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Research Article

Phytochemical Analysis and Gel Formulation Development from Ethanol-based *Terminalia catappa* Linn. Red Leaf Extract in Thailand: Impact on Stability and Release Properties

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ABSTRACT

Terminalia catappa Linn., commonly known as Hu-Kwang, is prevalent in Thailand and other tropical regions, drawing significant attention for its medicinal properties. It is notably effective against arthritis, dermatitis, hepatitis, and rheumatoid arthritis due to its anti-inflammatory properties. The primary objective of this study is to elucidate the phytochemical composition of *Terminalia catappa* Linn. red leaf extract and formulate it into a gel for potential therapeutic applications. *Terminalia catappa* Linn. red leaf extracts was obtained using a Soxhlet apparatus with 95% ethanol. Subsequently, the crude extract underwent a comprehensive analysis utilizing thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) techniques to identify its phytochemical constituents. A gel formulation containing the extract was developed as a main part of this study. The evaluation of all formulations involved a thorough assessment of their stability properties over three months at 4 °C, including the determination of total phenolic content, hydroxyl radical scavenging activity, and various physical characteristics. Additionally, the release kinetics of bioactive compounds from the gel formulations were examined using Franz static diffusion cells with cellophane membranes. Analysis of the TLC chromatogram revealed the presence of key phytochemicals such as quercetin, ursolic acid, and gallic acid in the *Terminalia catappa* Linn. red leaf extract. Further quantification through HPLC revealed concentrations of gallic acid, quercetin, and ellagic acid at 2.577, 0.708, and 3.920 mg/g, respectively. Notably, among the tested formulations, the one without enhancers demonstrated optimal attributes by manifesting a sustained release of bioactive compounds over 24 hours and maintaining stability for an extended duration. This study represents a significant advancement in understanding the phytochemical composition of *Terminalia catappa* Linn. red leaf extract and highlights the potential of its gel formulation as a therapeutic agent, offering stable release of bioactive compounds with total phenolic content and antioxidant property.

Keywords:
Terminalia catappa Linn.; phytochemical analysis; high-performance liquid chromatography; gel formulation; release property; stability test

1. INTRODUCTION

Terminalia catappa Linn. or Hu-Kwang is commonly found throughout Thailand. The plant has been used as traditional medicine, especially the leaves. There is anecdotal evidence supporting the use of using *T. catappa* L. leaves as herbal compress to relieve pain and inflammation in arthritis and dermatitis[1-4].

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Additionally, the red fallen leaves have traditionally been utilized in Taiwan for treating hepatitis[2,3].

Furthermore, research studies related to ethnobotanical usage have demonstrated that the red leaves were more effective than yellow and green leaves. Additionally, the ethanolic extract contributed more active compounds with stronger anti-inflammatory activity[4-6]. Moreover, the studies indicated that the red leaf extract exhibited antioxidant activity[3-6]. Notably, its anti-inflammatory property and antioxidant activity suggest that the plant has a potential for the treatment of arthritis, dermatitis, hepatitis, and rheumatoid arthritis[5,6]. These therapeutic effects are attributed to its rich phytochemical composition, which includes gallic acid, hydrolysable tannins such as punicalagin, corilagin, chebulagic acid, and ellagic acid, flavonoids such as kaempferol and quercetin, and triterpenoids like ursolic acid[7-11].

Previously, Fan *et al.* (2004) reported the anti-inflammatory activity of *T. catappa* L. ethanolic extract, noting its strong efficacy in this regard[5]. Additionally, Annegowda *et al.* (2010) discovered that the ethanolic extract of the leaves, obtained via Soxhlet extraction, possessed analgesic effect alongside anti-inflammatory properties[12].

Our previous study also demonstrated that the ethanol-based extract of *T. catappa* L. red leaf effectively scavenged hydroxyl radicals and exhibited anti-inflammatory properties in an EEP-induced ear edema rat model. Specifically, at doses of 1 and 3 mg/ear, compared to 1 mg/ear of phenylbutazone, significant inhibition of edema was observed within 15 minutes, with reductions of 53.85%, 61.54%, and 71.43%, respectively. This inhibition gradually decreased with prolonged application time[13]. These findings provide substantial support for the traditional medicinal use of *T. catappa* L. and advocate for further development efforts.

Therefore, the objectives of the present study were to elucidate phytochemical composition of ethanol-based *T. catappa* L. red leaf extract and formulate it into gel for potential therapeutic applications for inflammatory-related diseases such as arthritis and dermatitis.

2. MATERIALS AND METHODS

2.1. Chemical reagents

The 95% ethanol, Carbopol® Ultrez 21, propylene glycol, isopropyl myristate (IPM), menthol, butylated hydroxyl toluene (BHT), methyl paraben, and propyl paraben were purchased from O.V. Chemical and supply (Thailand). Ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA) was purchased from Rankem (India). Disodium hydrogen orthophosphate anhydrous (Na_2HPO_4), Sodium carbonate (Na_2CO_3), Sodium dihydrogen orthophosphate ($NaH_2PO_4 .2H_2O$), and concentrated sulfuric acid were purchased from Ajax Finechem (Australia). Corilagin, gallic acid and quercetin were analytical grade purchased from Sigma (Germany). Kaempferol and ursolic acid were analytical grade purchased from Fluka (Germany). Folin-Ciocalteau reagent, potassium persulfate, and hydrochloric acid were analytical grade purchased from Merck (Darmstadt, Germany). 1,10-phenanthroline and hydrogen peroxide (H_2O_2) were purchased from Merck (Germany). Ferrous sulfate anhydrous ($FeSO_4$) was purchased from Sigma (Germany).

2.2. Preparation of *T. catappa* L. red leaf extract

T. catappa L. red leaf were collected from the Faculty of Pharmacy, Chiang Mai University, during the months of July to August, and were accompanied by voucher specimen number TS-001. Subsequently, the leaf subjected to washing and drying in a hot-air oven at 40 °C for 24 hours, followed by grinding into powder form. The resultant dried powder was then sieved using No. 60 mesh sieve.

The dried *T. catappa* L. red leaf powder was extracted using a Soxhlet extraction technique by 95% v/v ethanol for 40 hours. Following extraction, the solvent was separated from the residue through filtration and subsequent evaporation steps. The extract was kept in a sealed container at 4 °C until required for further analysis.

2.3. Thin Layer Chromatography (TLC) analysis

TLC analysis was utilized to investigate phytochemical composition of the red leaf extract of *T. catappa* L. The TLC condition described by Males *et al.* (2004) were applied for this investigation[14,15]. The extract, along with four standards-gallic acid, kaempferol, quercetin, and ursolic acid-were applied onto a 5 × 11 silica gel 60 F254 TLC plate (Merck®, Germany).

The chromatographic plate was developed using a toluene:ethyl acetate:formic acid solvent system in a ratio of 36:12:5. Development proceeded until the mobile phase front reached a distance of 10 cm from the origin. Subsequently, the developed plate was uniformly sprayed with concentrated sulfuric acid and subjected to heating at 100 °C. Observations were conducted under both visible light and 365 nm UV light using a chromatographic viewer (UVP®, USA).

2.4. High performance liquid chromatography (HPLC) analysis

HPLC analysis was employed to examine the phytochemical composition of the red leaves of *T.catappa* L. and its extract. The HPLC method was

modified from the previous study of Kinoshita *et al.* (2007)[16]. The analysis of corilagin was conducted using the red leaf powder infusion in 90 °C water for three minutes. The HPLC separation was performed on a HP1100 Quaternary using an Agilant Zorbax SV-SB-C18 (5 µm, 4.6 x 150 mm). Mobile phase A consisted of acetronitrile:water:acetic acid (90:9:1), and mobile phase B consisted of water and acetic acid (99:1). The mobile phase was maintained at 1 mL/min with gradient program under ambient temperature. The gradient program was set as follows: 0-18 minutes, 0-95% A. The fingerprints were detected using UV-spectrophotometer at wave length at 280 nm.

The analysis of gallic acid, quercetin and ellagic acid was then conducted using a method for corilagin with some modification[16]. The extract was used for analysis instead of the plant powder. The analysis was conducted using an Agilent Zorbax Eclipse system paired with a corresponding photodiode array detector (Hewlett Packard, Waldbronn, Germany). This setup included a column-temperature controller and an autosampler for enhanced efficiency. Data processing and analysis were executed using the Agilent 3D ChemStation software. Chromatographic separation was performed on a XDB-C18 column (5 µm, 4.6 × 250 mm). For the analysis of gallic acid and quercetin, a mobile phase consisting of a mixture of water and acetic acid (96:4, v/v) as mobile phase A, and 100% v/v acetonitrile as mobile phase B, was utilized. The gradient program was set as follows: 0 - 40 minutes, 0 - 95% B. On the other hand, for ellagic acid, the mobile phase consisted of 0.5% v/v acetic acid (A) and a mixture of 0.5% v/v acetic acid with acetonitrile (B) in a ratio of 80:20. The gradient program was set as follows: 0 - 45 minutes, 0 - 95% B. The mobile phase was maintained at a flow rate of 1 mL/min with a gradient program. The column temperature was held constant at 25 °C throughout the analysis. Detection of chromatographic peaks was achieved using a UV-spectrophotometer at specific wavelengths: 280 nm for gallic acid, 375 nm for quercetin, and 254 nm for ellagic acid. The injection volume of each samples and standard solutions were 50 µL. The HPLC mobile phase, as well as the samples and standard solutions, were filtered through 0.45 µm membrane filter (ANPEL Laboratory Technologies, Inc., Shanghai, China) before use.

2.5. Formulation of gel containing *T. catappa* L. red leaf extract

Preformulation study was conducted to assess the physicochemical properties, including pH, solubility, and compatibility. All seven gel base formulations were prepared according to the details outlined in Table 1. Ethyl alcohol, propylene glycol, and isopropyl myristate were utilized as enhancers, with varying ratios.

To incorporate the extract into gel formulation, the concentration used in this study referred to our previous study on hydroxyl radical scavenging and anti-inflammatory activities as well as the Pauly's patent, showing that 0.1% to 3% of the extract exhibited anti-inflammatory activity[13,30]. Therefore, the crude extract was then incorporated into the gel base to make a concentration of the extract at 3 % w/w.

Table 1. The ingredients of gel base formulations.

Ingredients	Number of the formulas						
	1	**2**	**3**	**4**	**5**	**6**	**7**
Carbopol® Ultrez 21 (%w/w)	1	1	1	1	1	1	1
95% Ethyl alcohol (%w/w)	-	20	20	20	-	20	20
Propylene glycol (%w/w)	-	-	5	5	5	5	10
Isopropyl myristate (%w/w)	-	-	-	5	-	10	5
Menthol (%w/w)	5	5	5	5	5	5	5
BHT (%w/w)	0.05	0.05	0.05	0.05	0.05	0.05	0.05
EDTA (%w/w)	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Concentrated paraben* (%w/w)	1	1	1	1	1	1	1
Triethanolamine	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.
Distilled water q.s. to (g)	100	100	100	100	100	100	100
Ratio of EtOH : PG : IPM**	**0:0:0**	**20:0:0**	**20:5:0**	**20:5:5**	**0:5:0**	**20:5:10**	**20:10:5**

* Concentrated paraben is the mixture of 1% of 10% methyl paraben and 2% propyl paraben in propylene glycol.
**EtOH: 95% ethyl alcohol; PG: propylene glycol; IPM: isopropyl myristate

2.6. Evaluation of gel containing *T. catappa* L. red leaf extract

Physical Characteristics

The gel was assessed for odor and color. pH values were measured using a pH meter (Eutech® pH 510, Singapore). Additionally, viscosity was determined using a Rheometer plate and plate type (Brookfield®, England).

Total phenolic content determination

Total phenolic content (TPC) was using a modified Folin-Ciocalteu method according to the method of Javanmardi *et al.* (2003)[17]. Subsequently, the gel sample was diluted in ethanol with an accurate concentration, then 0.5 mL of the sample solution was combined with 5 mL of Folin-Ciocalteu reagent solution (diluted at a ratio of 1:10 with distilled water), followed by the addition of 4 mL of 7.5% w/v Na_2CO_3 solution. The resulting mixture was then stored in the dark for 30 minutes before absorbance measurement was taken at 765 nm using a UV-VIS spectrophotometer (Shimadzu®UV-2450, Japan). All experiments were conducted in triplicate, and gallic acid served as the standard substance. Total phenolic content is presented as gallic acid equivalent (GAE) in milligram per gram of dry extract.

Stability study

Accelerated stability testing of both the gel base and sample gel was conducted through a series of heating-cooling cycles, alternating every 48 hours between 45±2 °C and 4±2 °C for each cycle, totaling six cycles. The physical characteristics were assessed both before and after the completion of the cycles.

Following the stability assessment, a gel formulation was selected for further investigation. The chosen formulation underwent additional testing for hydroxyl radical (OH^-) scavenging activity, *in vitro* release study, and stability for three months.

The stability of the formulations at 4 °C was assessed over a period of three months. Twelve bottles of each selected formulation were prepared. At the 0, 1, 2, and 3-month intervals, three bottles from each formulation were collected and analyzed for physicochemical properties, hydroxyl radical (OH^-) scavenging activity, and total phenolic content.

2.7. Hydroxyl radical (OH^-) scavenging activity determination

The sample gel (n=3) was diluted in ethanol to an accurate concentration, then evaluated using the method outlined by Bai *et al.* (2011)[18] with slightly modification. In this procedure, the reaction mixture comprised 400 µL of sample solution, 250 µL of 10 mM $FeSO_4$, 250 µL of 10 mM 1,10-phenanthroline, 400 µL of 0.15% w/v H_2O_2, and 3000 µL of phosphate buffer at pH 7.2. The mixture was then incubated at 37 °C for 1 hour. Following incubation, the absorbance was measured at 536 nm using a UV-VIS spectrophotometer, and calculations were performed using the following equation.

$$\%\ \text{Hydroxyl radical scavenging activity} = \frac{(A_S - A_1)}{(A_0 - A_1)} \times 100$$

Where A_0 is an absorbance of blank solution; A_s is an absorbance of sample; and A_1 is absorbance of control. All experiments were performed in a triplicate.

2.8. *In vitro* release study of gel formulation

The study was carried out based on the method of Nuno *et al.* (2012)[19] with some modification. Cellophane membrane with the molecular weight cut-off at 1200 Dalton (Sigma®, USA) was utilized and soaked in receiver fluid (a mixture of DI water adjusted to pH 7.0±0.2 and 95% v/v ethanol in a ratio of 60:40) for 24 hours prior to use. Franz static diffusion cell (PermeGear®, India) with 15 mm surface diameter and 12 mL receptor volume was used. Approximately 1 g of each gel sample (n=3) was placed into the donor compartment. The receiver compartment of the cell was filled with receiver fluid and continuously stirred using a magnetic stirrer at a controlled temperature of 37 ± 2 ºC. Sample solutions from the receiver fluid were collected at 1, 2, 4, 6, 8, 12, and 24-hour intervals, with 1 mL of solution collected each time and replaced with fresh receiver fluid. The collected sample solutions were then analyzed for total phenolic content using Folin-Ciocalteu reagent.

2.9. Statistical Analysis

The mean values of the results obtained from all triplicate experiments were calculated, and the results were expressed as mean ± standard deviation. Subsequently, the data underwent statistical analysis using the T-test performed with SPSS software version 17 (IBM, NY, USA). A statistical difference was considered significant at $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1. *T. catappa* L. red leaf extract

The utilization of 95% ethanol as a solvent in Soxhlet extraction was noted. Previous reports have indicated that the resulting ethanolic extract contains a higher concentration of active compounds and exhibited

stronger antioxidant and anti-inflammatory properties[5]. Additionally, Annegowda *et al*. (2010) demonstrated that the ethanolic extract obtained via Soxhlet apparatus possessed analgesic and anti-inflammatory effects in both the early and late phases of Formalin-induced pain in mice[12]. Therefore, this technique was adopted for the present study.

The crude extract is characterized as a hard powder with a distinct odor and a dark brown color (Figure 1). The yield of the ethanol-based extract obtained using the Soxhlet extraction technique was determined to be 22.02% w/w. The percentage of yield obtained in this investigation surpasses that of previous studies on *T. catappa* L. leaves conducted by Madhavan *et al*. (2023)[20]. In their study, the yield of 95% v/v ethanol extract using seven-day maceration method was documented as 13.26%. Additionally, in the study conducted by Yunita *et al*. (2021), extract yields obtained from a three-day maceration method were recorded as 5.20%, 2.85%, and 2.25% utilizing 90% ethanol, ethyl acetate, and n-hexane as solvents, respectively[21]. Similar to the aforementioned results regarding extracted yields, it can be observed that Soxhlet extraction method using 95% ethanol as solvent could reduce extraction time and potentially increase the extract yield of nonpolar compounds.

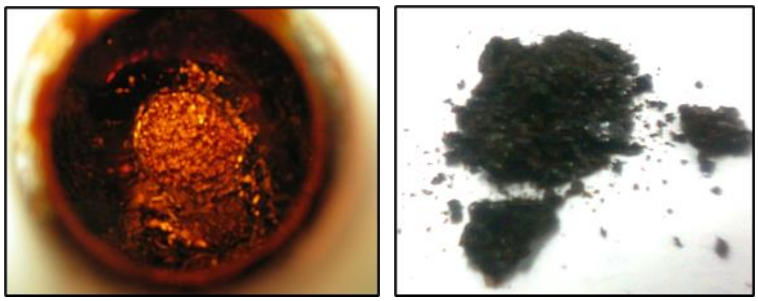

Figure 1. A: The extract viewed in round flask; B: The extract powder

3.2. Phytochemical composition of *T. catappa* L. red leaf extract

Previously, phytochemical screening was carried out for the water and ethanolic extracts of *T. catappa* L.[22]. The results showed that the phytochemical groups found in the extracts were hydrolysable tannins, flavonoids, and triterpenoids, as conform to the record of Duke (1992)[23].

To standardize naturally occurring bioactive compounds, ensuring product quality control is crucial. Therefore, TLC was performed for preliminary identification of the extract while HPLC was employed to investigate the phytochemical composition of *T. catappa* L. red leaf and its extract.

In this study, various concentrations of acetic acid and acetonitrile were utilized to identify the optimal conditions for detecting target compounds in the shortest possible time. The goal was to minimize waste generated from HPLC analysis and to pave the way for future application of this analytical method in identifying constituent compounds in this plant. Additionally, given that the extract is a crude extract, the use of a gradient method was expected to facilitate the separation of unpurified compounds more effectively.

One of the major compounds found in *T. catappa* L. leaves is corilagin, which exhibits solubility in both water and hydro-alcoholic solvents. Corilagin primarily contributes to anti-inflammatory activity with hepatoprotective effect. However, under acidic condition and elevated temperature, corilagin undergoes facile decomposition, yielding gallic acid and ellagic acid through hydrolysis reactions[16]. Since the extract in this study was obtained by Soxhlet extraction using 95% v/v ethanol with acidic nature, all of which contribute to the diminished yield of corilagin[24]. Consequently, analyzing corilagin may not be practical. Furthermore, quantifying gallic acid and ellagic acid, the hydrolysis byproducts of corilagin, was deemed more feasible. Accordingly, this study aimed to quantify corilagin in the leaf powder infusion in hot water for 3 minutes, using HPLC, in order to mitigate the risk of corilagin hydrolysis. Additionally, it sought to ascertain the presence of corilagin, a principal compound, in the red leaves of *T. catappa* L. sourced from Thailand.

In the present study, the developed TLC chromatogram was compared between the extract and 4 standards including mixture of the standards (1) quercetin (2), kaempferol (3), ursolic acid (4), gallic acid (5) and is shown in Figure 2A. There are 3 spots of the extract similar to quercetin, ursolic acid and gallic acid. The chromatogram showed that at least 13 phytochemicals were found in the extract as Rf value shown in Figure 2B.

The HPLC chromatogram of the *T. catappa* L. red leaf is shown in Figure 3. There were several components present, corilagin detected at retention time of 5.6 min and found with a concentration of 2.09 mg/kg of plant dried weight.

Furthermore, the HPLC chromatogram of the *T. catappa* L. red leaf extract (Figure 4) shows three phytochemical compositions, gallic acid, quercetin and ellagic acid, which were identified at retention times of 2.5, 23.0, and 24.9 min, respectively. It was observed that the extract had gallic acid, quercetin, and ellagic acid at concentrations of 2,577, 708, and 3,902 mg/kg of the extract, respectively. The results are in accordance with a previous study reporting that corilagin, gallic acid, quercetin and ellagic acid are active compounds present in *T. catappa* L. red leaf extract[7-10,25-27].

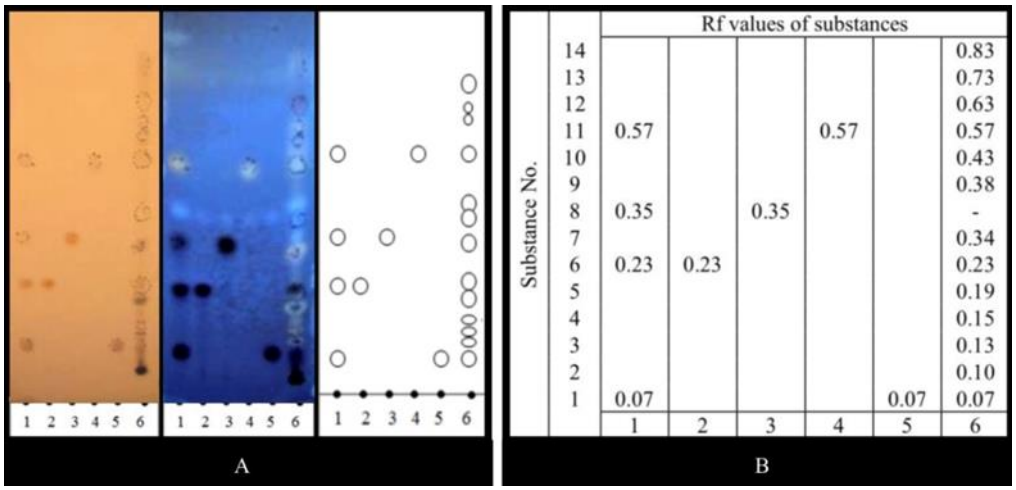

Figure 2. A: TLC chromatogram of *T. catappa* L. red leaf extract; B: Rf values of substances

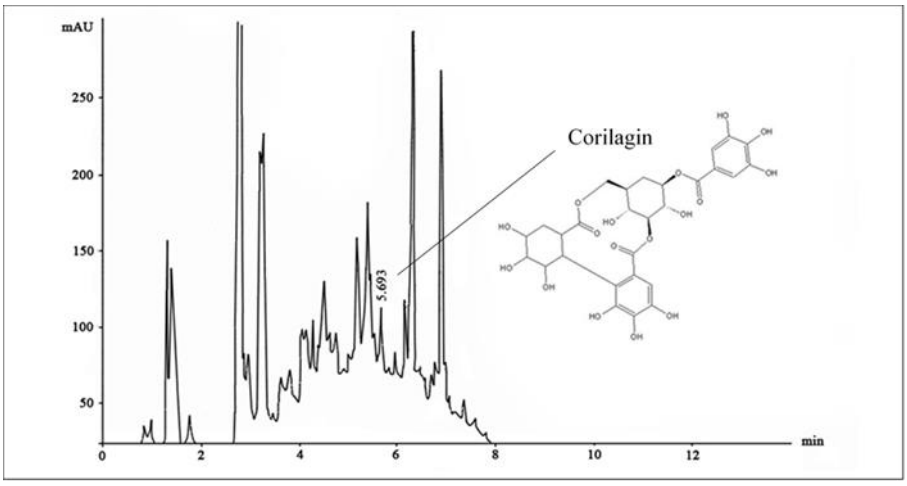

Figure 3. HPLC chromatogram of the *T. catappa* L. red leaf, prepared by infusion in 90 °C water for three minutes

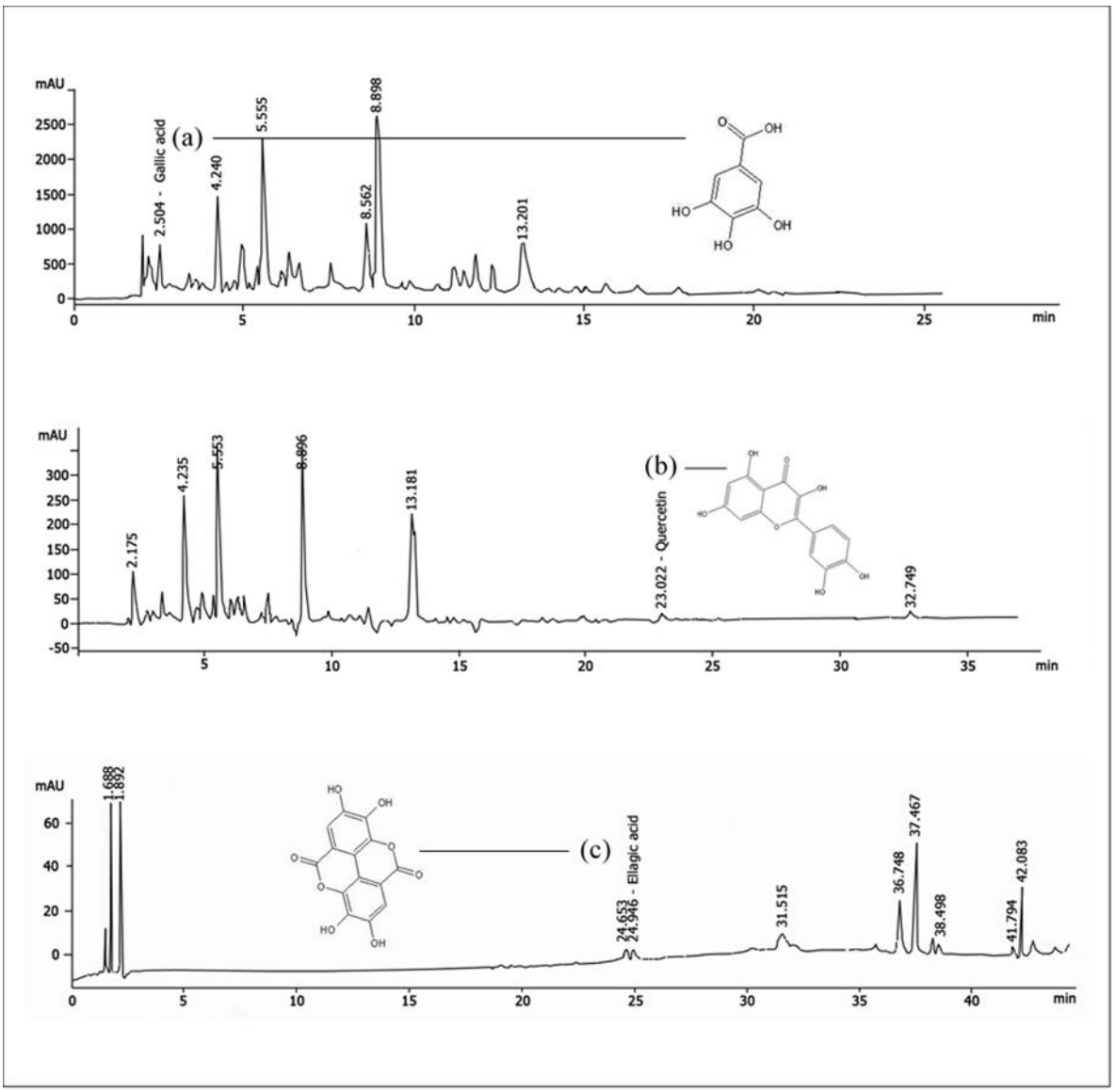

Figure 4. HPLC chromatogram of the *T. catappa* L. red leaf extract, which was *T. catappa* L. red leaf extract obtained by a Soxhlet extraction technique in 95% v/v ethanol. (a) is gallic acid, (b) is quercetin and (c) is ellagic acid

In addition, these compounds have been proposed for utilization as active biological markers to standardize and ensure the quality control of *T. catappa* L. red leaf extract.

Since corilagin, gallic acid, quercetin and ellagic acid have previously been reported to exhibit antioxidant and anti-inflammatory activities[7-10,25-27], it can be inferred that these compounds contribute to the potent biological activities of both *T. catappa* L. red leaves and its ethanolic extract. Corilagin has been reported to possess anti-inflammatory activities through the inhibition of the COX-2 enzyme and the suppression of inflammatory mediators, resulting in decreased levels of TNF-α, IL, and NO[25,26]. Gallic acid has demonstrated efficacy in reducing inflammation and allergic reactions by inhibiting histamine release and the synthesis of pro-inflammatory cytokines[7].

Additionally, gallic acid and ellagic acid significantly inhibit both COX-1 and COX-2 enzymes[7,9]. Moreover, quercetin reduces inflammation by inhibiting inflammatory mediators and reducing edema, including the inhibition of IL-6 synthesis from neutrophils[9,10].

However, in acid condition as well as high temperature, ellagitannins (including corilagin) is hydrolyzed into gallic acid and ellagic acid[28,29]. Therefore, gallic acid, quercetin, and ellagic acid were regarded as the principal bioactive constituents of ethanol-based *T. catappa* L. red leaf extract in this study. Moreover, these compounds may be recommended for application as biological markers for quality control or quantitative assessment of *T. catappa* L. red leaf extract.

3.3. Formulation and evaluation of gel formulations

Preformulation study showed that the pH of the extract at the concentration of 0.1% w/v in water was 3.9. The crude extract was acidic and soluble in 95% v/v ethanol and propylene glycol, and insoluble in isopropyl myristate. In acid-base reaction, the extract was not changed in acid solution but dissolved in basic solution. Moreover, the extract and Carbopol® Ultrez 21 were compatible.

Gel formulations were prepared using Carbopol® Ultrez 21 as a gelling agent with various ratios of enhancers (95% v/v ethanol, propylene glycol and isopropyl myristate). Menthol was a cooling agent in the formulation. Additionally, BHT and EDTA were added as an antioxidant and chelating agent, respectively. Concentrated parabens were used as preservatives, while triethanolamine was used as a gel stabilizer to neutralize Carbopol® Ultrez 21 and form a gel in the pH range of 7-8 before incorporating the extract. This was done to ensure the pH remained no less than 5, as the acidic property of the extract could lower the formulation's pH. Moreover, forming the gel before incorporating the extract can reduce the risk of a reaction between triethanolamine and the extract by hindrance effect. Consequently, triethanolamine was used in an equal amount in all gel bases so that the study could particularly focus on the impact of the enhancer on the stability and release profile of the formulations.

Our preceding investigation illustrated that the ethanol-based extract from *T. catappa* L. red leaves, at a concentration of 1 mg/mL, effectively scavenged hydroxyl radical with an efficacy of 82.72% (IC_{50} 0.6435 mg/mL) and demonstrated anti-inflammatory properties in an EEP-induced ear edema rat model. Specifically, when administered at doses of 1 and 3 mg/ear (converted to human-equivalent doses at 0.5 mg), compared to 1 mg/ear of phenylbutazone, a significant inhibition of edema was observed within 15 minutes, resulting in reductions of 53.85%, 61.54%, and 71.43%, respectively[13]. This discovery is consistent with the findings outlined in Pauly's patent (2002), where the extract, ranging from 0.1% to 3%, exhibited anti-inflammatory activity[30]. Consequently, the extract at a concentration of 3% w/w was formulated as a prototype topical preparation for the present study.

The gel base formulation demonstrated high viscosity within the pH range of 7-8. Gel formulation containing 3% w/w of the extract exhibited a brown color (Figure 5) and menthol odor. The total phenolic content determined in each the extract 3% gel formulation was slightly lower than that of the crude extract previously reported, measuring at 256.64 mg/g dry extract as GAE[22].

Once the extract was incorporated into the gel base, the viscosity decreased due to the acid property of the extract, lowering the pH value to below 6.5[31-33], particularly evident in formula No. 3-7 (Table 2). Furthermore, a significant decrease in viscosity occurred in formulations containing the extract, possibly due to the combined effect of the extract's acid property and the polarity of enhancer on Carbopol® Ultrez 21 swelling[29]

It is suggested to adjust final pH of all finished formulation to 6 and undertake additional research into the effects of enhancers and amine stabilizers on Carbopol® Ultrez 21 in formulations containing hydrolysable tannin-rich extract.

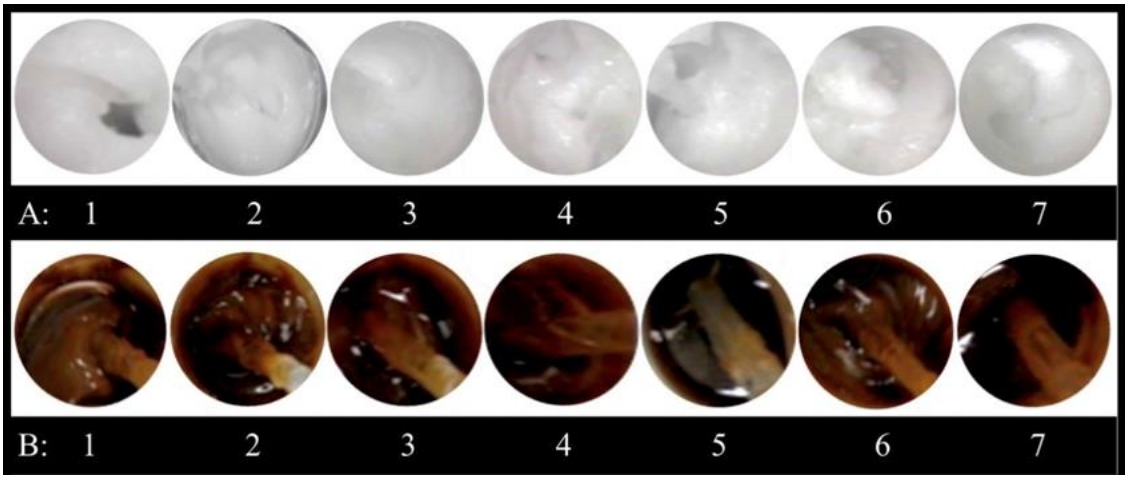

Figure 5. A: Gel base; B: gel containing the extract (1-7)

Table 2. The viscosity and pH value of the gel base (A) and gel containing the extract (B)

No.	Viscosity (Pa.s)		pH	
	Gel base (A)	Gel containing the extract (B)	Gel base (A)	Gel containing the extract (B)
1	18.323±0.248	8.665±0.360	7.93±0.06	7.38±0.12
2	17.557±0.485	8.040±0.264	7.90±0.20	7.49±0.08
3	18.141±0.057	3.906±0.077	7.66±0.09	6.14±0.15
4	17.941±0.378	6.011±0.370	7.53±0.12	6.31±0.05
5	18.536±1.099	4.684±0.194	7.40±0.02	5.98±0.04
6	15.777±0.420	2.918±0.416	7.64±0.09	4.72±0.05
7	18.768±0.728	2.021±0.093	7.46±0.23	5.73±0.06

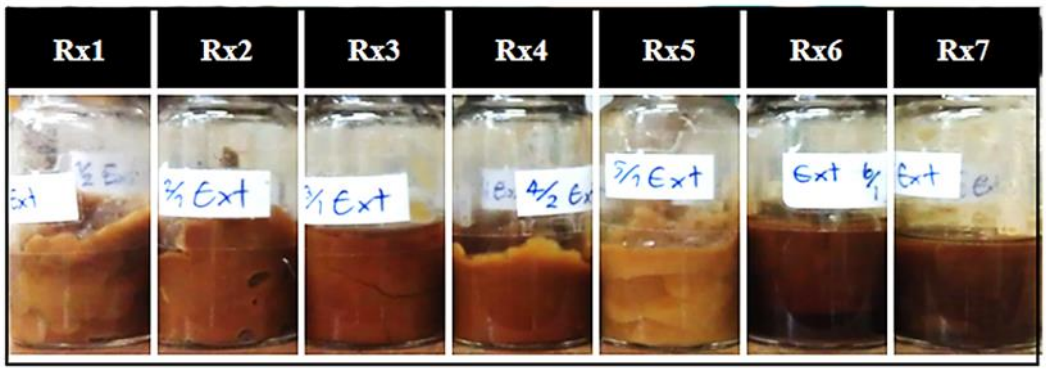

Figure 6. Gel containing the extract after heating cooling condition and syneresis occurred in Rx3, Rx4, Rx6, Rx7

Table 3. Physical properties and total phenolic content of the formulations after heating cooling condition

No.	Viscosity (Pa.s)		pH	
	Gel base (A)	Gel containing the extract (B)	Gel base (A)	Gel containing the extract (B)
1	16.3196±1.013	7.031±0.312	7.69±0.32	6.61±0.07
2	14.804±0.342	7.585±1.242	7.64±0.10	6.88±0.10
3	15.103±0.708	2.809±0.170	6.87±0.28	5.48±0.06
4	16.004±0.894	3.944±0.371	6.86±0.02	5.46±0.25
5	16.538±1.030	2.332±0.347	6.25±0.30	4.94±0.11
6	13.576±1.649	7.031±0.312	6.57±0.13	4.28±0.06
7	17.209±1.592	7.585±1.242	6.23±0.14	6.61±0.07

The study indicates that all gel base formulations exhibited no significant alterations in their physical characteristics. However, formulations 4 through 7 showed a significant decrease in pH values ($p < 0.05$), while viscosity remained relatively stable ($p > 0.05$). However, formulations incorporating the extract demonstrated significant alterations in pH, and viscosity, particularly evident in formulations Rx3-Rx7 (Table 3).

Formulations Rx3-Rx5 exhibited a decrease in viscosity correlated with pH reduction. Additionally, formulations utilizing isopropyl myristate (Rx4, Rx6, Rx7) and Rx3 exhibited signs of syneresis (Figure 6).

However, the influence of isopropyl myristate on syneresis remained unclear. Syneresis likely involves the hydrolysis of hydrolysable tannins such as punicalagin and corilagin into ellagic acid and gallic acid. This change might relate to high temperature and acidic condition inducing hydrolysis of tannin resulting in changes in physicochemical properties and impacting on solubility, hydrogen bond interactions, and Carbopol® Ultrez 21 swelling, ultimately leading to changes in viscosity and syneresis[34-36].

Based on this study, formulations Rx1, Rx2, and Rx5 did not exhibit syneresis, whereas formulations containing isopropyl myristate (Rx4, Rx6, Rx7) and Rx3 showed signs of syneresis. Consequently, these formulations were selected for further investigation, including *in vitro* release study, antioxidant activity assessment, and stability testing for longer period. This study was conducted only at 4 °C to determine if cool conditions could minimize hydrolysis reactions in the formulations. However, stability studies at room temperature and high temperatures should be further investigated to predict shelf life and determine optimal storage conditions for practical use.

3.4. Hydroxyl radical (OH·) scavenging activity of gel formulations

Hydroxyl radical, a highly active radical, plays a crucial role during the inflammatory process, capable of inducing inflammation, leading to pain, tissue destruction, and various diseases including arthritis, dermatitis, and rheumatoid arthritis[13]. In our previous study, hydroxyl radical (OH·) scavenging activity was conducted, revealing that the extract at 1 mg/mL exhibited potent scavenging activity against hydroxyl radicals, with the inhibition rate up to 82.70% (IC_{50} 0.6435 mg/mL) and also inhibited rat ear edema by more than 50% within 15 minutes[13]. This finding aligns with that of Pauly (2002), who reported that the ethanolic extract, at concentrations ranging from 0.1% to 3%, demonstrated anti-inflammatory activity[30]. Therefore, hydroxyl radical (OH·) scavenging activity was conducted in this study for a rough estimate of anti-inflammatory potential of the formulation.

The present study was conducted to investigate the hydroxyl radical (OH·) scavenging activity of a gel formulation containing 3% w/w of *T. catappa* L. red leaf extract.

The result shows that Rx1, Rx2 and Rx5 at 50 mg/mL (equivalent to 1.5 mg of the extract) exhibited hydroxyl radical (OH·) scavenging activity at 46.8, 38.24 and 48.47%, respectively. However, it is noteworthy that the extract solution from the previous study demonstrated stronger inhibition compared to the formulated gels[13]. Nevertheless, the formulations still show hydroxyl radical scavenging activity that is promising for anti-inflammatory effect.

3.5. The release profile of gel formulations

The 95% ethanol, isopropyl myristate and propylene glycol were utilized as enhancers to create a cosolvent system for the ethanol-based extract. The combination of isopropyl myristate with propylene glycol has been reported to exhibit synergistic enhancement[37]. However, formulations containing isopropyl myristate were found to be unstable after accelerated stability study in heating-cooling condition. Therefore, formulations Rx1, Rx2, and Rx5 were further evaluated for their *in vitro* release profiles by determining the total phenolic content, as depicted in Figure 7.

The composition of the receiver fluid significantly influences the dissolution of the extract. Our investigation involved testing different ratios of deionized (DI) water and 95% ethanol, specifically in proportions of 100:0, 70:30, 60:40, 50:50, and 0:100. Analysis of the results revealed that a receiver fluid ratio of 60:40 yielded the extract at a concentration suitable for measurement.

From Figure 7, it is observed that the total phenolic content released from Rx1 were similar to those from Rx5, but greater than those from Rx2. The variance in the release profile among the three formulations can be attributed to the choice of enhancer. Rx1 contained only ethyl alcohol from the extract solution, while Rx2 had a higher concentration of ethyl alcohol compared to Rx1. On the other hand, Rx5 contained only propylene glycol. The release profile suggests that the use of enhancers slightly impacted the release profile by impeding the release of active compounds from the gel. It is hypothesized that the formulation with a lower concentration of ethyl alcohol may release more total phenolic content from the gel to receiver fluid due to the affinity of active compounds to vehicle (water) is less than to the receiving fluid (DI water:95% ethanol at 60:40). Nevertheless, the total phenolic content was gradually released over time.

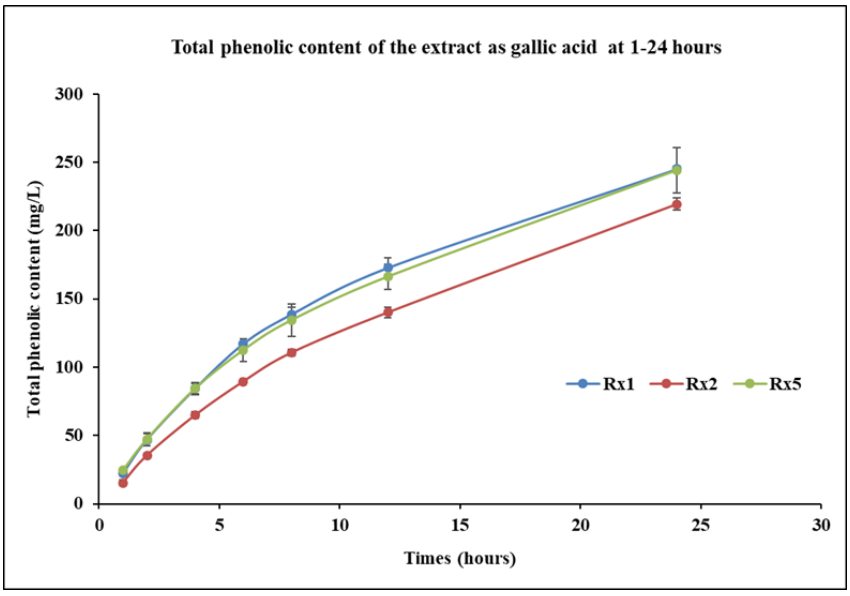

Figure 7. Total phenolic content of the extract as gallic acid released from gel at 1-24 hours

3.6. Stability test of gel containing *T. catappa* L. red leaf extract at 4 °C

As the extract is acidic and rich in hydrolysable tannins, stability testing was conducted under cool temperature at 4 °C for 3 months to limit the influence of temperature on hydrolysis. The stability of gels containing 3% w/w of *T. catappa* L. red leaf extract revealed that formulations Rx1, Rx2 (enhanced with 95% ethanol), and Rx5 (enhanced with propylene glycol) did not exhibit syneresis. After 3 months, Rx1 showed no change in viscosity, pH, total phenolic content, or hydroxyl radical scavenging activity. Rx2 showed a decrease in viscosity, with varied in total phenolic content, while the hydroxyl radical scavenging activity depended on changes in total phenolic content. This suggests that during storage at cool temperatures, active compounds such as hydrolysis of tannins correlate with an increase in total phenolic content, that might be a positive result from tannin hydrolysis into free phenol group and hydroxyl group, the major structure of action on antioxidant activity[16,24,38]. At the 3-month, the hydroxyl radical scavenging activity of Rx2 showed an increase compared to the initial level, despite no significant difference in total phenolic content. Meanwhile, Rx5, which exhibited the lowest pH value, demonstrated an increase in total phenolic content but a decrease in hydroxyl radical scavenging activity. This may indicate instability in acidic conditions (pH < 6.5), potentially reducing hydroxyl radical scavenging activity due to hydrolysis reactions of bioactive agents. This finding is consistent with the results reported by Annegowda *et al.* (2010)[12]. The data is presented in Table 4.

From this study, Rx1 is expected to maintain stability at 4 °C. It is suggested that gels containing *T. catappa* L. red leaf extract should have a pH > 6.5 and be stored in tightly sealed containers in a cool place. Furthermore, the impact of enhancers on stability should be further investigated.

Table 4. Stability study of gel formulation

Months	Viscosity (Pa.s)			pH			Hydroxyl radical scavenging activity (50 mg/mL of gel)		
	Rx1	Rx2	Rx5	Rx1	Rx2	Rx5	Rx1	Rx2	Rx5
0	4.599±0.326	8.456±0.091	6.445±0.512	6.45±0.04	6.46±0.04	6.37±0.02	43.82	35.39	53.70
1	7.268±0.174	8.958±0.552	7.274±0.486	6.56±0.01	6.46±0.07	6.39±0.01	49.69	39.29	35.01
2	5.346±0.475	6.314±0.212	7.913±0.091	6.53±0.13	6.43±0.23	6.27±0.23	20.00	30.32	37.47
3	5.602±0.419	5.409±0.480	7.138±0.120	6.64±0.02	6.54±0.06	6.28±0.14	43.02	43.42	44.71

4. CONCLUSION

Phytochemicals present in *T. catappa* L. red leaves include corilagin, gallic acid, ellagic acid, ursolic acid, and quercetin. The ethanol-based *T. catappa* L. red leaf extract was formulated into a gel at a concentration of 3% w/w for therapeutic applications. The formulation exhibited a brown color, menthol odor, and stability under cool conditions, particularly the formulation without enhancers. Rx1 consisting of the extract 3%, menthol, BHT, EDTA, and Carbopol® Ultrez 21, was the best formulation with good stability and the best releasing profile. The study highlights that the ethanol-based extract, rich in hydrolysable tannins, influences the development of Carbopol® Ultrez 21 gel formulations and the use of enhancers. These factors affect the physicochemical properties of the formulation, including viscosity, pH, and Carbopol® Ultrez 21 stabilization. These changes impact the release profile, antioxidant activity, biological activity, and stability of the formulation.

The results indicate that there is a limitation in using isopropyl myristate and propylene glycol as enhancers for Carbopol® Ultrez 21 gel containing alcohol-based *T. catappa* L. red leaf extract. Hydrolysable tannins are prone to hydrolysis at high temperature, affecting physicochemical properties and reducing gel volume. Consequently, formulation should be stored in tightly sealed container and cool storage condition. This study prompts for further investigation to optimize the isolation of the extract to obtain a topical gel preparation from *T. catappa* L. red leaf extract that is suitable for application and acceptable for inflammatory-related diseases such as dermatitis and arthritis. The formulation should undergo further development and evaluation *in vivo* for anti-inflammatory activity and *in vitro* permeation testing using a membrane mimicking human skin.

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Conflict of interest
The authors declare that they have no conflict of interest.

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