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Antioxidant, AChE inhibitory, and anticancer effects of *Verbascum thapsus* extract

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ABSTRACT

Verbascum thapsus (Mullein) is a medicinal plant used in folk medicine to treat various ailments. For this study, the biological functions of *Verbascum thapsus* (VT) methanol extract were determined in vitro. The plant's methanol extract was created through the maceration process. The phytochemical composition of plant extracts was investigated using liquid chromatography-electrospray ionization tandem mass spectrometry. The antioxidant capacity of the extract was determined using the 2,2-diphenyl-1-picrylhydrazil (DPPH radical) and 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS radical). Cell lines Caco-2 (human colorectal adenocarcinoma cells), LNCaP (Lymph Node Carcinoma of Prostate), and HEK293 (Human embryonic kidney 293 cells) were used to model colon, prostate, and non-cancerous cells. The cytotoxic activity of the plant extract on the proliferation of these cells was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) assay protocol. VT extract showed moderate DPPH and ABTS radical scavenging activities at 30 mg/ml concentration. With this, VT extract was determined to inhibit acetylcholinesterase (AChE) enzyme and had strong cytotoxic activity on cancerous cell lines. In addition, our findings clearly showed that the plant extract had greater cytotoxic activity on the viability of cancerous cells compared to non-cancerous (Human embryonic kidney cells; HEK293) cells. The current findings showed that *V. thapsus* might be a promising anti-cancer medication candidate for the treatment of human colorectal adenocarcinoma and colon cancer, as well as a good source antioxidans.

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Introduction

Plants produce secondary metabolites such as carotenoids, phenolic acids, anthocyanins, flavonoids, tocopherols, tannins, and stilbenes to defend themselves against biotic (living) and abiotic (non-living) threats (1-3). These metabolites have sedative, vasodilator, antithrombotic, antiviral, antiallergenic, anticarcinogenic, antioxidant, antiallergen, antiviral, antimutagenic, anti-inflammatory, and anticarcinogenic properties, as well as the ability to inhibit several of enzymes (4-6). Plants based chemicals and nanoparticles that prevent reactive oxygen species (ROS), limiting the development microbial infections (7-11).

It is critical to investigate the cholinergic hypothesis in orted to effectively treat Alzheimer's disease (AD), which causes some neurodegenerative illnesses and memory loss issues. According to the cholinergic hypothesis, cholinesterase enzymes must be inhibited to prevent the reduction of acetylcholine in the brain (12). It is well known that acetylcholinesterase (AChE) activity increases during the early stages of AD (12). AChE is found in the brain, muscles, spleen, plasma, erythrocytes, lungs, and spleen. A membrane-bound enzyme hydrolyzes cholinergic neurotransmitters (12-14). Furthermore, AChE inhibitors have an anti-inflammatory effect by reducing the release of activated cytokines from microglia in the blood and

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brain. In other words, it has been reported that there is a link between the inflammation and cholinergic system because acetylcholine reduces the release of cytokines in the parasympathetic anti-inflammatory pathway (15). It is widely acknowledged that conventional cancer therapies have several negative side effects and that patients are unable to receive adequate care due to the cancer cells' high resistance to cytotoxic and antineoplastic medications (16). Herbal therapy systems are widely used in wealthy countries such as Germany (77%), Canada (70%), France (49%), Australia (48%), and Belgium (31%), especially in chronic diseases such as cancer (17).

The herb *Verbascum thapsus* (VT) is said to be a remedy for various ailments and a food preservative. The biennial plant VT has small yellow blooms and is one of approximately 250 Verbascum species in the Scrophulariaceae family. It can grow to be two meters tall and hairy. VT is a plant that has long been used for medicinal purposes. The anti-inflammatory, analgesic, antiseptic, spasmolytic, expectorant, emollient, astringent, and diuretic properties of VT are known among people in Turkey, Greece, Western United States, India, Pakistan, and Italy (18-20). Alcoholic extracts and flower oils are also available in health stores in developing countries. Despite its benefits, the plant has been used as a fish poison (21), and VT cases of contact dermatitis have been reported (22). This paper describes the compounds found in the VT plant that have anticancer, antioxidant, and AChE inhibitory properties.

Materials and Methods

Chemicals

VT used in the study was obtained in June 2020 from Caldıran/Turkey (39°16′28.8"N and 44°02′28.8"E). Dr Zafer Telli, (Iğdır University) carried out the taxonomic identification. Methanol, ultra-pure water, and acetonitrile, ammonium formate were acquired commercially from Merck (Darmstadt, Germany) and Sigma Aldrich (Sternheim, Germany) for use in the LC-ESI-MS/MS study of the system (St. Louis, MO, USA). Agilent filters with a 25 mm diameter and 0.45 m pore size were used for sample filtration. Sigma-Aldrich commercially provided the chemicals and reagents for the antioxidant activity and enzyme inhibition tests.

MeOH Extraction of VT and LC-ESI-MS/MS analysis of components

The plant material was allowed to dry in a room condition and 20 g of samples were crushed up and extracted in 200 mL of methanol (1:10 (w/v)) for about two weeks at room temperature (25±2 °C). Then it was filtered using 125 mm pore diameter filter paper. The solvent in the filtered aqueous methanol extract was completely evaporated (Heidolph 94200, Bioblock Scientific) to obtain crude extract. A 10 mg/ml stock methanol solution was prepared using the crude extract. The prepared solution was filtered (with a pore diameter of 45 mm) before chromatography-electrospray ionization tandem mass spectrometry examination. With the aid of a 50% methanol and water solvent, this produced stock solution was then diluted to a 2 ppm concentration. The multiple reaction monitoring (MRM)-based LC-ESI-MS/MS equipment was used to analyze the phytochemical constituents in the plant methanol extract. The condition of the chromatography device was tuned to produce optimal chemical separation of the sample. Thus, a reversed-phase Poroshell 120 EC-C18 (100 mm 4.6 mm ID, 2.7 mm) analytical column was used. The temperature in the column was fixed at 25 °C. The elution efficiency was 90% (5 mM ammonium formate + water) – 10% (0.1% formic acid + acetonitrile) and 4.0 L injection volume and 0.4 mL/min solvent flow rate were determined (3).

Evaluation of Cytotoxic Activity

The MTT test was done to measure the cytotoxicity of the extract of VT on the HEK293, Caco-2, and LNCaP cell lines (23). The cell lines were cultured at 37 °C with 5% CO_2 and 95% humidity condition. The cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM), which contains 10% Fetal Bovine Serum (FBS) for cell development and reproduction as well as 1% penicillin/streptomycin to prevent contamination. Cells with a density of around 90% were passed and subcultured in Petri plates. During the passage procedure, the medium was removed, the cells were washed with phosphate-buffered saline (PBS), and Trypsin-EDTA was administered to the cells. Then the cells were incubated under standard incubation conditions for 2-3 minutes. The cells were collected after adding fresh medium and centrifuging for 5 minutes at 2000 rpm. After draining the supernatant, 1 ml of the medium was added to the pellet, and cells were counted under a light microscope using a Thoma slide and Trypan Blue dye. Then the cells were injected onto 96-well plates at a rate of $2x10^3$ cells per well and incubated for 24 hours. After the first 24 hours of incubation, extracts of various quantities were added and incubated for another 24 hours. Then, 10 µl of the MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) reagent (in 100 µl of media) was added after the media in the wells were removed. The medium was once more removed after 3 hours of incubation, and Dimethyl Sulfoxide (DMSO) was used to dissolve the formazan crystals that had developed. A microplate reader device was used to read the resulting color at 590 nm (Epoch, BioTek). For each experimental condition, three replicated wells were employed.

DPPH• and ABTS•+ Scavenging Activity

The DPPH• free radical and ABTS cation radical scavenging activities assays were employed for the assessment of the antioxidant capacity of VP methanol extract and standard antioxidants (24, 25). The ABTS radical cation (ABTS•+) dissolves in aqueous or organic solvents and reacts with the majority of antioxidants. Therefore, the ABTS test was used to identify hydrophilic and lipophilic antioxidants. The DPPH radical scavenging activity of samples was measured at 517 nm, and results were calculated using the following equation: DPPH• scavenging activity (%) = (AC-AS) / AC x 100 where AC denotes the control absorbance and AS the sample absorbance. Because the ABTS•+ radical dissolves in both aqueous and inorganic environments and is unaffected by ionic strength, it might be used to measure the antioxidant activity of both lipophilic and hydrophilic compounds. The ABTS+ stock solution was prepared by combining equal parts 2.45 mM potassium persulfate (Merck, India) and ABTS (Sigma Aldrich, India) aqueous solution (7 mM). The color changes (dark blue/green) in the antioxidant test after mixing with extract or standarts indicate that the ABTS•+, cation radical has lost its radical capabilities

(26). The ABTS•+ cation radical scavenging activity was evaluated at 734 nm and results were calculated using the following equation: ABTS•+ removal activity (%) = (AC-AS) / AC x 100 where AC is the absorbance of the control and AS is the absorbance of the sample.

Evaluation of AChE inhibitory activity

A spectroscopic method using acetylthiocholine iodide as substrate was used to evaluate AChE activity (27). 5,5′-dithio-bis (2-nitro-benzoic) acid (DTNB) was used to measure AChE activity. The sample (50-200 µl) and Tris-HCl buffer (100 µl) solutions were combined with acetylcholinesterase enzyme solution and left at 30 °C for 15 min. The mixes were treated with 50 µl. substrates and DTNB before being measured at 412 nm (28).

Statistical analysis

The statistical software tool Minitab was used to conduct the study's statistical analysis. The Bonferroni test was used to evaluate the multiple comparison difference between the groups and the ANOVA was utilized to discover differences between the two groups. Results were presented as the mean ± SD, and statistical significance was set at $P<0.05$.

Results

Phytochemical Compounds of VT

15 flavonoids and phenolics were examined in the VT extract by LC-MS/MS device based on multiple reaction monitoring (MRM) systems and also the extract MRM chromatogram as given in Figure 1. The major components were determined in the extract as fisetin (2094.94 µg/ml), p-coumaric acid (1416.41 µg/ml), and kaempferol (695.94 µg/ml), respectively. (Table 1).

As seen in the MRM chromatogram, p-coumaric and fisetin, which have the highest peak levels, were determined quantitatively in the plant extract (Figure 1).

Antioxidant Capacity

The free radical scavenging activities (RSA) and antioxidant properties of the sample were assessed using the DPPH and ABTS assays (35,36). Also, Table 2 shows the IC50 values of standards and extracts. The percentage of scavenging abilities was calculated by following equation:

$$RSA\% = \left(1 - \frac{Asample}{Acontrol}\right) X\ 100$$

Based on the fraction concentration necessary to scavenge 50% of the DPPH and ABTS radicals, the IC50 values were computed. Table 2 showed the data of the RSA (IC_{50}) of VT leaf and standard antioxidants. It was determined that the standard antioxidants (BHA, BHT, and Trolox) had free radical scavenging capabilities ranging from 71.16% to 87.41%.

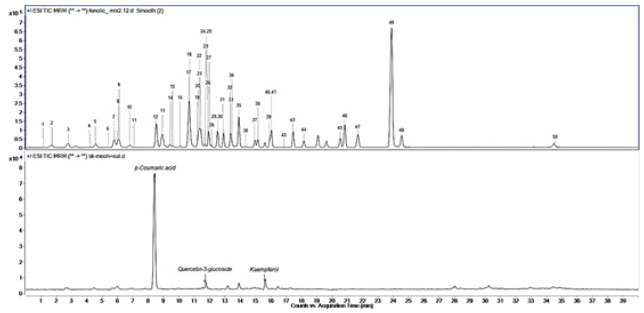

Figure 1. MRM chromatogram of the methanol extract obtained from Verbascum Thapsus.

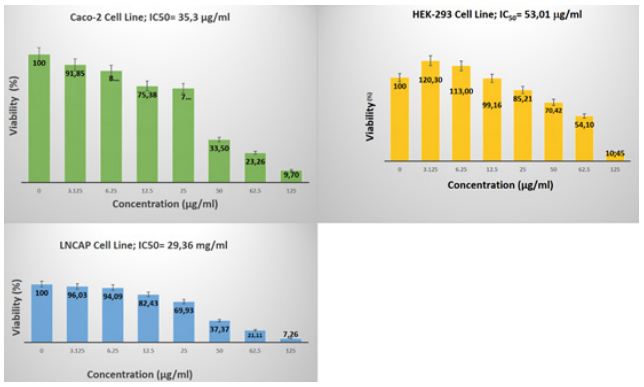

Figure 2. Cytotoxicity of *Verbascum thapsus* methanol extract on A) HEK293, B) Caco-2, and C) LNCaP cell line with *Verbascum thapsus* plant. Results are mean ±SD values for three independent experiments. (*$P<0.05$).

Cytotoxic activity of the VT Extract

In all tested cell lines, the treatments with the VT extract showed dose-dependent cytotoxic activities, as shown in Figure 2. Utilizing data on cell viability, half-maximal effective doses (EC_{50}) were computed. The EC50 dosages of VT extract on the tested cell lines (HEK293, LNCaP, and Caco-2) were calculated as 53.01 µg/mL, 29.36 µg/mL, and 35.30 µg/mL, respectively.

Figure 2 showed how VT extract affects both cancerous LNCaP and Caco-2 cell lines as well as non-cancerous HEK293 cell lines in terms of viability. When compared to the HEK293 cell line, it was determined that both cancerous cell lines (LNCaP and Caco-2) % viability was significantly reduced by doses of 50 g/mL. Our results are abundantly clear that VT extract had a stronger cytotoxic impact against Caco-2 and LNCaP cell lines than HEK293 when IC_{50} values were compared between cell types. The VT extract's highest cytotoxic activity (EC50) was measured in the LNCaP cell line. (Figure 2).

AChE Enzyme Inhibition

Increased AChE activity damages the cholinergic system because it causes acetylcholine to be hydrolyzed and

Table 2. Verbascum thapsus and standards for radical scavenging activity (% DPPH·, ABTS•+).

Antioxidant Compounds	DPPH· scavenging	DPPH· R2	ABTS•+ scavenging	ABTS•+ R2
BHA	71.16	0.98	75.34	0.99
BHT	72.04	0.99	77.78	0.99
Trolox	87.41	0.98	85.15	0.99
Verbascum thapsus (30 µg/mL)	10.33	0.99	35.59	0.99

Table 1. Chromatography-electrospray ionization tandem mass spectrometry analysis results of standard compounds and *Verbascum thapsus* extract.

Number	Standard Compounds	(Rt)*	R^2	RSD	Parent Ion (m/z)	Transitions (m/z)	LOD (µg/L)	LOQ (µg/L)	Recovery (%)	Verbascum thapsus Final Conc. (ng/ml)
1	Ascorbic Acid	1.11	0.99	1.78	175.1	114.9	7.75	23.5	96.70	ND
2	Shikimic acid	1.18	0.99	1.89	173.0	93.1	12.1	16.2	99.70	ND
3	Gallic acid	1.67	0.99	1.62	169.0	125.0	9.0	54.6	101.13	ND
4	Protocatechuic acid	2.75	0.96	1.41	290.9	138.8	21.9	38.6	99.72	166.75
5	Catechin	4.32	0.99	2.08	288.9	245.1	2.57	7.8	100.20	ND
6	4-Hydroxybenzoic acid	4.50	0.99	1.25	137.0	93.1	2.38	7.2	94.67	153.59
7	Chlorogenic acid	5.32	0.99	2.08	353.0	191.0	64.68	196.0	88.73	ND
8	4-Hydroxybenzaldehyde	5.67	0.99	2.19	121.0	92.0	1.91	5.7	98.00	59.12
9	Vanillic acid	5.83	0.99	1.89	167.0	151.8	2.54	7.7	95.60	103.72
10	Caffeic Acid	6.00	0.99	1.07	178.9	135.1	25.74	78	100.70	95.08
11	Syringic acid	6.99	0.99	1.16	197.0	181.8	4.27	12.8	101.90	ND
12	P-coumaric acid	8.41	0.99	1.94	163.0	119.0	3.0	9.1	100.50	1416.41
13	Salicylic Acid	8.84	0.99	1.43	137.0	93.1	6.0	8.3	99.80	ND
14	Taxifolin	9.19	0.99	1.48	304.8	258.9	9.2	12.1	99.70	ND
15	Polydatine	9.69	0.99	1.42	390.9	328.9	12.1	19.2	100.15	ND
16	Trans-ferulic acid	9.50	0.99	1.42	193.1	133.9	7.26	22.3	98.90	180.74
17	Sinapic acid	10.05	0.99	1.46	223.1	208.0	65.2	82.3	99.60	ND
18	Quercimeritrin	11.55	0.99	1.88	464.8	302.9	68.5	88..2	98.90	ND
19	Coumarin	8.41	0.99	2.19	147.1	91.3	214.2	247.3	97.509	ND
20	Scutellarin	11.11	0.99	1.33	462.8	286.8	16.2	21.2	99.30	ND
21	O-coumaric acid	8.40	0.99	2.12	163.0	119.1	31.8	40.4	99.80	ND
22	Cynarin	11.36	0.99	1.58	516.8	162.9	19.5	28.5	99.30	ND
23	Protocatechuic ethyl ester	10.57	0.99	1.44	181.0	107.9	15.4	22.2	98.90	ND
24	Hyperoside	11.55	0.99	1.78	464.8	302.8	140.0	162.0	99.90	6.29
25	Quercetin-3-glucoside	11.78	0.99	1.73	464.8	302.9	9.87	29.9	99.00	28.92
26	Rutin	11.75	0.99	2.08	608.9	399.4	28.5	85.0	99.30	ND
27	Resveratrol	12.07	0.99	1.08	464.9	302.8	7.1	9.1	99.80	ND
28	Naringin	11.89	0.99	1.27	227.0	142.9	24.37	73.8	98.60	172.09
29	Rosmarinic acid	12.26	0.99	1.58	358.9	160.7	16.2	21.2	99.90	ND
30	Quercetin-3-D-xyloside	12.42	0.99	1.67	432.7	299.5	9.87	29.9	100.00	ND
31	Hesperidin	12.55	0.99	1.58	611.0	302.9	19.0	26.0	99.70	ND
32	Neohesperidin	12.78	0.98	1.42	610.7	302.9	18.7	25.3	98.90	ND
33	Kaemerol-3-glucoside	13.18	0.99	1.33	448.8	286.9	10.4	15.6	98.90	1.73
34	Fisetin	15.62	0.99	1.24	286.8	137.1	10.1	12.7	100.10	2094.94
35	Oleuropein	13.61	0.99	1.15	539.1	275.1	24.6	30.6	100.20	ND
36	Baicalin	13.89	0.99	1.30	446.8	270.9	24.3	30.2	99.80	ND
37	Trans-cinnamic acid	14.32	0.99	1.34	147.1	103.1	215.1	240.2	99.70	ND
38	Ellagic acid	14.83	0.99	1.52	301.0	145.0	56.9	71.1	99.90	ND
39	Quercetin	14.82	0.99	1.67	300.7	150.9	15.5	19.0	99.90	17.86
40	Naringenin	14.96	0.99	2.38	270.9	119.1	2.6	3.9	100.40	ND
41	Silibinin	15.95	0.99	2.22	482.8	163.1	19.3	28.3	99.80	ND
42	Hesperetin	16.27	0.99	2.54	300.9	164.0	7.1	9.1	100.10	ND
43	Morin	15.83	0.99	2.20	302.8	153.0	22.3	28.4	100.20	ND
44	Kaempferol	15.62	0.99	1.95	284.1	116.1	12.39	37.5	99.00	695.94
45	Tamarixetin	17.27	0.99	1.96	315.0	299.9	24.7	35.1	99.50	29.21
46	Baicalein	18.04	0.99	1.81	271.0	123.0	23.9	32.7	99.40	ND
47	7-Hydroxyflavone	19.00	0.99	1.59	238.7	137.1	64.9	82.1	99.80	ND
48	6-Hydroxyflavone	19.67	0.99	1.51	239.0	103.1	5.9	8.2	100.20	ND
49	Biochanin A	20.72	0.99	1.22	284.9	151.1	212.4	244.2	99.50	ND
50	Chrysin	20.50	0.99	1.57	254.1	153.0	0.012	0.012	99.80	ND

*Rt: Retention time; R^2: Coefficient of determination; RSD: Relative standart deviation; LOD/LOQ (µg/L): Limit of detection/Quantification

amyloid proteins to be produced, which are the root causes of neurodegenerative diseases like Alzheimer's. The inhibition effects of VT methanol extract and Tacrin (standard inhibitor of AChE) on AChE were determined by calculating the IC50 value (Table 3).

Discussion

Many caffeic acid derivatives, iridoid glycosides, and flavonoids were found as chemicals from *Verbascum* species in the literature. Caffeic acid derivatives include chlorogenic acid, verbascoside, and luteolin; flavonoid-

Table 3. Half maximal inhibition concentration (IC50 values; µg/mL) of Verbascum thapsus on acetylcholinesterase.

Sample	AChE (IC50)	R^2
Verbascum thapsus	3.26 ± 0.12*	0.972
Tacrine	0.062±0.007	0.965

* Data are presented as mean values; ±standard deviation (SD) of triplicate values.

type substances include apigenin, luteolin, and their glycosidic derivatives were most known (29, 30). Numerous research indicated that the Verbascum species included luteolin, apigenin, diosmin, fisetin, quercetin, aucubin, and rutin in addition to protocatechuic acid, ferulic acid, chlorogenic acid, rosmarinic acid, p-coumaric acid and caffeic acid (31-34). The plant extract demonstrated lower DPPH and ABTS radical scavenging activities when compared to standard antioxidants. Studies using extracts from different Verbascum species reported differences in antioxidant properties (37-40). The plant extract demonstrated a strong inhibitory activity on cancerous cell lines when compared with non-cancerous HEK293 cells at high doses. Similar studies with reported EC50 values between 0.01 and 73.4 g/mL, supported our findings (41-43). Results obtained from the anticancer and cytotoxic studies indicate that phenolic chemicals in plants have inhibitory effects on cancerous cells (42, 44). Fisetin and p-coumaric acid, polyphenols having pharmacological properties, were identified in significant amounts in the plant extract, according to our findings. Fisetin is well recognized for exhibiting encouraging antitumor in several malignancies. According to reports, fisetin accomplishes this effect by preventing the growth and advancement of the cancer cell cycle and by causing cells to undergo (45). According to certain reports, p-coumaric acid similarly affects malignant cells in a similar manner as fisetin in the plant extract. Depending on dosage p-coumaric acid restricts cell migration and proliferation was reported (46). Many plants that are currently thought of as food have a variety of phytochemicals that are still unknown and beneficial for human health. This study investigated the anticancer, anticholinesterase, and antioxidant activities of Verbascum thapsus methanol extract. Also, this study analyzed the chemical composition of VT methanol extract using chromatography-electrospray ionization tandem mass spectrometry. Fisetin and p-coumaric acid were found as the two major organic components in the VT extract. These components are known to be responsible for many biological activities. These components are known to be responsible for many biological actions.

Conclusions

Growth inhibition of the VT extract on colone (Caco-2) and prostate (LNCaP) cell lines was significantly stronger than on healthy HEK293 (Human embryonic kidney 293 cells) cell lines. Furthermore, the outcomes shown that the VT methanol extract can scavenge both ABTS (. +) and DPPH free radicals and also has considerable inhibitory properties against the AchE. In the future, it's feasible that newly identified phytochemicals may be included in some food formulas as well as the chemical composition of drugs used to treat Alzheimer's and cancer. However, it has to be supported by a more thorough study. For possible anticancer and anticholinergic actions, novel compounds from diverse plant components must be identified and described.

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Interest conflict

The authors have no relevant financial or non-financial interests to disclose.

Authors' Contribution

All authors had equal roles in study design, work, statistical analysis, and manuscript writing.

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Ethics approval and consent to participate

No humans or animals were used in the present research.

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