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Total Phenolic Content, Antioxidant Properties and Element Concentrations of Methanol and Acetone Extracts of *Stachys tmolea* Boiss.

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Abstract

DPPH radical scavenging, total antioxidant status, phenolic and element concentrations of the different solvent extracts of *Stachys tmolea* Boiss. were determined in the study. It was found that, the methanol extract of the plant contains total phenolic substance is 68.91±1.4 mg Gallic acid equivalant/g extract. % DPPH radical inhibition was 64.79±1.98 and total antioxidant status was 2.28±0.21 mmol Trolox Equiv./L. The acetone extract contains total phenolic substance (27.26±1.2 mg GAE/g extract). % DPPH radical was 50.33±2.05, and total antioxidant status was 0.57±0.04 mmol Trolox Equiv./L. It is seen that, the methanol extract has properties close to the synthetic antioxidant butylated hydroxy anisole (total phenolic substance concentration is 82.33±2.1 mg GAE/g). It is determined that the species also contains elements such as Cu, Mn, and Fe that participate in superoxide dismutase and catalase. In conclusion, the methanolic extract of *Stachys tmolea* exhibits radical scavenging and antioxidative properties. The species is thought to be a plant that can evaluate in phytotherapy studies due to the important elements it contains.

Keywords
Stachys tmolea Boiss.; TAS; DPPH; Mineral

Stachys tmolea Boiss. in Metanol ve Aseton Ekstraktlarının Toplam Fenolik İçeriği, Antioksidan Özellikleri ve Element Konsantrasyonları

Öz

Bu çalışmada *Stachys tmolea* Boiss. farklı çözücü ekstraktlarının DPPH radikal süpürücü ve toplam antioksidan aktivite ile fenolik ve element konsantrasyonları belirlenmiştir. Bitkinin metanol özütünün toplam fenolik madde içeriği 68.91±1.4 mg GAE/g ekstre olarak tespit edilmiştir. % DPPH radikal inhibisyonu 64.79±1.98 ve toplam antioksidan aktivitesi 2.28±0.21 mmol Trolox eşdeğeri/L dir. Aseton özütü 27,26±1,2 mg GAE/g ekstre toplam fenolik madde içerir. % DPPH radikali inhibisyonu 50.33±2.05 ve toplam antioksidan aktivitesi 0.57±0.04 mmol Trolox eşdeğeri/L dir. Metanol ekstraktının sentetik bir antioksidan olan bütillenmiş hidroksi anizole (toplam fenolik madde konsantrasyonu 82.33±2.1 mg GAE/g) yakın özelliklere sahip olduğu görülmektedir. Türün ayrıca süperoksit dismutaz ve katalaz enziminin yapısındfa bulunan Cu, Mn, Fe gibi elementleri de içerdiği belirlendi. Sonuç olarak, *Stachys tmolea*'nın metanolik özütü, radikal süpürücü ve antioksidatif özellikler sergiler. Türün içerdiği önemli elementler nedeniyle fitoterapi çalışmalarında değerlendirilebilecek bir bitki olduğu düşünülmektedir.

Anahtar Kelimeler
Stachys tmolea Boiss.; TAS; DPPH; Mineral

1. Introduction

Plants are used for general health and disease prevention purposes. The primary aim of preventing the diseases that occur can be achieved by preventing these diseases. Preventing the disease, plant foods are becoming more reliable than synthetic substances. Natural antioxidants are gaining importance for this purpose, and their positive effects on human health are being investigated. The production of plant-derived medicines, multi-component herbal pills, dietary supplements, functional foods, and next-generation

botanical therapeutics containing recombinant proteins produced by plants is widespread. Many plants have been proven to have antioxidant, antimicrobial, and cancer-protective properties (Arai 1996).

Planting and production of plants of Lamiaceae plants this family are easy. In addition to the leaves grown for eating, some are used for decorative purposes. They are also important bee plants that provide nectar and pollen. Essential oils secreted from the glands on the leaves and the plant's stems are obtained commercially from many Lamiaceae species. Menthol, thymol and many other monoterpenes are used in medicine and the food industry (Topcu and Kusman 2014, Licina *et al.* 2013). They contain monoterpenes, sesqui, di- and triterpenoids, flavonoids and other phenolics. They are species with antioxidant, neuroprotective anti-inflammatory, antimicrobial, cholinesterase enzyme inhibitions (Perry and Howes 2011).

Stachys L. is the largest genera of the Lamiaceae family. Most species grow in rocky places (Bhattacharjee 1982). Subsequently, new species were described from Turkey. Stachys is represented in Turkey by 83 species belonging to the genus, 12 subdivisions, 15 divisions, and 2 subcategories (Bhattacharjee 1982, Duman 2005, Akcicek 2010). Infusions of Stachys, known as "mountain tea", are often used for therapeutic purposes (Ozturk *et al.* 2009). For anti-inflammatory (Khanavi *et al.* 2005, Skaltsa *et al.* 2001), antibacterial (Grujic-Jovanovic *et al.* 2004), anticancer (Amirghofran *et al*. 2006), anti-*Helicobacter pylori* (Stamatis *et al.* 2003) purposes their use is common. As a result of the content studies, germacrane D, linalyl acetate, β-caryophyllene, linalool, β-Pinene, and caryophyllene oxide were determined as the basic components of most of the species. *Stachys tmolea*, from the Lamiaceae family, is a perennial herbaceous plant. It grows on limestone gorges and moving cliffs, eroded stony shores, at an altitude of 200-1900 meters. It blooms in May-August. *Stachys tmolea* Boiss. grows in western, northwestern, and central Anatolia. Its plant has solitary or branched flowering stems about 1 m long. The trunk is usually soft hairy. Basal leaves are oblong in size, 2-14 cm × 0.5-4.5 cm. Its edges are carved or full, the apical part is sometimes pointed, sometimes oval, narrowed in the basal part (Kaya *et al.* 2001, Skaltsa *et al.* 2001). In this study, the antioxidant activity and element concentration of the methanol and acetone extracts of the endemic *S. tmolea* were determined.

2. Materials and Methods

2.1 Plant Material and Extraction Process

S. tmolea plant was collected from Kumalar Mountain, Çakmaktepe Pass, Şuhut/Afyonkarahisar (37 T 0719245, UTM 4511056) from an altitude of 1880 m. It was identified by Mustafa Kargioglu. The plant specimen, known locally as Sürmeli Çayçe, is kept in the Herbarium of Afyon Kocatepe University.

In the extraction of *S. tmolea*, mixtures consisting of above-ground parts such as flowers, leaves, branches, and stems were used. All plant parts were brought in small pieces (0.32 mm) and dried up at room temperature. 20 g of the powdered *S. tmolea* plant was taken and 400 mL of solvent was added to prepare the extract. The extracts were filtered, and the methanol/acetone was removed with a rotary evaporator. Prepared extracts were used to determine DPPH radical scavenging capacity and total phenolic content. The dried and powdered plant was taken and 10 mL of solvent was added and sonicated for the total antioxidant potential. After filtration on filter paper, it was centrifuged. The supernatant was taken, centrifuged again and used for analysis. The dried and powdered plant was taken and placed in the microwave oven to decompose the organic components to determine the mineral substance content of the plant (Aksoy and Senyer 2017).

2.2 Method

While determining the free radical scavenging capacity of methanol and acetone extracts obtained from the aerial parts of the plant, DPPH (2,2-diphenyl-1-picryl hydrazine) was used as a free radical. Ethanol and then DPPH solution was added to the samples and standards prepared at different concentrations. After 30 minutes at room

temperature, it was incubated in the dark. The absorbance at 517 nm was recorded against the blank consisting of ethanol. Extracts with antiradical properties cause a decrease in purple color due to DPPH. In this way, the reduced absorbance provides the remaining amount of DPPH solution. Chart of DPPH prepared by using the calibration determined the radical removal activities of the samples. Results were expressed as % inhibition (Thaipong *et al.* 2016).

Phenolic content concentration was determinated according to the Folin-Ciocalteu method. After adding 250 µl of Folin Ciocalteu reagent and 50 µl of extract solution to the tube, the total volume was made up to 3 ml with distilled water. 750 µl 20% (w/v) Na_2CO_3 after 5 min incubation, It was kept at room temperature. The absorbance was measured at 760 nm spectrophotometrically (Pharmaspec-1700, Shimadzu UV-VIS spectrophotometer). The results were calculated from the calibration curve of the gallic acid used as a standard and determined as the gallic acid equivalent. (Wojdylo *et al.* 2007).

Antioxidant characteristic was measured with kits (Rel Assay, Gaziantep, Turkey). The kits work according to the principle that ABTS (2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid) reacts with H_2O_2 in an acidic environment to oxidize $ABST^+$ and then the radical loses its initial blue/green color. The absorbance is measured at 660 nm (Erel 2004).

A microwave oven (Speed Wave ERGHOF) was used for the degradation of organic compounds in its structure before starting the element analysis of the species. 0.5 g of the *S. tmolea* plant was taken and added to the Teflon sample cups. Nitric acid, perchloric acid, and hydrogen peroxide solutions were added to the sample in certain proportions. Warming was carried out by placing it in the microwave oven. The samples taken into the microwave oven were kept at 90-150 ºC for specific periods. The samples, which came out of the oven and took room temperature, were transferred to 10 mL flasks. They were made up to 10 mL with 18.2 Ω ultrapure water. Element types and concentrations were measured in the samples taken into balloon jugs with ICP-OES (Spectro Genesis Kleve, Germany) (Aksoy and Senyer 2017).

2.3 Statistical Analysis

The analyzes were repeated in triplicate, and the average values were recorded. Statistical calculations of the results obtained using SPSS 15.0 and data analysis were expressed as "Mean ± Standard Deviation" values. Differences between groups were determined by one-way analysis of variance (one-way ANOVA). The difference between which groups was determined at $p<0.05$ significance value according to Duncan's multiple range test.

3. Results and Discussion

Primary metabolism in plants includes the metabolic pathways required for the cell to maintain its viability. In secondary metabolism, components responsible for plant defense are produced. Secondary metabolites are alkaloids, isoprenoids, terpenes, flavonoids, phenolic compounds, amino acids, plant amines, and glycosides (Güven and Gürsul 2014). Secondary metabolites have essential functions in plants. They begin to be synthesized as a defense mechanism when the plant is attacked by any stress factor (UV light, drought, salinity, herbicide, air pollution, heavy metals, etc.). When plants encounter environmental stress, an increase in the number of free radicals and other oxidative molecules is observed (Mazid *et al.* 2011). The most known free radicals are; superoxide and hydrogen peroxide and nitric oxide (NO). Reactive oxygen species (ROS) redox reactions are formed by water oxidation by mitochondrial or chloroplast electron transfer chains (Güven and Gürsul 2014). The survival of cells against this harmful molecule depends on their ability to operate their detoxification mechanisms. The result is the production of enzymatic and non-enzymatic antioxidants associated with phenolic secondary metabolism. Secondary metabolites can be found in roots, fruits, leaves, flowers, rhizomes, aerial parts, and seeds. These metabolites form the bioactive compounds of plants that can be used as functional foods (Güven and Gürsul 2014, Mazid *et al*. 2011).

Phenols constitute an important part of secondary metabolites. They exhibit biological activity due to the phenolic compounds they contain from the Lamiaceae family. There are studies on antioxidant, antimicrobial, analgesic, anti-inflammatory, and effects due to the flavonoids in their structures. Monoterpenes in essential oils, often to have anti-inflammatory properties. It is known that the saponins they contain have anti-inflammatory, analgesic and cytotoxic effects (Vaishali Rai *et al.* 2013). Phenolic antioxidant activity depends on the location of the hydroxyl groups in the aromatic rings of the compounds (Balasundram *et al.* 2006).

Lamiaceae (mint family) is a medicinal plant family. Lamiaceae is a diverse, cosmopolitan family. Species of the family live in different natural ecosystems. Most of the species have essential oils. Aromatic essential oils are mostly found in plants' leaves and above-ground parts. The species is valuable to the cosmetics, flavoring, fragrance, perfumery, pesticide and pharmaceutical industries. It is cultivated for flavor and edible leaves of some Lamiaceae species (Ozkan 2008, Carović-Stanko *et al.* 2016).

In the study in which the phenolic substances of the *Stachys lavandulifolia* species were determined by LC-MS/MS, quinic acid (2534±122 ppb), hyperoside (170±8 ppb), protocatechic acid (117±6 ppb) and p-coumaric acid (112±6 ppb) was determined as the most abundant phytochemical. They concluded that these compounds show antioxidant properties due to their polyphenolic structure and the removal of radicals by donating electrons due to the phenol groups in the aromatic rings in their structures. In addition, they stated that the high number of -OH of these compounds is an important factor that increases the total antioxidant level of the plant (Bingöl 2016).

In the study, the total phenolic content of the methanol and acetone extracts of the species was determined. The extracts of *S. tmolea* and standard antioxidant substances' total phenolic content are shown in Figure 1. The total phenolic content of acetone extract was 27.26±1.2 mg GAE/g extract; The total phenolic content of the methanol extract is 68.91±1.4 mg GAE/g extract. The total phenolic substance concentration of synthetic antioxidant butylated hydroxyanisole (BHA) is 82.33±2.1 mg GAE/g extract and natural antioxidant ascorbic acid (AA) is 146.76±1.06 mg GAE/g extract. Ascorbic acid's total phenolic substance concentration was found to be the highest. It is seen that the total phenolic substance concentration of BHA is higher than the extracts. When compared between the extracts; It is seen that methanol extract is more than acetone extract. The total phenolic substance concentration of the methanol extract was found to be close to BHA. In short, total phenolic content can be expressed as AA>BHA>STM>STA.

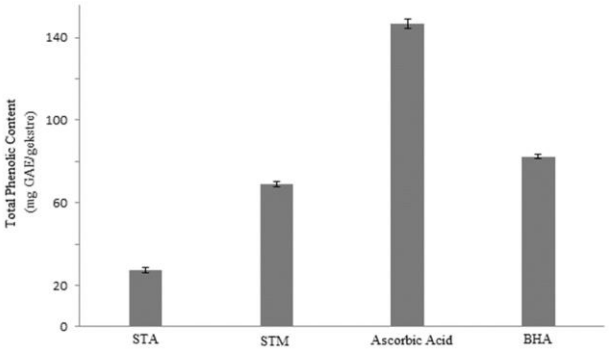

Figure 1. Total phenolic content of methanol and acetone extracts of *Stachys tmolea* plant and standard antioxidant substances. BHA: Butylated hydroxyanisole; STM: methanol extract of *Stachys tmolea*; STA: acetone extract of *Stachys tmolea*

Plant antioxidants are vital as neutralizing free radicals and reducing oxidative stress damage can help the body. Lamiaceae family plants contains rosmarinic acid, which exhibits antioxidant properties, and contains volatile terpenes (Wink 2003). There are many studies examining the antioxidant activities of the Lamiaceae family (Ahmad *et al.* 2012, Lagouri *et al.* 2013, Trakoontivakorn *et al.* 2012, Sodré *et al.* 2012). Albayrak *et al.* (2013) stated in their study on thyme, rosemary and sage, mint, lemon balm and basil that the total phenolic substance concentration and antiradicalic activity of these species are quite high. Diphenyl-1-picrylhydrazil (DPPH) is a widely used free radical to measure the radical scavenging activity of plants. This method is often used because

it is fast, simple, and inexpensive. The DPPH method is based on the reduction of a stable free radical, DPPH. The single electron free radical DPPH achieves maximum absorption at 517 nm (purple color). DPPH, a stable free radical, is reduced to DPPH-H by reacting in the presence of a hydrogen donor. In the DPPH-H form, lightening in color (yellow color) is observed according to the number of captured electrons. This method is one of the most accepted models to evaluate any plant's free radical scavenging activity, hydrogen donors, and antioxidant activities. In the thesis study of Bingöl (2016), DPPH and ABTS radical scavenging activities and FRAP, CUPRAC, ferric thiocyanate activities of ethanolic and aqueous extracts of Stachys *species Stachys lavandulifolia* were investigated. At the end of the study, it was found that the ethanolic extract of the species (50.1%) had a more DPPH radical scavenging activity than the aqueous extract (14.6%) (Bingöl 2016).

In this study, DPPH radical scavenging activities of methanol and acetone extracts of *Stachys tmolea* species were investigated. The DPPH free radical removal activity of the plant's methanol and acetone extracts and standard substances is given in Figure 2 as % inhibition. As seen in Figure 2, the DPPH free radical scavenging activity of natural antioxidant ascorbic acid is higher than synthetic antioxidant butylated hydroxy anisole. These standard antioxidants have higher DPPH Free radical scavenging activity than extracts. When the extracts were examined, it was seen that the methanol extract had higher DPPH free radical scavenging activity than the acetone extract. All samples' DPPH free radical scavenging activity can be shown as AA>BHA>STM>STA.

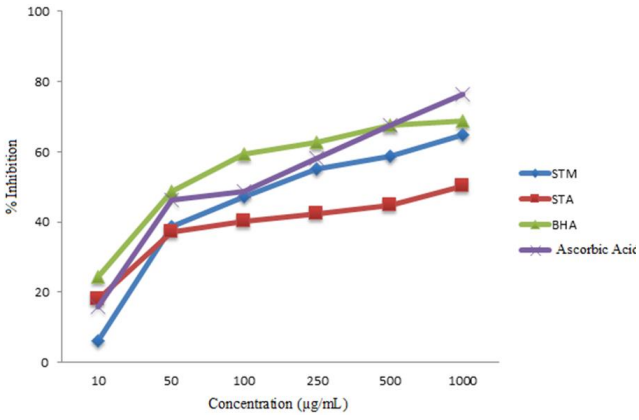

Figure 2. % inhibition values of methanol and acetone extracts of Stachys tmolea and standard substances. BHA: Butylated hydroxyanisole; STM: methanol extract of Stachys tmolea; STA: Acetone extract of Stachys tmolea

The IC_{50} values of the methanol and acetone extracts of the *Stachys tmolea* plant and standard substances are given in Figure 3. The IC_{50} dose indicates the plant extract/antioxidant concentration that inhibits half of the 1 mM DPPH radical. Among the standard antioxidants, the IC_{50} dose of ascorbic acid is lower than the IC_{50} dose of BHA, consistent with the DPPH radical scavenging activity. However, this was not observed among the extracts. The IC_{50} dose of STM is higher than the IC_{50} dose of STA. In general terms, the IC_{50} doses of all samples can be represented as AA<BHA<STA<STM.

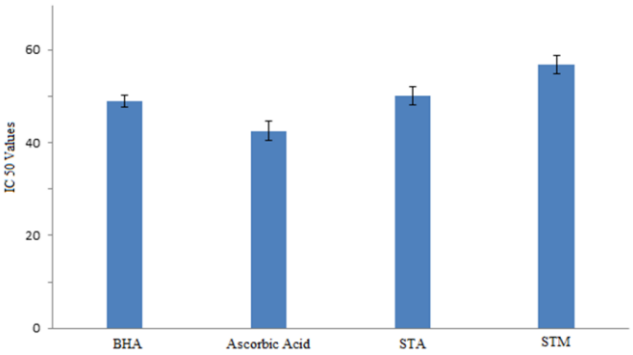

Figure 3. IC_{50} values of methanol and acetone extracts of *Stachys tmolea* and standard substances. BHA: Butylated hydroxy anisole; STM: methanol extract of *Stachys tmolea*; STA: Acetone extract of *Stachys tmolea*

Purification and determination of each component of a natural antioxidant source alone are both expensive and time-consuming assays. Considering the relationships between antioxidant compounds in a mixture, methods measuring total antioxidant capacity gain importance. This study determined the total antioxidant status of methanol and acetone extracts of *Stachys tmolea*. The total antioxidant status of the methanol and acetone extracts of the *Stachys tmolea* is shown in Figure 4.

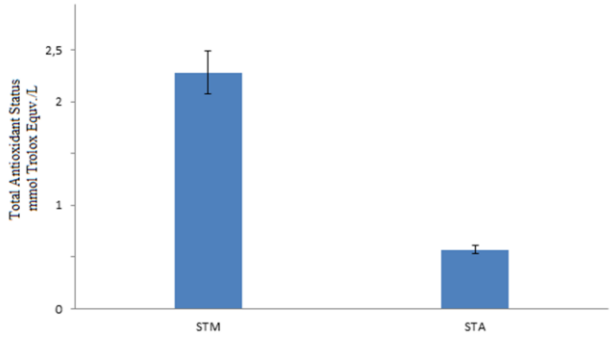

Figure 4. Total antioxidant status of methanol and acetone extracts of *Stachys tmolea*. STM: methanol extract of *Stachys tmolea*; STA: acetone extract of *Stachys tmolea*

The total antioxidant status of the methanol extract is 2.28±0.61 mmol Trolox Equiv/L; The total antioxidant status of the acetone extract is 0.57±0.04 mmol Trolox Equiv/L. It is seen that the total antioxidant status of the methanol extract is considerably higher than the total antioxidant status of the acetone extract.

Air, water, and soil are necessary for plants' development and life stages. The elements in the soil, it has an important place in plant development. The soil should have a sufficient amount of nutrients and organic matter content and have a particular pH value. These substances that make up the soil can vary greatly in quantity. As a result of this change, differences occur in the soil's physical, chemical, and biological properties. The nutrients contained in the soil affect the plant species growing in that region. The mineral substance contents of plants differ in different developmental stages and climatic conditions of the plant. The acidity or alkalinity of the soil (pH) is also a feature that affects the utilization of nutrients and plant growth. Soil pH, and the variability of calcium and magnesium are adequate on the solubility of aluminum, iron, phosphate, and microelements (Gentili *et al.* 2018). In another study, anatomical and ecological analyzes of six Stachys species (*S. annua*, *S. setifera*, *S. sosnowskyi*, *S.cretica*, *S. iberica* and *S. tmolea*) were made. In this context, the element concentrations of the species were also examined. N, P, K, Ca, Mg, Na, S concentrations in the leaves, stems and roots of *Stachys tmolea* are given in Table 1 (Leblebici 2011).

Table 1. N, P, K, Ca, Mg, Na, S concentrations in the leaves, stems and roots of *Stachys tmolea* (Leblebici 2011)

	N (ppm)	P (ppm)	K (ppm)	Ca(ppm)	Mg(ppm)	Na(ppm)	S(ppm)
Leave	3,38	2320	23760	22435	5625	317	2416
Stem	0,30	870	14180	7850	1450	184	494
Root	0,67	680	7125	1610	1175	154	992

Table 2 shows the elements contained in *Stachys tmolea* and their amounts. It is seen that the K, Ca, Mg and Na concentrations determined in the previous study are quite high compared to the concentrations determined by us. *Stachys tmolea* samples taken from the Eskişehir-Kütayha region were collected in the study. This situation may be caused by regional, climatic, seasonal, geographical and geological, genetic, and ecological differences. In the study, apart from these elements, aluminum, boron, barium, beryllium, bismuth, cobalt, chromium, copper, iron, gallium, lithium, manganese, nickel, and lead concentrations were determined and contributed to the literature.

Table 2 Elements contained in *Stachys tmolea* and their amounts

	Concentration (ppm)		Concentration (ppm)		Concentration (ppm)		Concentration (ppm)
Al	781.22±35.4	**Ca**	8357.1±98.42	**Ga**	2.29±0.28	**Na**	209.39±4.78
B	18.06±2.1	**Co**	0.137±0.02	**K**	4616.32±66.87	**Ni**	2.57±0.25
Ba	52.14±1.8	**Cr**	1.82±0.26	**Li**	0.569±0.04	**Pb**	3.90±0.18
Be	0.119±0.03	**Cu**	6.03±1.08	**Mg**	845.98±13.68	**Zn**	19.28±1.04
Bi	3.34±0.7	**Fe**	997.82±21.39	**Mn**	35.76±1.36		

Multielement analyses of *Stachys tmolea* quantified by ICP-OES. Concentrations are given as mean ± standard deviation.

4. Conclusions

There are very few studies on *Stachys tmolea* Boiss, which is endemic to Turkey in the literatüre. As a result of this study, in which the DPPH radical scavenging activity, total phenolic content, total antioxidant status, and mineral substance levels of the methanol and acetone extracts of the plant were determined, it was determined that especially the methanol extract has a strong radical scavenger, high phenolic content and antioxidant status. These properties of the methanol extract were found to be close to the synthetic antioxidant BHA. In addition, it is crucial to find elements such as Cu, Fe, and Mn, which are added to the mineral substances and antioxidant enzyme structure in the plant. It can be said that especially the methanol extract of the plant stands out with its antioxidative properties.

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