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STUDIES ON THE EFFECT OF *SIDA ACUTA* AND *VETIVERIA ZIZANIOIDES* AGAINST THE MALARIAL VECTOR, *ANOPHELES STEPHENSI* AND MALARIAL PARASITE, *PLASMODIUM BERGHEI*

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ABSTRACT

The methanolic extracts of *Sida acuta* and *Vetiveria zizanioides* leaves and root was investigated for antimalarial activity against *Plasmodium berghei* infections in mice. The median lethal dose was determined to ascertain the safety of the extract in mice. The antimalarial activities during early and established infections were evaluated. Phytochemical screening was also investigated to elucidate the possible mechanism of the antimalarial and antivectorial properties. The extracts of *Sida acuta* and *Vetiveria zizanioides* leaf and root demonstrated that a significant antiplasmodial activity in all the three groups (test for root and leaves and one control includes three groups) of the antimalarial evaluations. Plant extracts treatment showed higher mortality against mosquito larvae, lethal dose (Lc_{50} and Lc_{90}) was also worked out for the larval instars of malarial vector, *Anopheles stephensi.* Phytochemical screening revealed that the presence of some vital insecticidal and antiplasmodial constituents such as terpenoids, flavonoids and alkaloids. The leaf and root extract of *S. acuta* and *V. zizanioides* showed markedly significant antimalarial activity and antivectorial activity effects even at low concentrations. O *S. acuta* and *V. zizanioides* are promising in mosquito control and also safe for the non-target organisms. This integrated application could be useful as alternative synthetic insecticides. These agents should preferentially to be applied in mosquito control strategies to reduce the mosquito populations and prevent the malaria.

Key Words: *Sida acuta, Vetiveria zizanioides,* Antimalarial, antivectorial, *Plasmodium berghei,* Phytochemical, traditional medicine.

INTRODUCTION

Malaria is transmitted by the female *Anopheline* mosquito. In India this species is mainly predominant in urban and rural populations. Malaria is a parasitic disease caused by a protozoan of the genus *Plasmodium*. Most of the lethal cases are caused by *Plasmodium falciparum*, the most virulent of the four *Plasmodia* species that infect humans. Malaria is prevalent in tropical countries with an incidence of 300 million per year and a mortality rate of 8, 81,000 per year. Currently, multi-drug resistance

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has become one of the most important problems impeding malaria control efforts (Htut *et al*., 2009; Sendagire *et al*., 2005). This has led to attempts to discover other antimalarial agents, mainly from plant sources. Medicinal plants may provide Antimalarial drugs directly, as in the case of quinine from cinchona bark, or they may supply template molecules on which to base further new structures by organic synthesis artemisinin from *Artemisia annua*. Pesticides have become a potential hazard of the manufacturer, consumer and the environment. Air, water and food have become contaminated with pesticides as result of their extensive misuse. The risks to humans may be short-term or long-term, depending on the exposure period to these chemicals. The main groups of pesticides of concern are insecticides, herbicides, fungicides and a few soil fumigants. The important and fundamental contribution of the Green Revolution is the application of pesticides to control a wide variety of insectivorous and herbaceous pests that would otherwise diminish the quantity and quality of crops and also food products.

Anopheles stephensi is the principle vector of malaria and it lays egg on clean water. To control malaria the vector mosquito should also be controlled. Repellents play an important role in protecting humans from the bites of insect pests. The phyto-chemicals derived from plant sources can act as antiplasmodial, larvicides, insect growth regulators, repellents and ovipositional attractants and have deterrent activities (Babu *et al*., 1996). *Sida acuta* Burm F. (Malvaceae), locally known as "arivalmukku pachilai" is an erect, branched small perennial herb or small shrub which grows abundantly on cultivated fields, waste areas, roadsides and open clearing in Tamilnadu, India. The bark is smooth, greenish, the root is thin, long, cylindrical and very rough; leaves are lanceolate, the flowers are yellow, solitary or in pairs; seeds are smooth and black. In Indian traditional medicine, the root of *S. acuta* is extensively used as a stomachic, diaphoretic and antipyretic. It is regarded as cooling, astringent, tonic and useful in treating nervous and urinary diseases and also disorders of the blood, bile and liver (Khare *et al*., 2002). *Vetiveris zizanioides or* Vetiver (name derived from Tamil) is a member of the grass family (Poaceae), is native to India (common name: khus), but is widely cultivated in tropical regions of the world. Practical uses of vetiver include the plant (root system) for erosion control (Dalton *et al*., 1996) and the essential oil from the roots for perfumery and aromatherapy. Traditional medicine uses are extensive and occur throughout the world. Constituents of the essential oil of *V. zinzanoides* with known biological activity against insects include sesquiterpenes (3-4 %), sesquiterpenols (18-25%) and sesquiterpenones (7-8%) (Chauhan and Raina, 2005). Hence in the present study an attempt has been made to evaluate the mosquitocidal and antiplasmodial effect of *Sida acuta* and *Vetiveria zizanioides.*

MATERIALS AND METHODS

Collection of eggs

The eggs of *An. stephensi* were collected from field of lab National Institute of Communicable Diseases, Government of India, Mettupalayam, Tamil Nadu, India. The eggs were then brought to the laboratory and transferred to 18 x 13 x 4 cm size enamel trays containing 500 ml water and kept for larval hatching. They were hatched and reared have been still maintained from many generations in the laboratory. The eggs and larvae obtained from this stock were used for different experiments.

Maintenance of larvae and pupae

The larvae reared in plastic cups. They were daily provided with commercial fish food (Lyimo *et al*., 1992). Water was changed alternate days. The breeding medium was regularly checked and dead larvae were removed at sight. The normal cultures as well as breeding cups used for any experimental purpose during the present study were kept closed with muslin

cloth for preventing contamination through foreign mosquitoes. The pupae were collected from culture trays and were transferred to glass beakers containing 500 ml of water with help of a sucker. The glass beaker containing pupae was then kept in 90 x 90 x 90 cm size mosquito cage for adult emergence.

Collection of plant materials and Preparation of phyto extracts

The plants, *Sida acuta* and *Vetiveria zizanioides* was collected in its natural habitat in and around Bharathiar University campus, Coimbatore, Tamil Nadu, India and the herbs were air-dried and ground to provide a fine powder. Extracts were then prepared by soxhlation of the powder with methanol solvent. 200gms of the powder was soxhlated with 1,000 ml of methanol for 24 hours (Vogel, 1978). Upon evaporation under reduced pressure, methanolic extracts were obtained.

Qualitative phytochemical analysis

The preliminary qualitative phytochemical studies were performed for testing the different chemical groups present in *S. acuta* leaf and *V. zizanioides* root methanol extracts of extracts (Nkere and Iroegbu, 2005).

Parasites and Inoculums

Plasmodium berghei were used to assess the in-vivo intrinsic antimalarial activity. The test protocol was based on the 4-day suppressive test described by Peters et al. (Peters *et al*., 1975). Parasite strain was maintained by serial passage of blood from mouse to mouse. A standard inoculum of 1×10^7 of parasitized erythrocytes from a donor mouse in volumes of 0.1ml was used to infect the experimental animals intraperitoneally.

Animals Ethical Clearance Approval

Male albino mice weigh between 27–30g were used for this study. The animals were fed Standard mouse cubes and clean drinking water *ad libitum*. Animals were caged in groups of five. The animals were housed in the Animal House in KMCH, College of Pharmacy, Coimbatore, Tamil Nadu, India. Approval for the study was obtained from the Animal Ethics Committee (CPCSEA/KMCRET/20/2009).

Larval and Pupal Toxicity test

Laboratory colonies of mosquito larvae and pupae were used for the larval and pupal toxicity test. Twenty-five numbers of first, second, third and fourth instar larvae were introduced into the 500 ml glass beaker containing 249 ml of de-chlorinated water and 1ml of desired concentrations of plant extracts was added separately. Larval food was given for the test larvae.Similar procedure was followed for the pupal toxicity assay also. At each tested concentration 2 to 5 trials were made and each trial consisted of five replicates. Mixing 1ml of acetone with 249 ml of de-chlorinated water set up the control. In the plant extracts the larvae exposed to de-chlorinated water without acetone served as control. The control mortalities will be corrected by using Abbott's formula (Abbott, 1925).

$$\text{Corrected mortality} = \frac{\text{Observed mortality in treatment - Observed mortality in control}}{\text{100 - Control mortality}} \times 100$$

$$\text{Percentage mortality} = \frac{\text{Number of dead pupae}}{\text{Number of pupae introduced}} \times 100$$

LC_{50}, LC_{90}, regression equation and 95% confidence limit of lower confidence of limit (LCL) and Upper Confidence Limit (UCL) were calculated from toxicity data by using probit analysis (Finney, 1971).

Acute Toxicity Tests

The oral acute toxicity of the methanol extract was estimated in albino mice (27 - 30g) by medium lethal dose (LD_{50}) described by Lorke's method (1983). A total of twenty albino mice of both sexes were employed, acclimatization period of 24 h was allowed. Prior to the test the mice underwent fasting for whole night. The extract was weighed and dissolved in distilled water. The test was carried out. In the first, the extract was administered orally at doses of 500, 1000, 1500 and 2000 mg/kg to three groups of 5 animals each received respectively.

The animals were monitored for 24 h and numbers of deaths per groups were recorded. Then, the mice were observed continuously for one hr after the treatment; intermittently for four hrs, and thereafter over a period of 24 hrs (Center for Drug Evaluation and Research, 1996). The mice were observed for gross behavioral changes such as feeding, hair erection, lacrimation, mortality and other signs of toxicity manifestation (Pillai and Santhakumari, 1984). The mice have free access to food and clean water during the experiment.

Test on early malaria infection (4-day suppressive test)

Test on early malaria infection followed by Makinde *et al*. (1989). Twenty five mice were divided into five groups of five mice each. Each mouse received standard inoculum of 1×107 *Plasmodium berghei var Anka* infected erythrocytes through the intra-peritoneal route at the commencement of the experiment (day 1). Group's 1-3 mice received 400, 600 and 800 mg extract/kg body weight i.p. respectively. While the 4th group which served as the positive control received 5mg chloroquine/kg body weight, mice in 5th group received 1ml distilled water and served as the negative control. On the fifth day (i.e., day 5) two drops of blood samples from the animals' caudal vein were taken and transferred on slides, thus, making thin film from each mouse and staining with Giemsa stain, Then, each stained slide was examined under the microscope with an oil immersion objective of 100x magnification power to evaluate the percent suppression of each extract with respect to the control groups so that the average percentage (%) parasitaemia could be evaluated as

$$= \frac{\text{Parasitaemia in the control group 1 - Parasitaemia in the drug treated group}}{\text{Parasitaemia in the control group}} \times 100$$

Analysis of results

Blood films were made from the tail of each mouse, fixed with methanol, stained with Giemsa stain and examined under microscope in order to assess the activity of the drug/extract. Percentage parasitaemia in each field was calculated as described above while Average percentage chemosuppression was calculated as:

$$= \frac{\text{Average Parasitaemia in negative control - Average Parasitaemia in drug treated group}}{\text{Average Parasitaemia in negative control}} \times 100$$

The Student's t-test and ANOVA (one- or two-way) were used to test the differences between groups. Differences between means at 5% level ($P \leq 0.05$) were considered significant.

RESULTS

Table 1 illustrates the larval and pupal mortality of *A. stephensi* after the treatment of methanol extract of *V. zizanioides* root. The considerable mortality was evident after the treatment of VZME for the immature stages of *A. stephensi* such as I, II, III, IV instars and pupa. Mortality was increased as the concentration was increased. The concentration employed here was 30, 50.75,100 and125 ppm. Among the different larval stages, the I instar larvae was more susceptible than the other instar larvae. The plant extract also showed considerable pupal mortality. It was recognized that the fourth stage larvae and pupae of mosquitoes were more tolerant to toxicant than early instar. Similar trend has been noticed for all the larval instars and pupae of *A. stephensi* at different concentrations of VZME treatment. The LC_{50} and LC_{90} values were represented as follows: LC_{50} value of I instar was 81.10 %, II instar was 99.09 %, III instar was 107.9 %, IV instar was 120.8 % and pupa was 138.4 %, respectively. The LC_{90} value of I instar 205.6 %, II instar was 228.3 %, III instar was 237.3 %, IV instar was 235.6 % and pupa was 244.7%, respectively.

Table 2 illustrates the larval and pupal mortality of *A. stephensi* after the treatment of methanol extract of *S. acuta* leaf. The considerable mortality was evident after the treatment of SAME for the immature stages of *A. stephensi* such as I, II, III, IV instars and pupa. Mortality was increased as the concentration was increased. The concentration employed here was 30, 50.75,100 and125 ppm. Among the different larval stages, the I instar larvae was more susceptible than the other instar larvae. The plant extract also showed considerable pupal mortality.

It was recognized that the fourth stage larvae and pupae of mosquitoes were more tolerant to toxicant than early instar. Similar trend has been noticed for all the larval instars and pupae of *A. stephensi* at different concentrations of SAME treatment. The LC_{50} and LC_{90} values were represented as follows: LC_{50} value of I instar was 4.99 %, II instar was 5.99 %, III instar was 7.75 %, IV instar was 9.15 % and pupa was 10.84 %, respectively. The LC_{90} value of I instar 17.91 %, II instar was 21.24 %, III instar was 23.32 %, IV instar was 24.33 % and pupa was 23.1 %, respectively.

The mortality rate and the acute toxicity symptoms of orally administered *S. acuta and V. zizanioides* leaf and root extract increased as the dose increased from 500 to 1500 mg/kg (Table 3). The main observed behavioural signs of toxicity were asthenia, piloerection, ataxia, anorexia, urination, diarrhea, lethargy and coma. There were no signs of toxicity as above said. According to Horn (1956) and Rhiouani *et al*. (2008), plants or plant products with LD50 values higher than 2,000–3,000 mg/kg are considered free of any toxicity. This supports the logical usage of this plant in folk medicine practices. All the treated mice were carefully observed for 24 hours for any signs of toxicity (behavioural changes and mortality). D/T: dead/treated mice; none: no toxic symptoms were recorded during the observation period; latency: time to death (in hours) after the dose administration.

Table 4 illustrates the early malaria infection or Peters four days chemo suppressive activity test for the Methanol leaf extract of *S. acuta* against the *P. berghei*. It produced a dose dependent chemo suppression activity and the highest suppression of parasitaemia was observed at the dose of 800mg/kg body weight of mice. Percentage suppression was observed to increase as extract concentration increased. After four days treatment with the different doses, the mean parasitaemia of the test groups ranged from 16.6±0.8% to 36.3±1.1% while the corresponding value of the negative control group being 54.6±0.7%. The mice treated with CQ (Chloroquine) were absolutely clear from the parasites on day four.

Table 5 illustrates the early malaria infection or Peters four days chemo suppressive activity test for the Methanol root extract of *V. zizanioides* against the *P. berghei*. It produced a dose dependent chemo suppression activity and the highest suppression of parasitaemia was observed at the dose of 800mg/kg body weight of mice. Percentage suppression was observed to increase as extract concentration increased. After four days treatment with the different doses, the mean parasitaemia of the test groups ranged from 29.5±1.0% to 41.3±0.9% while the corresponding value of the negative control group being 50.2±0.7%. The mice treated with CQ (Chloroquine) were absolutely clear from the parasites on day four.

Table 6 illustrates the preliminary phytochemical analysis of *S. acuta* leaves and *V. zizanioides* root extract revealed the presence of various chemical compounds in methanol extract. The tests revealed the presence of all the compounds in *S. acuta* leaf extract and at the same time the main compounds like alkaloids and Anthraquinone were mildly present in *V. zizanioides* and certain compounds like Cardenolide were found to be absent in *V. zizanioides*. The appearance of frothing foam indicated the presence of saponins and the cream colored precipitate revealed the presence of alkaloids, the appearance of bluish green colour confirmed the presence of tannins. The sulfuric acid test with the appearance of orange colour precipitate indicated the presence of flavonoids. The Chloroform test confirmed the presence of terpenoids with the appearance of reddish brown colored precipitate and the Borntrager's test and HCL Test indicated the presence of anthraquinone with changing the extract to deep red color.

S.No.	Treatment	Doses (mg/kg/day)	*Average parasitemia in percentage	chemo-suppression (%)	Significance
1.	Extracts	400	36.3±1.1	33.5	$P < 0.05$
2.	Extracts	600	27.4±0.7	49.8	$P < 0.05$
3.	Extracts	800	16.6±0.8	69.6	$P < 0.05$
4.	Chloroquine	5	1.0±0.0	100	-
5.	Distilled water	1ml	54.6±0.7	0	-

vector, *Anopheles stephensi.*

	95% Confidence limit		
	LCL $LC_{50}(LC_{90})$	UCL $LC_{50}(LC_{90})$	X^2
	69.98(172.4)	93.36(269.6)	0.188
	87.24(188.8)	116.5(306.9)	0.041
III	95.13(195.5)	128.6(321.3)	0.078
IV	107.3(197.3)	143.3(308.2)	0.041
Pupa	122.4(205.3)	166.3(319.2)	0.360

Chi square value Significant at P <0.05 level.

Values were presented as Mean ± SD, N= 5

N= Number of experiments

and pupa	30	50	75	100	125	(LC_{90})	Equation	$LC_{50}(LC_{90})$	$LC_{50}(LC_{90})$	
I	40±1.2	45±3.6	53±4.1	60±4.8	72±5.3	4.99(17.91)	Y= -0.496+0.099X	3.50 (14.34)	6.12 (26.09)	0.51
II	37±1.2	44±3.4	50±4.0	57±4.6	67±5.0	5.99(21.24)	Y= -0.504+0.084X	4.53(16.24)	7.49 (34.90)	0.10
III	31±0.7	38±1.2	46±3.6	53±4.0	61±4.8	7.75(23.32)	Y= -0.639+0.082X	6.39 (17.60)	10.14(39.4)	0.18
IV	27±0.7	33±1.0	40±1.2	47±3.6	56±4.1	9.15(24.33)	Y= -0.772+0.084X	7.64 (18.37)	12.42(40.8)	0.07
pupa	18±0.0	24±0.7	33±1.0	42±1.2	50±3.6	10.84(23.1)	Y= -1.132+0.104X	9.22 (18.21)	14.21(34.5)	0.04

Chi square value Significant at P <0.05 level.

Values were presented as Mean ± SD, N= 5.

N= Number of experiments.

X^2= Chi-Square value.

Table 3. Acute oral toxicity of the methanolic leaf and root extracts of *S. acuta* and *V. zizanioides* administered orally to mice.

Dose mg/kg	Mortality		Toxic symptoms
	D/T	Latency(h)	
0	0/5	-	None
500	0/5	-	None
1000	0/5	-	None
1500	0/5	-	None

*Values were presented as Mean ± SEM, n=5.

Table 4. Effects of ethanolic leaf extract of *V.zizanioides* on early malaria infection.

S.No.	Treatment	Doses (mg/kg/day)	*Average parasitemia in percentage	% chemo-suppression	Significance
1.	Extracts	400	41.3±0.9	17.7	$P < 0.05$
2.	Extracts	600	35.7±0.9	28.8	$P < 0.05$
3.	Extracts	800	29.5±1.0	41.2	$P < 0.05$
4.	Chloroquine	5	1.0±0.0	100	-
5.	Distilled water	1ml	50.2±0.7	0	-

* = Values were presented as Mean ± SEM, n= 5

Table 5. Effects of Methanolic leaf extract of *S. acuta* on early malaria infection.

DISCUSSION

Today, the environmental safety of an insecticide is considered to be of paramount importance. The search for herbal preparations that do not produce any adverse effects in the non-target organisms and are easily biodegradable remains a top research issue for scientists associated with alternative vector control (Chowdhury *et al*., 2008). Development of resistance to various types of insecticides such as organochlorides, organophosphates and carbamates (Singh and Bansal, 2001; Bansal and Singh, 2002; Shanmugasundaram *et al*., 2008) poses serious threat to the conventional control measures for vectors. Such insecticides pollute water bodies, air and land.

Methanolic leaf extract of *Cassia fistula* was tested for larvicidal activity *against Cx. quinquefasciatus* and *An. stephensi* (Govindarajan *et al*., 2008). The leaf extract of *Acalypha indica* with different solvents viz, benzene, chloroform, ethyl acetate and methanol were tested for larvicidal, ovicidal activity and oviposition attractancy against *Anopheles stephensi.* The larval mortality was observed after 24 h exposure. The LC_{50} values are 19.25, 27.76, 23.26 and 15.03 ppm, respectively (Govindarajan *et al*., 2008). The extract was found to be more lethal to the larvae of *An. stephensi* than *Cx.quinquefasciatus* with LC_{50} values of 17.97 and 20.57 mg/ l, respectively. The extracts of *Quercus iusitania* var. *infectoria* galls (Oliv.) showed larvicidal activity and their possible use in biological control of *Culex pipines* (Redwane *et al*., 2002). The LC_{50} values are 335 and 373 ppm for the 2nd and 4th instar larvae. The leaf extract of *Cassia fistula* with different solvents viz., methanol, benzene and acetone were studied for the larvicidal, ovicidal and repellent activity against *Aedes aegypti*. The 24 h LC_{50} concentration of the extract against *Aedes aegypti* were observed at 10.69, 18.27 and 23.95 mg/l respectively (Govindarajan, 2009). Similarly the allopathic drug causes many side effects as they are synthetic formulation it creates resistance for the disease and affects the organs too. According to Hilou *et al*. (2006) the stem bark extracts of *Amaranthus spinosus* exhibited higher suppressive activity (with ED_{50}=789.36±7.19 mg/kg) on *P. berghei berghei* in mice. Recently Aarthi and Murugan (2011) studied the *in vivo* antimalarial activity of *Mimosa pudica* and *Phyllanthus niruri* which showed a good activity against the *P.berghei.* Traoré *et al*. (2008) have reported that the extracts of *C. alata* (leaf) showed a significant effect against *P. falciparum* (IC_{50}=80.11 µg/mL) and *P. berghei* (ED_{50}= 112.78 mg/kg). Similar result was obtained in our study also that the leaf extract of S. acuta exhibited higher suppressive activity (with ED_{50}=16.6±0.8 mg/kg) on *P. berghei.*

Hence, in the present study, we sought to determine whether a methanol extract from *S. acuta and V. zizanioides* could be used for vector control as well as parasite control. We observed a functional response by all immature life stages of *A. stephensi* to the methanolic extract of leaves and root of the plant species used in the study. Among the two plant, *S. acuta* was more potential than the *V. zianioides*. Allegorically both allayed the growth of the immature of the mosquito vector and the Antimalarial activity was seen to have more high in *S. acuta*.This biological activity is attributed due to the compounds in the leaves, including alkaloids; flavonoids, phenols, and steroids that together or independently produce morbidity and mortality effects in *P. berghei* and *A. stephensi*. According to literature survey the chemical compound particularly the Cryptolepine was found to present in the leaf extract of *S. acuta* and this might have induced the antiparasitic and antivectorial activity. Allegorically the *V. zizanioides* consists of sesquiterpenoid compounds which is naturally insecticidal and not antiplasmodial and hence the antiplasmodial activity was found to be very less in the root extract of *V. zizanioides*. The application of herbal drugs to different human and animal disease conditions dates back to human history. Plants have been the basic source of sophisticated traditional medicine systems for thousands of years and were instrumental to early pharmaceutical drug discovery and industry

(Elujoba *et al*., 2005). In the present study the methanol extract does not produce any toxic effect to the test animal mice and hence the dose was randomly fixed as 400mg/kg, 600mg/kg and 800mg/kg. The activity against the *P. berghei* (ANKA, CQ sensitive) was merely dose dependent and when the dose increased from 400 to 800mg/kg the chemo-suppression was also increased. Similarly the modern drug (CQ) expressed a 100% suppression of the parasites growth. The initial low percentage chemo-suppression observed in the extract-treated group compared to the chloroquine-treated group may be due to the fact that the extract at the dose administered had not accumulated sufficiently to bring about considerable chemo-suppression (Adebayo *et al*., 2003) or it has a lower speed of action compared to chloroquine. However, the prolonged administration of the extract led to the total clearance of the parasites. This results from accumulation of enough active compounds to effect total clearance of the parasites. After the addition of dosage the percentage chemo-suppression of the extract was favorably comparable to that of chloroquine.

CONCLUSION

Nature nurtures all creatures and use of green products protects the environment and avoids the global warming also. Use of bio-pesticide instead of synthetic pesticides for mosquito control and employing the herbal drug for malaria will reduce the pollution in the environment and avoids the health hazards.

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