with EAC cells. c) Effect of *Sidaacuta* Chloroform and Toulene extracts on body weights of animals induced with EAC cells. Values expressed as Mean ± SEM, One way ANOVA followed by Dunnett's post hoc test. *p < 0.05 is considered significant

CONCLUSION

Natural products are slowly, but surely being touted as the game changers in the field of medicine, with particular impetus towards the anti-cancer potential of medicinal plants. Findings exist in abundance baring the vital aspects of medicinal plant research and the need of these plants to gain further in-roads in cancer therapy [11]. One such plant, with ample scope is *Sida acuta*, its use being well known in traditional medicine and therefore its selection in this study [12].

The current study showcased the anti-cancer potential of *Sida acuta in vitro* and specifically, the chloroform and toluene extracts *in vivo*. Preliminary phytochemical evaluations suggested the presence of flavonoids in appreciable levels versus the other phytoconstituents. The only logical alternative was to further test the veracity of this claim by *in vitro* cytotoxicity tests. By means of the MTT assay on Hep-2, HeLa, A-549 and Vero cell lines, the IC_{50} values were obtained providing preliminary confirmation of the anti-cancer capability of the plant extracts. In addition, theresults of the DPPH scavenging assay demonstrated a moderate scavenging activity for the toluene, chloroform and ethyl acetate extracts.Furthermore, the cytotoxicity test (CTC_{50}) on DLA and EAC cells helped in identifying the chloroform and toluene extracts to be highly potent and therefore, the eligible candidates for *in vivo* evaluation.

By employing the DLA model of cancer development, we were able to establish the *in vivo* efficacy of the chloroform and toluene extracts. A significant increase in life span was observed in the mice treated with the higher dose of both extracts with negligible changes in the body weights.

The anti-cancer evaluation of the whole plant extract of SA yielded two worthy candidates for further screening. Isolation of the active phytoconstituents and extended studies elaborating on their mechanistic action may eventually lead to a novel molecule for anti-cancer therapy. However, the potency of these extracts needs to verified by means of standard toxicity tests to ensure that healthy cells of the body are not destroyed inadvertently.

In conclusion, the results of the present study on plant *Sida acuta* showed promising *in vitro* cytotoxicity and anti-cancer activity. Further higher studies of these plant extracts may prove significance in many diseases. Isolation of active constituents may lead to invention of a new lead molecule for treatment of many diseases.

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