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ANALGESIC AND ANTI-INFLAMMATORY ACTIVITY OF SEED EXTRACTS OF *DIPLOCYCLOS PALMATUS* (L) C. JEFFREY.

PARAG KADAM AND SUBHASH L. BODHANKAR*.

Department of Pharmacology, Poona College of Pharmacy,
Bharati Vidyapeeth Deemed University, Pune 411038, India.

ABSTRACT

Aim: To investigate the analgesic and anti-inflammatory properties of seed extracts of *Diplocyclos palmatus* (L) C. Jeffrey.

Methods: Three extracts of different polarity of *Diplocyclos palmatus* (L) C. Jeffrey seeds were tested for their analgesic and anti-inflammatory activities. Analgesic activity was evaluated using the hot plate and acetic acid-induced writhing in mice. Anti-inflammatory activity was evaluated using carrageenan induced rat paw edema and cotton pellet granuloma in rats.

Results: Pretreatment with ethanolic extract of *Diplocyclos palmatus* (L) C. Jeffrey at 100, 200 and 400 mg/kg showed significant dose dependent analgesic and anti-inflammatory effects in acetic acid induced pain model, carrageenan induced rat paw edema and cotton pellet granuloma models, while petroleum ether extract and aqueous extract of *Diplocyclos palmatus* (L) C. Jeffrey at 100, 200 and 400 mg/kg showed weak analgesic and anti-inflammatory effects.

Conclusion: The findings in this study suggest that the ethanolic seed extract of *Diplocyclos palmatus* (L) C. Jeffrey possesses analgesic and anti-inflammatory activities.

KEY WORDS: Analgesic, Anti-inflammatory, *Diplocyclos palmatus* (L) C. Jeffrey, Carrageenan, Cotton pellet.

SUBHASH L. BODHANKAR
Department of Pharmacology, Poona College of Pharmacy,
Bharati Vidyapeeth Deemed University, Pune 411038, India.

INTRODUCTION

Diplocyclos palmatus (L) C. Jeffrey syn *Bryonia lacinosa* (N.O. Cucurbitaceae) plant locally known as 'Shivlingi' is distributed throughout India, an annual climber with bright red fruit and is reported to be highly medicinal [1]. Locally in India its seeds are being used for promoting conception in women. Plant is used against snake-bite. Its leaves are used in inflammation [2]. Roots are used for treatment of asthma. The seeds are used for increasing sperm count also as an aphrodisiac [3]. The main active constituents of the plants are Bryonin, a bitter principle [4]; punicic acid, source of seed oil [5]; non ionic glucomannon [3]; and goniothalamin [6]. Non-steroidal anti-inflammatory drugs (NSAIDs), steroidal drugs, and immuno-suppressant drugs, which have been used usually in the relief of inflammatory diseases by the people of the world for a long time. However, these drugs were often associated with severe adverse side effects, such as gastrointestinal bleeding and peptic ulcers [7]. Recently, many natural medicines derived from plants, marine organisms were considered as the effective and safer for the treatment of various diseases including inflammation and pain [8]. The objective of the study was to evaluate the analgesic and anti-inflammatory activities of extracts of the seeds of *Diplocyclos palmatus* (L) C. Jeffrey in rodents.

MATERIALS AND METHODS

(i) Procurement of Plant material

Seeds of *Diplocyclos palmatus* (L) C. Jeffrey were obtained from a commercial supplier of Pune and then identified and authenticated by Department of Botany, Agharkar Research Institute, Pune, India and voucher specimen (No. S - 131) is deposited at that Institute.

(ii) Extraction Procedure

The seeds of the plants were dried in shade and powdered. Dried powder (300 g) was subjected to successive extraction in soxhlet extractor as per standard procedure using petroleum ether (40-60^{o}C) and ethanol at their boiling point for 48 h. The marc obtained from the ethanol extraction was further utilized for aqueous extraction by maceration for 48 hrs. The percentage yields of petroleum ether extract of *Diplocyclos palmatus* (L) C. Jeffrey (PEEDP) was 3.5 g, ethanol extract of *Diplocyclos palmatus* (L) C. Jeffrey (EEDP) was 4.5 g and aqueous extract of *Diplocyclos palmatus* (L) C. Jeffrey (AEDP) was 8 g. All the extracts were subjected to phytochemical and pharmacological screening.

(iii)Phytochemical analysis

Preliminary phytochemical studies of all the extracts were performed for carbohydrates, proteins, amino acids, fats and oil, steroids, volatile oil, glycosides, alkaloids, flavonoids, triterpenoids, tannins and phenolic compounds using standard procedures [9].

(iv) Drugs and Chemicals

Carrageenan (Sigma-Aldrich, St. Louis, MO, USA), Acetic acid (Pure Chem. Ltd., India), petroleum ether (Merck, India), ethanol (Qualigens, India), formaldehyde (British Drug House, India) and Tween 80 (Research Lab, India) were purchased from authorized vendors.

(v) Experimental animals

Female wistar rats weighing 200-250 g and female Swiss albino mice weighing 20-25 g were used for the study. The animals were procured from National Toxicology Centre, Pune and housed in the animal house maintained under standard hygienic conditions, at 25 ± 2^{o}C, humidity (60 ± 10%) with 12 hour day and night cycle, with food and water *ad libitum*. The study was carried out as per CPCSEA norms after obtaining approval (CPCSEA/01/2011) from the Institutional Animal Ethical Committee of college.

(vi) Acute oral toxicity study

Healthy female Swiss albino mice were subjected to acute toxicity studies as per OECD guidelines-425. The animals were fasted overnight and divided into groups with 5 animals in each group. Extracts (EEDP, PEEDP and

AEDP) were administered orally at one dose level of 2000 mg/kg body weight. The mice were observed continuously for behavioral and autonomic profiles for 2 hrs and for any sign of toxicity or mortality up to 48 hrs [10].

(vii) Eddy's hot plate method in mice.

The mice were divided into eleven groups of 6 mice each. Group 1 served as Vehical control (2% Tween 80). Group 2 served as standard (pentazosin, 30 mg/kg, p.o.). Group 3, 4 and 5 were treated orally with EEDP 100, 200 and 400 mg/kg body weight respectively. Group 6, 7 and 8 were treated orally with PEEDP 100, 200 and 400 mg/kg body weight respectively. Group 9, 10 and 11 were treated orally with AEDP 100, 200 and 400 mg/kg body weight respectively. The animals were individually placed on the hot plate (UGO Basile, Italy) maintained at 55 ± 1^{o}C, one hour after their respective treatments. The response time was noted as the time at which animals reacted to the pain stimulus either by paw licking or jump response, whichever appeared first. The cut off time for the reaction was 15 seconds [11].

(viii) Acetic acid induced writhing in mice.

Acetic acid solution at a dose of 10 ml/kg (0.6%) was injected i.p. and the number of writhes during the following 15 min period was observed. Significant reductions in number of writhes by drug treatment as compared to vehical treatment (vehical control) animals were considered as a positive analgesic response. The mice were divided into eleven groups of 6 mice each. Group 1 served as vehical control (2% Tween 80). Group 2 served as standard (acetylsalicylic acid, 100 mg/kg, p.o.). Group 3, 4 and 5 were treated orally with EEDP 100, 200 and 400 mg/kg body weight respectively. Group 6, 7 and 8 were treated orally with PEEDP 100, 200 and 400 mg/kg body weight respectively. Group 9, 10 and 11 were treated orally with AEDP 100, 200 and 400 mg/kg body weight respectively. The percent inhibition of writhing was calculated as follows:

% Inhibition = (VC-VT/VC) 100

VT, number of writhes in drug treated mice. VC, number of writhes in control group of mice [12].

(ix) Carrageenan induced paw edema in rats.

Female wistar rats were divided into eleven groups of 6 rats each. Group 1 served as vehical control (2% Tween 80). Group 2 served as standard (diclofenac, 10 mg/kg, p.o.). Group 3, 4 and 5 were treated orally with EEDP 100, 200 and 400 mg/kg body weight respectively. Group 6, 7 and 8 were treated orally with PEEDP 100, 200 and 400 mg/kg body weight respectively. Group 9, 10 and 11 were treated orally with AEDP 100, 200 and 400 mg/kg body weight respectively. The test samples or vehical were administered orally to experimental animals once a day for a period of 7 days. One hour after the last administration, acute paw edema was induced by subplanter injection of 0.1 ml of 1% freshly prepared carrageenan suspension in normal saline into the right hind paw of each rat. The paw volume was measured before (0 h) and at intervals of 1, 2, 3, 4, 5, 6 and 24 hr after carrageenan injection using plethysmometer (UGO Basile, Italy). Then percentage of inhibition of edema was calculated for each group with respect to the control group as follows,

% Inhibition of paw edema = (VC-VT/VC) 100

VC and VT represent average paw volume of control and drug treated animals respectively [13].

(x) Cotton pellet granuloma in rats

Eleven groups of animals were used in this model. Subacute inflammation was produced using cotton pellets. Under aseptic precautions, an incision was made on the back of each rat in each group and sterile cotton pellets (50 ± 1) mg were implanted subcutaneously in the axilla under ether anesthesia. Group 1 served as vehical control (2% Tween 80). Group 2 served as standard (diclofenac, 10 mg/kg, p.o.). Group 3, 4 and 5 were treated orally with EEDP 100, 200 and 400 mg/kg body weight respectively. Group 6, 7 and 8 were treated orally with PEEDP 100, 200 and 400 mg/kg body weight respectively. Group 9, 10 and 11 were treated orally with AEDP 100, 200 and 400 mg/kg body weight respectively for six days. On the seventh day, animals were sacrificed; stomach and pellets were removed along with the granulation tissue and pellets were dried at 60^{0}C for 24 hrs.

The net dry weight of the granuloma was determined. Stomach was cut open in greater curvature and ulcer scoring was done. Histopathological studies were performed on stomach to confirm ulcer score [14].

(xi) Statistical analysis

The data was analyzed by one way ANOVA followed by Dunnett's test, two way ANOVA followed by Bonferroni's post hoc test. All statistical analyses were performed using Graph Pad Prism software (San Diego, CA). Data was considered statistical significant at $P<0.05$.

RESULT

1. Phytochemical screening

The PEEDP contained fats and oils, volatile oil, flavonoids, alkaloids, tannins and phenolic compounds. The AEDP contained carbohydrates, proteins, amino acids, glycosides, alkaloids, tannins and phenolic compounds. The EEDP contained fats and oils, steroids, volatile oil, saponins, glycosides, flavonoids, alkaloids, tannins, phenolic compounds and triterpenoids.

2. Acute oral toxicity

Administration 2000 mg/kg of all the three extracts of *Diplocyclos palmatus* (L) C. Jeffrey did not produce any behavioral abnormalities and mortality. So the dose selected for further study was 100, 200 and 400 mg/kg for each extracts.

3. Hot plate test

All the three extracts did not exhibit analgesic activity. In contrast, pentazocin at the dose of 30 mg/kg, p.o. significantly ($P<0.001$) increased pain threshold of mice compared to the vehical control group at 60 and 90 min.

4. Acetic acid writhing response

As shown in Table 1, the EEDP significantly ($P<0.01$) inhibited the acetic acid induced writhing response at the dose of 400 mg/kg, while 100 and 200 mg/kg showed non-significant inhibitory response. However there was no significant inhibition in the acetic acid induced writhing on treatment with AEDP (100, 200 and 400 mg/kg) and PEEDP (100 and 200 mg/kg). Acetylsalicylic acid (ASA) at 100 mg/kg markedly decreased the number of writhing ($P<0.001$), and inhibited the writhing response by 40 %.

Table 1

Effect of oral administration of Diplocyclos palmatus (L) C. Jeffrey on acetic acid induced writhing in mice.

Group	Treatment	No. of writhes (Mean ± SEM)	% Inhibition
1.	Vehical control	65 ± 1.40	
2.	Acetylsalicylic acid 100 mg/kg	39 ± 1.60 ***	40
3.	EEDP 100 mg/kg	64 ± 1.50	1.54
4.	EEDP 200 mg/kg	60 ± 1.30	7.69
5.	EEDP 400 mg/kg	57 ± 1.40 **	12.31
6.	PEEDP 100 mg/kg	66 ± 1.90	-1.54
7.	PEEDP 200 mg/kg	62 ± 1.10	4.62
8.	PEEDP 400 mg/kg	60 ± 0.60	7.69
9.	AEDP 100 mg/kg	66 ± 1.80	-1.54
10.	AEDP 200 mg/kg	61 ± 1.40	6.15
11.	AEDP 400 mg/kg	61 ± 1.30	6.15

Data are expressed as mean ± S.E.M.; n=6 mice per group. One way ANOVA followed by Dunnett's test when compared with normal control *P<0.05, **P<0.01, ***P<0.001.

5. Carrageenan induced rat paw edema

The EEDP, PEEDP and AEDP were administered for 7 days before the injection of carrageenan caused dose dependent inhibition of increase in paw edema from 1 h to 5 h. The inhibitory effect of the EEDP was recorded with a dose of 200 and 400 mg/kg at 3rd h (7.75 %), (9.55 %) and at 5th h (29.50), (30.98) respectively. Diclofenac (10 mg/kg) were administered 1 h before the injection of

carrageenan caused significant (P<0.001) inhibition of increase in paw edema at 5th h. The inhibitory effect of the diclofenac at 10 mg/kg was recorded (9.82 %) at 3rd h and (31.49 %) at 5th h. The inhibition elicited by the EEDP was comparable to that of diclofenac. However there was no significant inhibition in increase in paw edema on treatment with PEEDP and AEDP. (Table 2)

Table 2
Effect of Diplocyclos palmatus (L) C. Jeffrey on inhibition of right hind paws edema on carrageenan induced inflammation in rats.

Group	Change in paw edema volume			% Inhibition at		
	1 h	3 h	5 h	1 h	3 h	5 h
Carrageenan control	1.11 ± 0.061	3.06 ± 0.052	3.94 ± 0.072			
Diclofenac 10 mg/kg	1.06 ± 0.011	2.76±0.064**	2.70 ± 0.064******	4.07	9.82	31.49
EEDP 100 mg/kg	1.11 ± 0.018	3.07 ± 0.034	3.88 ± 0.075	-0.15	-0.49	1.52
EEDP 200 mg/kg	1.08 ± 0.014	2.82 ± 0.039*	2.78 ± 0.035*****	2.56	7.75	29.50
EEDP 400 mg/kg	1.06 ± 0.011	2.76±0.044**	2.72 ± 0.044******	4.22	9.55	30.98
PEEDP 100 mg/kg	1.10 ±0.005	3.09 ± 0.035	4.06 ± 0.113	0.90	-1.09	-3.09
PEEDP 200 mg/kg	1.09 ± 0.015	3.09 ± 0.044	3.91 ± 0.048	1.06	-0.98	0.80
PEEDP 400 mg/kg	1.08 ± 0.010	3.05 ± 0.045	3.67 ± 0.071*	1.96	0.27	6.90
AEDP 100 mg/kg	1.08 ±0.013	3.18 ± 0.042	3.83 ± 0.076	2.71	-3.98	2.75
AEDP 200 mg/kg	1.08 ± 0.011	3.09 ± 0.047	3.83 ± 0.023	2.26	-1.25	2.75
AEDP 400 mg/kg	1.07 ± 0.018	3.08 ± 0.051	3.83 ± 0.055	3.32	-0.87	2.75

Data are expressed as mean ± S.E.M.; n=6 rats per group. Two way ANOVA followed by Bonferroni's post hoc test when compared with carrageenan control *P<0.05, **P<0.01, ***P<0.001.

6. Cotton pellet granuloma

The effects of EEDP, PEEDP, AEDP and diclofenac on cotton pellet induced granuloma in rats are shown in Table 3. EEDP (400 mg/kg) and diclofenac (10 mg/kg) significantly (P<0.001) inhibited the granuloma formation, while EEDP (200 mg/kg) and PEEDP (400 mg/kg) significantly (P<0.01 and P<0.05, respectively) inhibited the granuloma formation compared with control group. However there was no significant inhibition in granuloma formation on treatment with EEDP (100 mg/kg), PEEDP (100 and 200 mg/kg) and AEDP (100, 200 and 400 mg/kg).

Table 3
Effect of oral administration of Diplocyclos palmatus (L) C. Jeffrey on cotton pellet granuloma in rats.

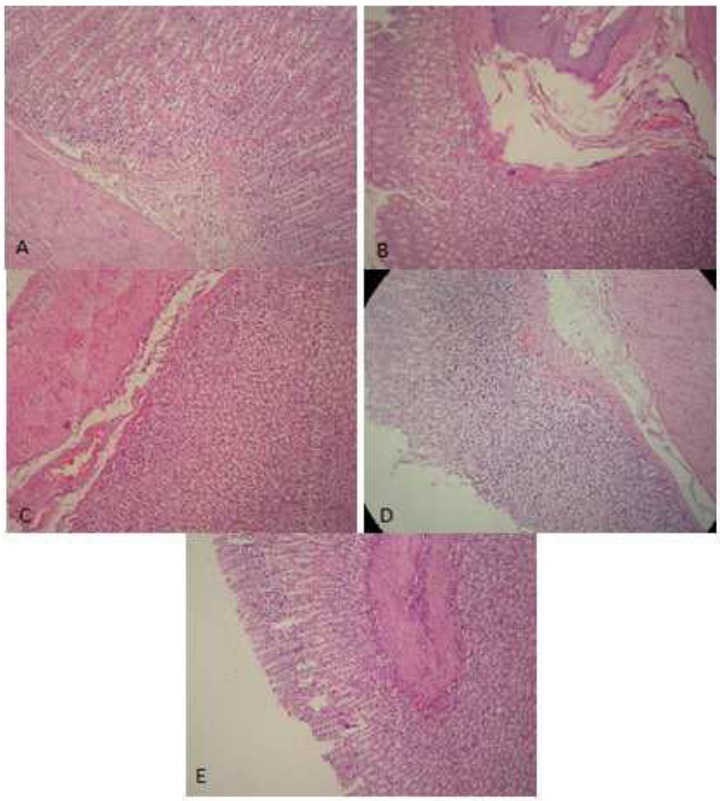
Group	Treatment	Dry granuloma weight (mg) (Mean ± SEM)	% Inhibition
1.	Vehical control	92.2 ± 2.55	
2.	Diclofenac 10 mg/kg	25 ± 1.69***	72.82
3.	EEDP 100 mg/kg	91 ± 2.4	1.09
4.	EEDP 200 mg/kg	78 ± 3.1**	15.22
5.	EEDP 400 mg/kg	42 ± 1.8***	54.35
6.	PEEDP 100 mg/kg	92 ± 3.3	0
7.	PEEDP 200 mg/kg	87 ± 2.32	5.43
8.	PEEDP 400 mg/kg	82 ± 2.99*	10.87
9.	AEDP 100 mg/kg	96 ± 2.5	-4.35
10.	AEDP 200 mg/kg	87.2 ± 1.11	5.43
11.	AEDP 400 mg/kg	84.8 ± 2.29	7.61

Data are expressed as mean ± S.E.M.; n=6 rats per group. One way ANOVA followed by Dunnett's test when compared with normal control *P<0.05, **P<0.01, ***P<0.001.

All the extracts at doses 400 mg/kg showed a less ulcer score compared to the standard group (diclofenac 10 mg/kg). Histopathology of stomach of normal rats showed intact gastric mucosa, no

ulceration was observed. Diclofenac treated rats showed ulceration and congestion. EEDP, PEEDP and AEDP at 400 mg/kg treated rats showed lesser ulceration and no congestion. (Figure 1)

Figure 1
Histopathology of stomach.



(A) Normal control (B) Diclofenac 10 mg/kg treated (C) EEDP 400 mg/kg treated. (D) PEEDP 400 mg/kg treated, (E) AEDP 400 mg/kg treated.

DISCUSSION

The present investigation was designed to evaluate the use of *Diplocyclos palmatus* (L) C. Jeffrey for pain and inflammation. Three extracts of different polarity of *Diplocyclos palmatus* (L) C. Jeffrey seeds were tested for their analgesic and anti-inflammatory activities.

Analgesic activity

In this study, the analgesic activity of *Diplocyclos palmatus* (L) C. Jeffrey was investigated using the hot plate and mouse writhing tests. The writhing model is a sensitive method for screening peripheral analgesic efficacy of compounds and it is more sensitive to non-steroidal analgesics [15]. The effect of acetic acid is due to the liberation and increased level of several mediators such as histamine and serotonin which act by stimulation of peripheral nociceptive neurons [16]. The results of this study suggested that the EEDP mediate its analgesic activity peripherally and this could be attributed to the blocked of mediators like histamine and serotonin [17]. It has been reported that aspirin and other NSAIDs inhibit a number of writhes in this model by inhibition of cycoloxygenase enzyme in peripheral tissues, thus interfering with the mechanism of transduction in primary afferent nociceptors by blocking the effect or the synthesis and/or release of inflammatory mediators [18]. The results of experiments in hot plate test indicated

absence of hot plate central analgesic activity of test extracts.

Anti-inflammatory activity

The anti-inflammatory activity of *Diplocyclos palmatus* (L) C. Jeffrey, in this study was investigated using the carrageenan induced paw edema and cotton pellet granuloma tests. Carrageenan is a family of linear sulphated polysaccharides extracted from the red seaweed marine alga *Chondrus crispu.* Lambda carrageenan is used in animal models of inflammation to test analgesics, because dilute carrageenan solution (1 %) injection causes swelling and pain [19]. Carrageenan has been widely used as a noxious agent able to induce experimental inflammation for the screening of compounds possessing anti-inflammatory activity. This phlogistic agent, when injected locally into the rat paw, produced a severe inflammatory reaction, which was discernible within 30 min [20]. The development of edema induced by carrageenan is a biphasic event: the early phase (90–180 min) of the inflammation is due to the release of histamine, serotonin and similar substances. The later phase (240–360 min) is associated with the activation of kinin-like substances and the release of prostaglandins, proteases and lysosome [21]. EEDP might inhibit the later phase of the carrageenan-induced edema by reducing the release of prostaglandins, proteases and lysosome; while the PEEDP and AEDP might inhibit particularly the later phases by restraining the kinin-like substances and prostaglandins productions.

Chronic inflammation is the reaction arising when the acute response is insufficient to eliminate the pro-inflammatory agents. Chronic inflammation includes a proliferation of fibroblasts and infiltration of neutrophils with exudation of fluids. It occurs by means of development of proliferative cells which can either spread or form granuloma. Efficacy of anti-inflammatory agents in chronic inflammatory states is indicated by their ability to inhibit the increase in the number of fibroblasts during granular tissue formation [22]. In the cotton pellet-induced granuloma formation, EEDP was effective in inhibiting the transudative phase and proliferative phase of inflammation. Non-steroidal anti-inflammatory agents such as aspirin give inhibition only whereas steroidal anti-inflammatory agents have a strong inhibition on both the transudative and proliferative phase [23]. EEDP was effective in both carrageenan-induced paw edema as well as cotton pellet granuloma and it can be assumed that it is effective in all the phases of inflammation i.e., acute, subacute and proliferative phases.

Evaluation of the ulcerogenic effect of the extracts on the rat stomach revealed a lesser ulceration of the gastric mucosa. Ulceration of the gastric mucosa by anti-inflammatory drugs usually indicates that prostaglandin synthesis inhibition may be involved in their mechanisms of action. Inhibition of the synthesis of prostaglandin, a group of prostanoid mediators of inflammation and intact gastric mucosa is largely responsible for the anti-inflammatory and gastric ulceration effects of NSAIDs. Consequently, the irritant effect of the extract on the rat gastric mucosa suggests that the extract, like diclofenac, may inhibit prostaglandin synthesis [24]. Phytochemical analysis of the *Diplocyclos palmatus* (L) C. Jeffrey seeds has mainly demonstrated the presence of saponins, flavonoids, terpenoids and steroids. Steroids can decrease inflammation and reduce the activity of the immune system, while triterpenoids impairs histamine release from mast cells and exerts anti-inflammatory effects [25]. Flavonoids are often used for their antioxidant effect against free radicals. There are also strong indications that they have antiviral, anti-inflammatory and anti-hypertensive properties [26]. We propose that the analgesic and anti-inflammatory activity of the EEDP seeds could be due to combined effect of flavonoids, saponins, steroids and triterpenoids, which are the major components of the ethanol extract of this species.

CONCLUSION

The analgesic and anti-inflammatory properties of *Diplocyclos palmatus* (L) C. Jeffrey can be considered an effective agent to treat inflammatory diseases. This plant, mainly its seeds, demonstrated a high activity for ethanol extract at doses (200 and 400 mg/kg). The study corroborated the analgesic effects of this species, justified and supported scientifically its ethnopharmacological use as an anti-inflammatory agent to treat pain and inflammation.

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