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Research Article

# Evaluation of peripheral and central analgesic activity of ethanolic extract of *Clerodendrum infortunatum* Linn. in experimental animals

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## ABSTRACT

**Background:** *Clerodendrum infortunatum* Linn. (Verbenaceae) is an important and widely used medicinal plant. Though variously used in Ayurveda, Unani, and Homeopathy system of medicine in the case of ailments such as diarrhoea, skin disorders, venereal and scrofulous complaints, wounds, post-natal complications, as anti-helminthic, and external applications on tumors, the plant needs thorough investigation for its specific medicinal activity. This study evaluates both the central and peripheral analgesic effect of the ethanolic extract of the leaves of *C. infortunatum* Linn. (EECI) in the experimental animals.
**Methods:** Acute toxicity test was done following the Organization of Economic Co-operation and Development guidelines. EECI (100 mg/kg, 200 mg/kg, and 400 mg/kg body weight [b.w.] p.o) was evaluated for central analgesic activity by the tail flick method and peripheral analgesic activity by the acetic acid (0.7%) induced writhing test, respectively. Using aspirin (300 mg/kg b.w. and 100 mg/kg b.w.) as the standard drug.
**Results:** EECI significantly decreased the number of writhing in writhing test at all the doses ($p<0.01$) and increased the reaction time in tail-flick method ($p<0.01$) at all the doses. EECI in the dosage of 400 mg/kg b.w. produced effects which was comparable with that of the standard drug aspirin ($p<0.001$) in writhing test ($p<0.001$) and tail flick method ($p<0.001$).
**Conclusion:** The study showed significant central and peripheral analgesic activity of EECI which may be attributed to the inhibition of prostaglandin synthesis, phospholipase A2, and tumor necrosis factor alpha. *C. infortunatum* Linn. as a commercial source of analgesic drug should be subjected to further research.

**Keywords:** *Clerodendrum infortunatum*, Analgesia, Writhing test, Tail flick method

## INTRODUCTION

One of the most common reasons an individual seeks the advice of a physician is because he or she is in pain. Pain was called by Sherrington, "the physical adjunct of an imperative protective reflex." Painful stimuli generally initiate potent withdrawal and avoidance responses. It is a protective mechanism and occurs whenever any tissues are being damaged, causing the individual to react to remove the pain stimulus. Pain differs from other sensations in that it sounds a warning that something is wrong, preempts other signals, and is associated with an unpleasant affect.[1,2]

The International Association for the Study of Pain (IASP) defines pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage." This is to be distinguished from the term nociception which the IASP defines as the unconscious activity induced by a harmful stimulus applied to sense receptors. Pain, therefore, is a perception similar to vision and hearing and is a symptom that cannot be objectively assessed.[1,3,4] Pain sensation involves several components: motivational, emotional, sensory-discriminative, affective, and cognitive aspects. Perception of pain and response to analgesic drugs are complex processes that involve multiple biochemical pathways. Each of these pathways is influenced by significant genetic factors that may modify pain perception and/or response to analgesics. There is a wide range of inter-individual variability in the perception of pain, as well as in the dosage of analgesics that will provide pain relief.

Drugs mostly used for the management of pains are either opioids which are commonly used for the management of acute post-operative pain or non-opioids, and these drugs have been reported to possess potential toxic effect such

as gastrointestinal bleedings. Currently, pain treatment is far from perfect, and there is a need for new, efficacious analgesics with minimum adverse effects.[5]

As presently available synthetic analgesic and anti-inflammatory drugs pose several health problems during their clinical use, search to develop new and more effective drugs with fewer side effects is necessary. The use of natural products is growing in the world especially in developing countries like India where over 75% of the population relies mainly on plants and plant extracts for healthcare.[6]

*Clerodendrum infortunatum* Linn. (Verbenaceae) is an important and widely used medicinal plant, reported to contain active bitter substance like clerodin, has been widely used as tonic and anthelmintic agent in the country sides of North India. Though, variously used in Ayurveda, Unani system of medicine and homeopathy in case of ailments such as diarrhoea, skin disorders, venereal and scrofulous complaints, wounds, post-natal complications, and as external applications on tumors, the plant needs thorough investigation for its specific medicinal activity. Traditionally, the plant is used as an antipyretic and antihelmentic. Leaves of the plant are prescribed for tumor, certain skin diseases and scorpion sting. The antioxidant, antimicrobial, anti-malaria, anthelmintic, and analgesic activities of the plant have further created an upsurge in investigations on the plant.[7,8]

The current study was undertaken to evaluate the peripheral and central analgesic activities of ethanolic extract of *C. infortunatum* Linn. (EECI) in the experimental animals.

## METHODS

Materials used in the study includes:

- Chemicals: Aspirin, acetic acid and normal saline
- Herbs: EECI
- Animals: Wistar albino rats of either sex for tail flick method
- Swiss albino mice of either sex for writhing test and acute oral toxicity study
- Equipments: Mouth gags, feeding tube, tuberculin syringe, analgesiometer, Soxhlet apparatus (Inco Ltd.), insulin syringe and Ryle's tube, beaker, glass jar, glass rod, marking pen.
    1. Aspirin: Soluble form of aspirin was obtained from Nice Laboratory reagents, Cochin, India. In this study, aspirin was taken as standard drug. It belongs to salicylates (non-steroidal anti-inflammatory drugs)
    2. Acetic acid: Acetic acid was used to induce writhing in mice. It was obtained from Nice Laboratory reagents, Cochin. 10 ml/kg of 0.7% v/v acetic acid was used to induce writhings. 0.7% v/v acetic acid was prepared by adding 0.7 ml of acetic acid in 100 ml of distilled water. The solution was prepared freshly before each experiment
    3. Normal saline: It was used to prepare 1% w/v carrageenan solution and also used as a drug in control animals
    4. Leaves of *C. infortunatum* Linn.: Fresh leaves of *C. infortunatum* Linn. were collected from Amrutha Vana Centre for Herbal Gardens and Landscaping Services, Govt. of Karnataka, Bangalore, India (in the month of August 2012). The plant identity was authenticated by botanist Prof. Jadimath.

### *Animals*

All the animals were obtained from the Animal House, Department of Pharmacology, S N Medical College, Bagalkot.

Wistar albino rats of either sex weighing 150-250 g were selected for the experiment. Pregnant rats, animals with infection, animals with injuries, deformities were excluded from the study. Animals showing >20-30 sec of reaction time in tail flick experiment were also excluded. 60 albino rats were used for the tail flick test and carrageenan-induced paw edema test (30 animals each).

Swiss albino mice of either sex weighing 25-30 g were selected for the experiment (15 animals for acute toxicity test and 30 animals for writhing test). Pregnant animals, animals showing infections, injuries, and abnormalities were excluded from the study.

All the animals were maintained at 12:12 hrs dark: light cycle, 25±2°C, and 35-60% humidity. All the animals were received standard laboratory diet (VRK Nutritionals, Pune) and water was provided *ad libitum*.

### *Equipments*

1. Mouth gag: To facilitate the introduction of oral feeding tube into the stomach of the animals
2. Oral feeding tube: Was used to administer the test and standard drugs, normal saline into the stomach of the animals
3. Tuberculin syringe: Used to inject 0. 7% v/v acetic acid into the peritoneal cavity of mice and also used to inject 1% w/v carrageenan intradermally into the rat hind paw
4. Analgesiometer: Was used to measure the reaction time m tail flick method. This is performed mainly to identify pain stimulus threshold in rodent against a radiant heat. It is used to screen the central analgesic activity (spinal reflex). The instrument has two parts: (1) restrainer: Where the animals are placed leaving the tail outside, (2) heat source: made up of nicrome wire where the distal part of the tail was placed to find out the reaction time
5. Soxhlet apparatus: A solid material containing some of the desired compound is placed inside a thimble made

from thick filter paper, which is loaded into the main chamber of the Soxhlet extractor. The Soxhlet extractor is placed onto a flask containing the extraction solvent. The Soxhlet is then equipped with a condenser. The solvent is heated to reflux. The solvent vapor travels up a distillation arm and floods into the chamber housing the thimble of solid. The condenser ensures that any solvent vapor cools, and drips back down into the chamber housing the solid material.

The chamber containing the solid material slowly fills with warm solvent. Some of the desired compound will then dissolve in the warm solvent. When the Soxhlet chamber is almost full, the chamber is automatically emptied by a siphon side arm, with the solvent running back down to the distillation flask.

This cycle may be allowed to repeat many times, over hours or days. During each cycle, a portion of the non-volatile compound dissolves in the solvent. After many cycles the desired compound is concentrated in the distillation flask.

The advantage of this system is that instead of many portions of warm solvent being passed through the sample, just one batch of solvent is recycled. After extraction the solvent is removed, typically by means of a rotary evaporator, yielding the extracted compound. The non-soluble portion of the extracted solid remains in the thimble and is usually discarded.

6. Stopwatch: It is used to find out the reaction time (in sec) in tail flick method.

***Methodology***

*Preparation of extract*

The leaves of the *C. infortunatum* were dried under shade for a period of 4 weeks. The dried plant material was milled to a fine powder using the mechanical grinder. The powder plant material was extracted with absolute ethyl alcohol using Soxhlet extraction apparatus. Dried powder (300 g) was extracted in a Soxhlet extractor with ethanol for about 8-9 hrs at 45°C. Extract was collected and dried using rotary flash evaporator at 40-45°C and crude residue was collected. The solvent was completely removed under reduced pressure and semisolid mass was obtained. The yield was calculated as 30 g. The extract was stored in well-closed glass container at 5°C in refrigerator for further study.[8-10]

*Acute oral toxicity study*

It was done according to Organization for Economic Co-operation and Development guidelines 425 (up and down procedure). All the five mice were administered 2000 mg/kg of EECI orally and observed continuously for a period of 14 days, every hourly for 24 hrs, and every day for 14 days for its movements, grooming activity, exploring activity, writing reflex, eye movements, and convulsion etc.[11]

**Figure 1: *Clerodendrum infortunatum* Linn.**

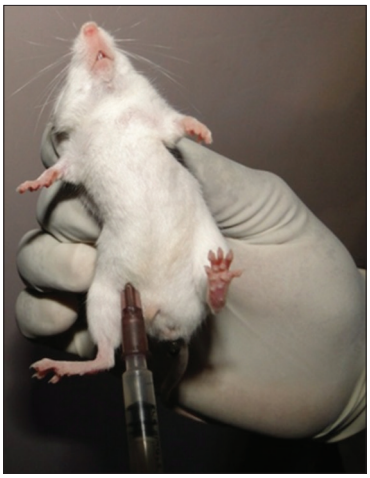

**Figure 2: Intraperitoneal injection of mouse with 0.7% v/v acetic acid for writhing to test the peripheral analgesic activity.**

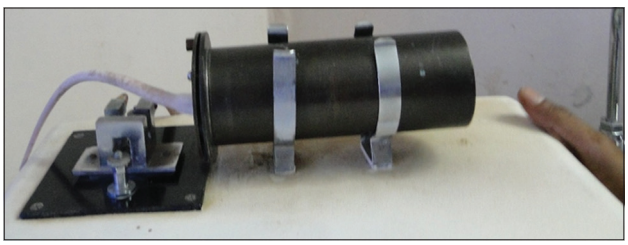

**Figure 3: Tail-flick test for central analgesic activity.**

*Phytochemical analysis*

Qualitative phytochemical analysis of plant extracts: the leaf extracts were analyzed for flavonoids, alkaloids, glycosides, saponins (SN), tannins, proteins and amino acids, sterols and triterpenoids, carbohydrates, fixed oils, anthraquinone, steroids, and resins.

1. Flavonoids: Alkaline reagent test - To the test solution add few drops of sodium hydroxide solution; formation of an intense yellow color, which turns to colorless on addition of few drops of dilute acid, indicates presence of flavonoids

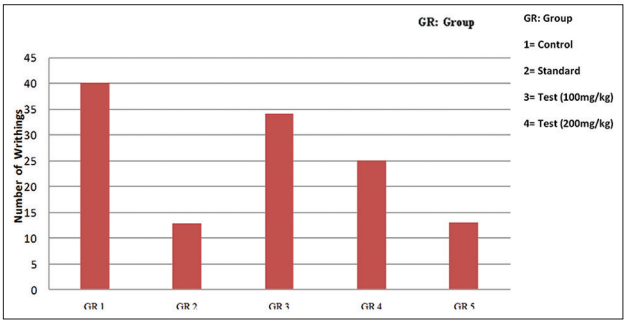


**Figure 4: Number of writhings in acetic acid induced writhing test.**

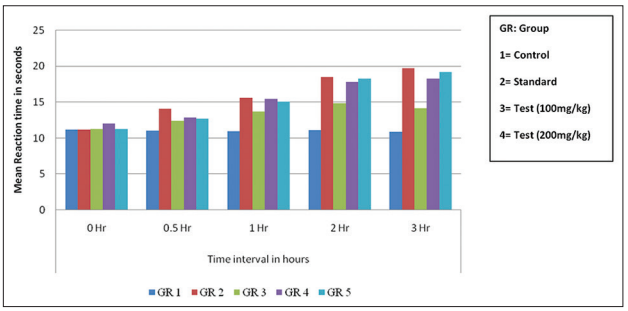


**Figure 5: Reaction time (sec) in tail flick method.**

**Table 1: Number of writhing in standard, control and test groups.**

| Group | Mean±SD | Percentage of inhibition |
|---|---|---|
| Control | 40.17±2.40 | - |
| Standard (100 mg/kg) | 12.67±1.21 | 96.8 |
| Test (100 mg/kg) | 34.00±2.40 | 15.3 |
| Test (200 mg/kg) | 25.00±2.42 | 37.7 |
| Test (400 mg/kg) | 13.00±1.41 | 67.6 |

F=215.8; df=4; p≤0.0001. *Post-hoc* Dunnett's test applied and p<0.05. All the groups were compared against the control group. SD: Standard deviation

2. Alkaloids: Tannic acid test - Alkaloids give buff color precipitate with 10% tannic acid solution
3. Glycosides: Keller killiani test - To the extract add 0.4 ml of glacial acetic acid containing a trace amount of ferric chloride. Transfer to a small test tube. Add carefully 0.5 ml of concentrated sulfuric acid to the side of the test tube, blue color appears in the acetic acid layer
4. SNs: Froth test - 1 ml solution of drug in water is placed in a semi-micro tube and shaken well and note for a stable froth
5. Tannins: Ferric chloride test - Test solution gives blue green color with ferric chloride
6. Proteins and amino acids: Millon's test - Test solution with 2 ml of Millon's reagent (Mercuric nitrate in nitric acid containing traces of nitrous acid), white precipitate appears, which turns red upon gentle heating
7. Sterols and triterpenoids: Libermann–Bachard test - Extract is treated with few drops of acetic anhydride, boiled, and cooled. Concentrated sulfuric acid is added from the sides of the test tube. Formation of a brown ring at the junction of two layers is seen. If the upper layer turns green indicates the presence of steroids and formation of deep red color indicates the presence of triterpenoids.
8. Carbohydrates: Benedict's test - Treat the test solution with few drops of Benedict's reagent (alkaline solution containing cupric citrate complex). Boil on water bath. Reddish brown precipitate forms if reducing sugars are present
9. Fixed oils: Stain test - Press a small quantity of extract between two filter papers. The presence of stain on filter paper indicates the presence of fixed oils
10. Anthraquinones: Borntragers test - About 0.5 g of the extract was taken into a dry test tube and 5 ml of chloroform was added and shaken for 5 mins. The extract was filtered and the filtrate was shaken with equal volume of 10% ammonia solution. A pink violet or red color in the ammonical layer was observed for the presence of anthraquinone
11. Resin: 5 ml of distilled water was added to the extract and observed for turbidity
12. Steroids: 2 ml of acetic anhydride was added to 0.5 g of extract and 2 ml of $H_2SO_4$ was added along the sides of the test tube and the result was observed for red color ring formation.[12,13]

### *Analgesic activity*

#### *Peripheral analgesic activity*

Acetic acid induced writhing: following 12 hrs fasting 30 healthy Swiss albino mice of 25-30 g were randomly divided into five groups of six animals each. Group I received 0.5 ml of normal saline (control group), Group II received 100 mg/kg of aspirin (standard group),[14] Group III, IV, V received EECI (test groups). All the drugs were given orally. After 1 hr all the animals received 10 ml/kg of 0.7% v/v acetic acid injection intraperitoneally (i.p.). Number of writhings was counted between 5 and 20 mins after acetic acid injection.[15]

#### *Central analgesic activity*

Tail flick method: the test was carried out in healthy Wistar rats. 30 animals weighing 150-250 g were randomly divided into five groups of six animals each after 12 hrs fasting. Group I received 0.5 ml of normal saline (control group), Group II received 300 mg/kg of aspirin (standard group),[16] and Groups III, IV, V received EECI (test groups). All the drugs were given orally. After ½ hr, 1 hr, 2 hrs, 3 hrs the tail flick response was carried out and the reaction time was measured by placing the distal 1/3rd of the tail about 1 cm from the radiant heat source. The time taken by the animal to withdraw the tail was

**Table 2: Reaction time (s) in tail flick test in standard, control and test groups.**

| Group | Mean±SD | | | | |
|---|---|---|---|---|---|
| | 0 hr | ½ hr | 1 hr | 2 hrs | 3 hrs |
| Control | 11.2±0.61 | 11.08±0.73 | 11.01±0.82 | 11.14±0.52 | 10.91±0.87 |
| Standard (300 mg/kg) | 11.23±2.36 | 14.10±1.56 | 15.60±2.08 | 18.5±2.59 | 19.75±3.02 |
| Test (100 mg/kg) | 11.3±0.94 | 12.4±1.03 | 13.7±0.9 | 14.9±0.51 | 14.2±1.49 |
| Test (200 mg/kg) | 12.04±1.16 | 12.9±2.20 | 15.5±1.10 | 17.8±0.91 | 18.3±1.08 |
| Test (400 mg/kg) | 11.3±1.37 | 12.7±1.2 | 15.1±1.58 | 18.3±0.75 | 19.2±0.40 |
| F | 0.36 | 3.36 | 11.7 | 34.6 | 32.2 |
| Df | 4 | 4 | 4 | 4 | 4 |
| p | 0.83 | 0.02 | 0.0001 | 0.0001 | 0.0001 |

SD: Standard deviation

taken as the reaction time. Cut off time was kept as 20-30 sec. The animals showing reaction time of >20-30 were excluded from the study.[17]

### *Statistical analysis*

All results are expressed as the mean ± standard error of the mean. The results were analyzed for statistical significance ($p<0.05$, $p<0.01$) by one-way (ANOVA) followed by Dunnett's test using computerized Graph Pad InStat version 3.05, Graph pad software, the U.S.A.

## RESULTS

### *Acetic acid induced writhing test for evaluating peripheral analgesic activity*

In the present study, the test drug at the dose of 400 mg/kg body weight (b.w.) produced 13±1.41 writhing movements in 20 mins duration. The percentage of protection from writhing test with test drug at 400 mg/kg was 67.6%. Standard drug aspirin produced 12.67±1.21 writhings and 96.8% of protection at the dose of 100 mg/kg.

The results obtained with the test, and standard drugs were significant when compared to the control. The test drug, however, was found to be equally effective as that of standard drug aspirin (100 mg/kg b.w.) showing statistically significant analgesic activity ($p<0.05$).

### *Tail flick test for evaluating central analgesic activity*

The above results show the mean of reaction time of control to be 10.91±0.87 sec at 3rd hr. Standard drug aspirin at the dose of 300 mg/kg body weight showed the mean reaction time of 19.75±3.02 sec at 3rd hr and test drug in the dose of 400 mg/kg showed the mean reaction time of 19.2±0.40 sec at 3rd hr, which is comparable to that of the standard drug. EECI at a dose of 200 mg/kg, 400 mg/kg showed significant activity from 30 mins, and at 100 mg/kg showed significant activity from 1st h onward.

## DISCUSSION

*C. infortunatum* is an important medicinal plant used in Indian folk medicine in the treatment of bronchitis, asthma, fever, diseases of the blood, inflammation, burning sensation, and epilepsy. Pharmacological actions include antimicrobial, antioxidant, analgesic, anticonvulsant and antipyretic activities.[7]

Peripheral analgesic activity of EECI was evaluated by using writhing test in mice according to the method of Koster et al.[18,19]

The extracts derived from leaves of *C. infortunatum* Linn. exhibited significant analgesic activity in albino rats by inhibiting acetic acid induced writhing which is a model of visceral pain. Intraperitoneal injection of acetic acid produces pain through activation of chemosensitive nociceptors or irritation of the visceral surface, which lead to the liberation of histamine, bradykinin, prostaglandins, and serotonin.[20,21]

Acetic acid induced writhing is a highly sensitive and useful test for analgesic drug development but not a selective pain test as it gives false positive result with sedatives, muscle relaxants, and other pharmacological activities.[22]

In the present study, the test drug at the dose of 400 mg/kg b.w. produced 13±1.41 writhing movements in 20 mins duration. The percentage of protection from writhing test with test drug at 400 mg/kg was 67.6%. Standard drug aspirin produced 12.67±1.21 writhings and 96.8% of protection at the dose of 100 mg/kg.

The results obtained with the test and standard drugs were significant when compared to the control. The test drug, however, was found to be equally effective as that of standard drug aspirin (100 mg/kg body weight) showing statistically significant analgesic activity ($p<0.05$).

In a study by Pal Dilipkumar et al. in 2009, SN isolated from *C. infortunatum* leaves exhibited protection from writhing

induced by 1.2% v/v acetic acid in adult Swiss albino mice. SN was administered i.p. at doses of 30, 50, 75, and 100 mg/kg and standard drug used were aspirin, paracetamol and morphine sulphate.[23]

Although the writhing response test is very sensitive it has poor specificity in analgesic screening. Tail flick test was conducted to confirm and study the possible analgesic mechanism of *C. infortunatum* Linn.

Central analgesic activity was evaluated by using the tail flick test which is considered to be a spinal reflex induced by heat according to Schumacher et al., Wolff et al.,[24,25] but could also involve higher neural structures (central analgesic activity).[26]

In the tail flick method, a mean of reaction time of control was 10.91±0.87 sec at 3rd hr. Standard drug aspirin at the dose of 300 mg/kg body weight showed the mean reaction time of 19.75±3.02 sec at 3rd hr and test drug in the dose of 400 mg/kg showed the mean reaction time of 19.2±0.40 sec at 3rd hr which is comparable to that of the standard drug. EECI at a dose of 200 mg/kg, 400 mg/kg showed significant activity from 30 mins, and at 100 mg/kg showed significant activity from 1st hr onward.

Preliminary phytochemical screening of the plant extract exhibited the presence of flavonoid, alkaloids, tannin, SNs, sterols, and fixed oils.

In earlier studies *C. infortunatum* Linn. Leaves on preliminary chemical analysis were found to contain SN, clerodin (a bitter diterpene) 4, 6 and some enzymes. Leaves also contain a fixed oil which consists of glycerides of lenoleic, oleic, stearic and lignoceric acid. Luperol and β-sitosterol from roots. Clerosterol identified as 5, 25-sigmastadien_3β-ol, clerodolone as lup_20 (30)-en- 3β-diol-12-one and clerodone as 3β-hydroxylupan- 12-one and a steroidal glycoside from roots.[7]

Previous phytochemical investigation of the plant revealed the presence of alkyl sterols and 2,-(3, 4-dehydroxyphenyl) ethanol 1-O-α-2 rhamnopyranosyl-(1→3)-β-D-(4-O-caffeoyl) glycopyranoside (acteoside) in this plant.[8] The plant was also found to contain triterpenes, steroids and flavonoids.[7]

Various flavonoids, both glycosides, and aglycones were previously reported as having potent anti-inflammatory and analgesic activity. It is suggested that some flavonoids block both cyclooxygenase and lipoxygenase pathway of the arachidonate cascade at high concentration, while at low concentration only lipoxygenase pathway is blocked.[27] Flavonoids produces analgesic action by opioid like action.[28] Furthermore, there are few reports on the role of tannins in analgesic and anti-inflammatory activity. Previous studies suggested that alkaloids also involve in analgesic action through non-narcotic action.[29,30]

In the present study flavonoids, tannins and alkaloids might be attributed to the central analgesic and peripheral analgesic activities. In conclusion, the extract from *C. infortunatum* Linn. possesses both peripheral and central analgesic activity. The present study also substantiates the traditional use of *C. infortumatum* Linn. for the treatment of various inflammatory ailments. The plant can be recommended for the further studies to isolate the active ingredients.

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