

IN VITRO ANTIOXIDANT ACTIVITY AND IN VIVO ANALGESIC, CYTOTOXIC AND CENTRAL NERVOUS SYSTEM DEPRESSION EFFECTS OF ETHANOLIC EXTRACT OF *LEUCAS ZEYLANICA* (L.) W.T. AITON IN SWISS ALBINO MICEMd. Mohsin Mian^{1*}, Fahmida Fyza Redi² and Abdur Rahman³¹Dept. of Pharmacy, Southeast University, Banani, Dhaka, Bangladesh.²Dept. of Pharmacy, North South University, Bashundhara, Dhaka, Bangladesh.³Dept. of Pharmacy, University of Asia Pacific, Green Road, Dhaka, Bangladesh.***Corresponding Author: Md. Mohsin Mian**

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Article Received on 27/11/2016

Article Revised on 18/12/2016

Article Accepted on 08/01/2017

ABSTRACT

Aim: *Leucas zeylanica* (L.) W. T. Aiton can be traced through the medicinal history as a safe ethno medicinal herb and has been put to treat headache, abdominal pain, and convulsion in India, Bangladesh and China in folkloric medicine. The aim of the study was to evaluate CNS depression, cytotoxic, analgesic and antioxidant activities of whole plant extract of *L. zeylanica* to justify the traditional uses as per scientific basis. **Methods:** CNS depressant activity of ethanolic extract was evaluated by hole cross test at dose of 100 and 200 mg/kg. Analgesic activity was determined by acetic acid-induced writhing and formalin-induced paw licking and biting test at 150 and 300mg/kg dose and cytotoxicity and antioxidant activity was assessed by Brine shrimp lethality bioassay and DPPH (1, 1-diphenyl-2-picrylhydrazyl) free radical scavenging assay respectively. The analysis was conducted at the end of 2015. **Result:** The extract of *L. zeylanica* showed a significant dose dependent CNS depressant action by lowering the movement of mice mostly at 60 and 120 minute. The extract also possessed 60.73% ($P < 0.01$) and 81.94% ($P < 0.01$) inhibition in acetic acid induced writhing test and formalin induced paw licking and biting test respectively. Cytotoxicity screening illustrated moderate to potent toxicity to *Artemia salina* with LC_{50} value 11.063 μ g/ml. Non-enzymatic antioxidant effect was exhibited in a concentration-dependent manner. **Conclusion:** The study demonstrated analgesic, cytotoxic, central nervous system depression and antioxidant effect of *L. zeylanica* plant.

KEYWORDS: Analgesic study, CNS activity, antioxidant potential, cytotoxicity, *Leucas zeylanica*.

INTRODUCTION

Medicinal plants which are now available and have become extincted all over the world are a huge source of compounds having therapeutic value and have been being used directly or with modification to treat various ailments since early age of humanity.^[1, 2] According to World health organization (WHO), around 80% of the world population now-a-days is using herbal medicine for some aspects of their primary health care by manipulating almost 70,000 plant species.^[3,4] It is much difficult to synthesize new pure bioactive stereoisomers. Besides, available pharmacologically active compounds exert many undesirable side effects and are not economically friendly. So it has been obligated to consider natural products like medicinal plants as major potential sources of innovative therapeutic agents through investigation and research.^[5] *Leucas zeylanica* (L.) W. T. Aiton belongs to family Lamiaceae. In English this species is named as Ceylon Slitwort, in Hindi Kulunphul and in Bangladesh Kusha or Dholkolosh.^[6] It is native to Southeast Asian countries and an erect, hispid, annual herb.^[7] It is used as

Ayurvedic medicine to treat malaria, cough, burning and abdominal pain and urination. In addition it is used for headache, for scabies, scorpion and snake bites and to treat convulsion caused by fever in some areas such as India, Bangladesh, Srilanka.^[8,9] Though spotlighted plant *L. zeylanica* is used traditionally to treat various diseases but not scientifically justified. Literature survey denotes that there is no report on cytotoxic, CNS depression and analgesic effect of *L. zeylanica*. Since there is a huge lack of existing literature on the activities of the plant, hence we undertook the present study as a primary biological investigation and to develop reports on *L. zeylanica* plant for its CNS depression, cytotoxic, analgesic and antioxidant activities.

In vertebrate species, the central nervous system (CNS) consisted of brain and spinal cord which controls almost all our bodily functions both behavior and movement by releasing various neurotransmitter.^[10] Along with other inhibitory neurotransmitters GABA is the major inhibitory transmitter of brain. Various pharmacologically and clinically active substances such

as benzodiazepines, barbiturates, alcohol and anticonvulsants amplify the release and action of GABA.^[11] In this present study CNS depressant activity test was done for the ethanolic extract of *L. zeylanica* by the hole cross test considering diazepam as standard.^[12]

Cancer is the most hypercritical health burden of modern era both in developed and developing countries because of its treatment procedure.^[13] Cytotoxic botanicals are the source of active anticancer compounds that helps in the treatment of cancer with fewer side effects.^[14] Cytotoxicity is being toxic to cell and cause necrosis or lyses of cell; busulfan, camptothecin, vincristine, paclitaxel are some widely used plant derived cytotoxic substances.^[15] We tested cytotoxicity of ethanolic plant extract of *L. zeylanica* by using the Brine-shrimp lethality bioassay.^[16] Analgesics selectively relieve pain by acting on central or peripheral pain mechanisms, without significantly altering consciousness.^[17] In our study analgesic activity was determined by using two custom methods and diclofenac-Na was taken as standard on mice.^[18] Antioxidants such as tocopherol, polyphenols and glutathione interact with and neutralize free radicals, thus preventing them from causing damage and safe from many chronic diseases, such as cancer, diabetes, aging, and other degenerative diseases.^[19, 20] Antioxidants are sometimes called “free radical scavengers”.^[21, 22] Antioxidant property was tested by DPPH free radical scavenging ability.^[23]

MATERIALS AND METHODS

Plant materials collection and identification

Fresh whole plants of *L. zeylanica* were collected from Sector-15, Uttara, Dhaka, Bangladesh on 20th July, 2015. Then the plants were taxonomically identified by the expert and director of Bangladesh National Herbarium, Dhaka, Bangladesh. (Accession number: DACB-42282).

Extract preparation

Washed plant parts were subjected to air drying for 7 days then pulverized into powder by a grinding machine. About 300gm of powdered material was taken in amber glass containers, soaked in 850ml of ethanol. The containers were kept for a period of 10days accompanying occasional shaking. Then filtration was done by mean of Whatman filter paper (Bibby RE200, Sterilin Ltd., UK) and evaporated at 40°C-50°C temperature. Finally, about 35.07gm extract of *L. zeylanica* was found and kept in a refrigerator.^[24]

Preparing Experimental animal

150 mice of either sex were collected from the International Center for Diarrheal Disease and Research, Bangladesh (ICDDR, B), were kept in standard environmental condition (Relative Humidity 55-65% and probable Temperature 25° C and 12 hours: Light and Dark cycle) and fed the ICDDR, B formulated food and water until the average weight of the mice became 28-30 gm.

Ethical approval

The experimental protocols on animals were approved by Institutional Animal Ethics Committee of Southeast University, Dhaka, Bangladesh.

Reference Drugs and Chemicals

For all investigations, appropriate reference drugs and chemicals were used as positive control: diazepam for CNS depression test, diclofenac-Na for analgesic tests; both are purchased from BASF Aktiengesellschaft, Germany. Dimethyl sulfoxide (DMSO), 1, 1-diphenyl-2picrylhydrazyl (DPPH) all were purchased of SIGMA Company. All other chemicals were of analytical grade.

Phytochemical Screening of the Extract

The extract of *L. zeylanica* (whole plant) was undertaken to qualitative analysis to identify various phytoconstituents such as alkaloids, gums, steroids, flavonoids, reducing sugars, saponins, tannins, glycosides, carbohydrate and phenolic contents. All the identification tests were done by using standard procedures.^[25, 26]

Oral toxicity studies

The acute oral toxicity of plant extract in male Swiss albino mice was studied as per reported method.^[27]

CNS depressant activity test

The purpose of this study was to examine the effect of the ethanolic extract of *Leucas zeylanica* on the emotional behavior of the mice. The method was described by Takagi Watanabe & Saito.^[12] A cage of 30x20x14cm³ was prepared by plywood with a steel partition in the middle of it. A hole of 3 cm diameter was made at a height of 7.5 cm in the center of the partition. The number of passages of a mouse through the hole from one chamber to other was counted for a period of 3 min on 0, 30, 60, 90 and 120 min after the oral treatment with normal saline (10ml/kg), experimental crude extract (at dose of 100 mg/kg and 200 mg/kg) and diazepam (10mg/kg).

Cytotoxicity screening

Following the method (Brine-shrimp lethality bioassay) cytotoxicity of the ethanolic extract of *L. zeylanica* was investigated on a species of brine shrimp, *Artemia salina*. Eggs of the *A. salina*, were collected from an aquarium shop, Katabon, Dhaka, Bangladesh. In artificial sea water (38gm NaCl in 1liter distilled water) the egg was hatched for 48 h to be matured into nauplii.^[28, 29] 1 mg of experimental crude extract was dissolved in dimethyl sulfoxide (DMSO) and the sea water is added by the way of serial dilution technique to attain concentrations of 10µg/ml, 20µg/ml, 40µg/ml, 60µg/ml, 80µg/ml and 100µg/ml. Standard vincristine sulfate served as the positive control.^[30] Then 5 ml sea water along with 10 live brine shrimp nauplii was added to the pre-marked vials.^[31] After 24 hours, the vials were inspected using a magnifying glass to count each alive nauplii.

ANALGESIC ACTIVITY TEST

Acetic acid-induced writhing method

The analgesic activity of the plant extract of *L. zeylanica* was determined by Acetic acid induced writhing method on mice.^[18] Following this method experimental animals were randomly selected for 4 groups (Control, Standard, Group-I and Group-II) consisted of 4 mice. The plant extract was administered orally in two different doses (150 and 300 mg/kg body weight) and tween-80, diclofenac-Na (10mg/kg-body weight) to the mice of test group-I, II and control group, standard group respectively by using a feeding tube. After 15 min, acute pain was induced by intra-peritoneal (i. p.) administration of 20 μ l of 1% acetic acid solution to standard group and after 30 min rest of the mice were administered. Then the mice were placed in observation table and counted the number of writhing for 15minutes.^[32] The incomplete writhing was counted as half writhing.^[33]

Formalin induced paw licking and biting test

Analgesic activity of the plant extract of *L. zeylanica* on mice was determined by using formalin test.^[34] At first experimental animals were randomly selected to create 4 groups (Control, Standard, Test group-I and Test group-II), each consisted of 4 mice. The plant extract was administered orally in two different doses (150 and 300 mg/kg body weight) and tween-80, diclofenac-Na (10mg/kg-body weight) to the mice of Test group-I, II and control group, standard group respectively by a feeding tube. After 15 min acute pain was induced by injecting 20 μ l of 5% formalin into the dorsal surface of the right hind paw to standard group and after 30 min to rest of the mice. Then the number of licking and biting was counted for 30 min. The first 5 min is considered as the early phase and the period between 15 and 30 min as the late phase.

IN VITRO ANTIOXIDANT STUDY

DPPH (1, 1-diphenyl-2-picrylhydrazyl) Free Radical Scavenging Assay

The method, DPPH (1, 1-diphenyl-2-picrylhydrazyl) free radical scavenging assay to determine antioxidant activity was developed by Brand Williams.^[35, 36] A solution of 0.1mM DPPH in methanol was freshly prepared daily before the test and 3ml of the solution was mixed with 1ml methanol solution of *L. zeylanica* plant extract in different concentrations in 5 test tubes. After incubation for 30 minutes the absorbance was measured

at 760 nm using a spectrophotometer against blank.^[37,38] The percentage scavenging activity was calculated from $[(A_0 - A_1)/A_0] \times 100$, where A_0 is the absorbance of the control and A_1 is the absorbance of the extract/ standard.

STATISTICAL ANALYSIS

All the data presented after the analysis were as means \pm standard error of means (SEM). The data of analgesic activity test and CNS depressant test were analyzed by one-way ANOVA followed by least significant difference (LSD) *post hoc* analysis. *P* values <0.01 , <0.05 and <0.001 were considered as statistically significant. The linear regression analysis was used to calculate IC_{50} values using Microsoft Office Excel 2010 in case of antioxidant activity test and cytotoxicity screening.

RESULTS

Phytochemical Screening

The data of preliminary phytochemical screening of ethanol extract of *L. zeylanica* whole plant is provided in Table 1.

Table 1: Phytochemical analysis of ethanolic *L. zeylanica* whole plant extract

Chemical constituents	Existence in LZE
Alkaloids	+
Steroids	+
Flavonoids	+
Tannins	+
Glycosides	+
Phenolic contents	+
Gum	–
Saponins	–
Reducing sugar	–

LZE: *Leucas zeylanica* extract, + indicates presence; – indicates absence of the phytochemical constituents which were screened using various identification methods.

CNS depression activity measurement

The acquired results of hole cross test of *L. zeylanica* are presented in Table-2. The number of hole crossed from one side of the box to another by mice was counted as 1 movement. The depression was most indicatory at 200mg/kg body weight in 5th observation in 120 min compared to the standard diazepam.

Table 2: Effect of LEZ on hole cross test in mice

Animal Group	Observation				
	0 minute	30 minute	60 minute	90 minute	120 minute
Control	8.75 \pm 1.84	8.50 \pm 2.25	8.00 \pm 0.7	6.50 \pm 1.04	6.25 \pm 2.39
Standard	5.25 \pm 2.39	4.00 \pm 1.22	3.5 \pm 0.28	3.25 \pm 1.65*	2.75 \pm 1.33*
LZE (100mg/kg B. wt.)	6.5 \pm 2.79	5.75 \pm 1.75	5.75 \pm 0.47*	4.57 \pm 0.50*	3.75 \pm 0.75*
LZE (200mg/ kg B. wt.)	7.00 \pm 0.41	5.00 \pm 1.75	6.5 \pm 0.70*	3.75 \pm 1.49*	3.60 \pm 1.18*

Values of movement observation expressed as mean \pm SEM (n=5), *significant at $P < 0.05$ when compared to control group. B. wt: Body weight, LZE: *Leucas zeylanica* extract. (n=5 mice).

RESULTS OF THE CYTOTOXICITY TEST

The ethanolic extract of *L. zeylanica* sample gave a significant percentage of mortality compared to the standard. Then from the regression curve of log of

concentration vs. percentage of mortality of nauplii illustrated in Figure-2 LC₅₀ value was determined. The LC₅₀ value of standard and *L. zeylanica* plant extract was accordingly 9.33 and 11.063 µg/ml.

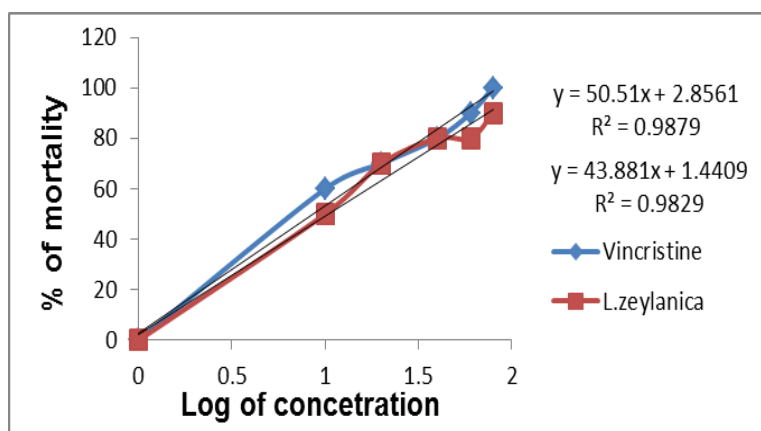


Figure 1: Graphical presentation of percentage (%) of mortality of *Artemia salina* nauplii and Log of concentration of *Leucas zeylanica* extract, vincristine sulphate (standard) in Brine shrimp lethality bioassay

RESULTS OF THE ANALGESIC ACTIVITY TEST

Acetic acid induced writhing method

All the experimental data of plant extract of *L. zeylanica* of writhing test are presented in table 3.

Table 3: Analgesic Activity of LEZ by acetic acid induced writhing test in mice

Animal Group	Writhing count				Mean±SE	% of writhing inhibition
Group-I: (Control)	40	37	47	39	40.75±2.17	0
Group-II: (Standard)	3	20	13	10	11.5±3.52*	71.77
Group-III: LZE (Dose 150mg/kg B. wt)	30	22	27	24	25.75±1.75*	36.80
Group-IV: LZE (Dose 300mg/kg B. wt)	23	13	19	9	16±3.109*	60.73

Values of writhing count expressed as mean±SEM (n=5), *significant at $P < 0.01$ when Compared to control group. B. wt: Body weight, LZE: *Leucas zeylanica* extract. (n=4 mice).

Formalin induced paw licking and biting method

The results of plant extract of *L. zeylanica* on analgesic activity by formalin test are given in table 4. The *L. zeylanica* pretreated animals showed a significant ($p < 0.05$), ($p < 0.01$), ($p < 0.001$) dose-related reduction of paw licking and biting when compared with control and

L. zeylanica treated with the dose of 300 mg/kg showed better activity than standard, diclofenac-Na. At the dose of 300 mg/kg, the plant extract showed 81.94% ($p < 0.001$) inhibition where as standard showed 76.38% inhibition.

Table 4: Analgesic Activity of LEZ by formalin induced paw licking and biting test in mice

Animal Group	Early Phase	Inhibition (%)	Late Phase	Inhibition (%)
Control	23.25±2.39	-	18.00±1.73	-
Standard	7.5±1.94***	79.56	4.25±1.80***	76.38
LZE (Dose 150mg/kg B. wt)	13.75±1.44*	40.86	8.25±1.75**	54.16
LZE (Dose 300mg/kg B. wt)	9.5±1.93***	59.14	3.25±1.65***	81.94

Values of paw licking and biting count expressed as mean ± SEM (n=5), ***, **, * significant at $P < 0.001$, $P < 0.01$, $P < 0.05$ respectively when compared to control group. B. wt: Body weight, LZE: *Leucas zeylanica* extract. (n=4 mice)

DPPH radical scavenging activity

Data of antioxidant test by DPPH radical scavenging method are set in the table 5 and from the regression curve of concentration vs. percentage of inhibition IC_{50}

value was determined. The IC_{50} values of standard and *L. zeylanica* extract are $13.425\mu\text{g}/\text{mg}$ and $15.94\mu\text{g}/\text{mg}$ respectively.

Table 5: DPPH free radical scavenging activity of BHT (Standard) and LZE

Concentration($\mu\text{g}/\text{ml}$)	% of inhibition of BHT	% of inhibition of LZE
7.812	48.25	48.22
15.625	58.5	50.17
31.25	60.44	57.50
62.5	75.17	78.22
125	89	81.75

BHT: Butylatedhydroxy Toluene, LZE: *Leucas zeylanica* extract.

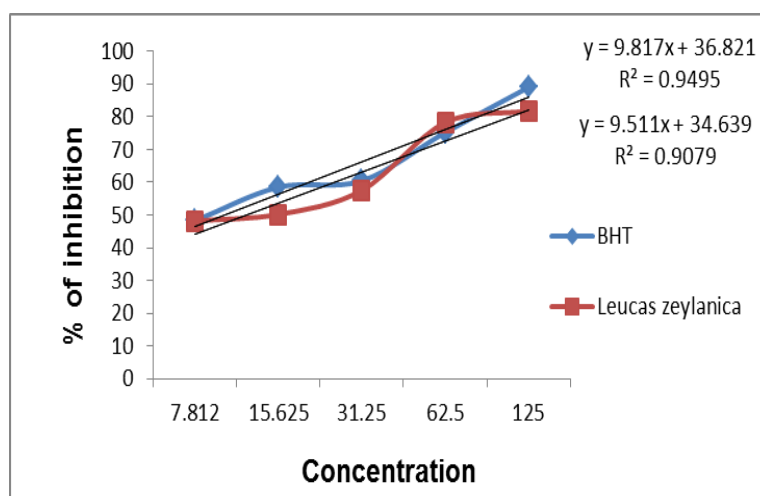


Figure 2: Standard curve of inhibition in different concentration for butylated hydroxyl toluene (BHT) and *Leucas zeylanica* extract in DPPH free radical scavenging activity for non-enzymatic antioxidant test

DISCUSSION

The most admissible process of evaluating drug action on CNS objectively is to observe its effect on locomotors activity, increase of the activity refers alertness and decrease is considered as sedation.^[39, 40] The plant extract of *L. zeylanica* decreased the locomotors activity of the mice in hole cross test significantly ($p < 0.05$) in 120 minute at the dose of $100\text{mg}/\text{kg}$ and continued up to 90 and 120 minute at the dose of $200\text{mg}/\text{kg}$. The effect of the extract is very much close or similar to the effect of standard diazepam. Benzodiazepines bind to the binding sites of GABA receptors and the binding enhances GABA-ergic transmission and causes membrane hyperpolarization.^[41] From recent researches it has been established that various flavonoids and neuroactive steroids were found to be ligands for the GABA receptors in the central nervous system; that can act as benzodiazepine-like molecules.^[42] Phytochemical screening of ethanol extract of *L. zeylanica* indicates the presence of steroids, flavonoids.

Cytotoxic compounds refer to as antitumor would be detected significantly by brine shrimp lethality bioassay. Showing toxicity to *Artima salina* is comparable to the toxicity to human solid tumor cell lines.^[43, 44] In comparison to the standard vincristine sulfate, the

percentage mortality increased as the log concentration increased. The LC_{50} value for the standard is $9.33\mu\text{g}/\text{ml}$ and for the crude extracts of *L. zeylanica* was $11.063\mu\text{g}/\text{ml}$ respectively. This result illustrates the plant as moderate to potent toxic to biological system.

Acetic acid induced writhing method for analgesic test is considered to determine peripherally acting nociception induced by the release of free arachidonic acid from the tissue phospholipid which enhance levels of PGE2 and PGF2 α .^[45] By viewing the result, it seems that the plant extract has inhibitory effect on the cyclooxygenase pathway to control peripheral pain. Among various phytoconstituents flavonoids are known to inhibit the prostaglandins synthesis.^[46] Hypothetically the plant extract shows analgesic action due to the presence of flavonoid. On the contrary, the formalin test is another significant analgesic test which is better related to clinical pain.^[47, 48] caused by both central and peripheral activities. This type of nociception has two phases; first phase is neuropathic pain caused by the direct stimulation of sensory nerve fibers and second one is inflammatory pain by various prostaglandins, TNF α like mediators.^[49, 50] The percentage of inhibition of the plant extract at $300\text{mg}/\text{kg}$ is 81.94% ($p < 0.001$) which is greater than comparing with the percentage of inhibition

of standard diclofenac-Na. The extract showed analgesic effects especially to neuropathic pain by controlling the increase of Ca^{2+} through TRPA1, presumably evoked by formalin on CNS. The presence of flavonoids, saponins, tannins and steroids might give analgesic effect either singly or synergistically.^[51]

Oxidative stress is the root cause of aging and various human diseases like arteriosclerosis, stroke, diabetes, cancer and neurodegenerative diseases such as Alzheimer's and Parkinson's disease now-a-days.^[52] During investigation the *L. zeylanica* plant extract was able to convert violet color DPPH solution to yellow color in a concentration dependent manner which revealed the ability of the extract to reduce stable free radicals of DPPH. The maximum percentage of scavenging free radicals of *L. zeylanica* extract is 81.75% which is very close to the BHT standard. The lower IC_{50} value indicates higher antioxidant capacity^[36] and extract of *L. zeylanica* showed almost similar radical scavenging activity as compared to standard BHT. The present results suggest that the tested plant extract has moderate to potent antioxidant activity. This antioxidant potential of *L. zeylanica* could be correlated with the *in vivo* documented effects of several other botanicals that contain significant proportions of polyphenols and flavonoids.^[53]

CONCLUSION

The results obtained from our current investigation establish the potential of the selected plant as CNS depressant agent and analgesic. Besides, the findings also demonstrate the scientific rationale for the folkloric uses of the plant in management of pain. From the study, we can also gaze the ethanolic extract of *L. zeylanica* possess moderate cytotoxicity and antioxidant activity which encourages the use of the plant in the management of tumor cells and in oxidative stress. Nevertheless, further research is needed towards fractionation of the extract and isolation, identification of active principles present in the extract as well as their mechanism of actions. Hence, the present study concludes that the selected plant *Leucas zeylanica* could be a prestigious candidate for further chemical and pharmacological investigation for better use as medicine.

ACKNOWLEDGEMENT

The authors gratefully acknowledge the logistic support provided by the Southeast University, Bangladesh to carry out this research.

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