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Effect of employing different extraction techniques and solvents on the total phenolic content and antioxidant properties of an Iranian endemic species *Zeravschania khorasanica* (Apiaceae)

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Abstract: *Zeravschania khorasanica*, a species endemic to the eastern part of Iran, possesses distinct characteristics that distinguish it from its two closely related species. This research employed five different extraction techniques to identify the active components, total phenolic content, and *in vitro* antioxidant activity of the extract. Furthermore, hydro-distillation was utilized for GC/MS analysis to determine the composition of the essential oil. The total phenolic content was estimated using the Folin-Ciocalteu assay and the antioxidant capacity was evaluated using the DPPH radical scavenging test. The findings revealed that ethanolic Soxhlet extraction yielded the highest efficiency in extracting total phenolic content (88.19±1.99 gallic acid mg/100g). In contrast, water maceration extraction demonstrated the highest antioxidant activity (68.1±5.48 %). Interestingly, the study uncovered that there is no significant positive correlation between the phenolic content and the antioxidant activity of the plant. Additionally, HPLC analysis identified three phenolic constituents in the extract. The Soxhlet extraction method yielded the highest levels of chlorogenic acid (5.8 ppm), caffeic acid (4.1 ppm), and salicylic acid (10.3 ppm). As per the GC/MS analysis, a total of eleven compounds were identified. The predominant compounds were elemicin at 58.19% and trans-α-bergamotene at 25.78%.

Keywords: Antioxidant activity, apiaceae, essential oil, extraction techniques, phenolic content, *Zeravschania khorasanica*.

INTRODUCTION

Apiaceae (Umbelliferae) are deemed one of the largest flowering plant families worldwide, comprising 434 genera and approximately 3780 species (Ahmad *et al.*, 2017; Duran *et al.*, 2010; Lamamra *et al.*, 2017; Thiviya *et al.*, 2022). Iran, with its diverse flora, is considered one of the significant centers of variation, hosting over 100 genera and nearly 400 species within the family Apiaceae (Amiri and Joharchi, 2016). Aromatic plants are extensively applied as sources of nutrition, flavor, and medicine (Amiri and Joharchi, 2016; Thiviya *et al.*, 2022). Moreover, this plant family is known for producing secondary products with various biological properties (Martins *et al.*, 2016). The essential oils (EO_s) found in the secretory glands of plants are vital for their environmental resistance and play a crucial role in their survival. These oils act as a defense mechanism against various environmental stressors and pathogens, helping the species adapt and thrive in their respective habitat (Lefahal *et al.*, 2018; Martins *et al.*, 2016).

Extraction is the process of separating secondary metabolites from inert materials and active plant materials using a suitable solvent. Most medicinal plants possess antioxidant properties (Azwanida *et al.*, 2015; Abubakar and Haque, 2020). Medicinal plants can be extracted using various techniques (Abubakar and Haque, 2020). The genus *Zeravschania* Korovin currently consists of 14 herbaceous species (Lyskov *et al.*, 2022; Terentieva *et al.*, 2021). Five of these species were recently described (Lyskov *et al.*, 2022). The genus *Zeravschania* has a significant distribution in Iran, with its range extending from the western to eastern regions of the country, and reaching into central Asia (Kljuykov *et al.*, 2019; Lyskov *et al.*, 2022). *Zeravschania* was initially recognized as a monotypic genus consisting only of the species *Z. regeliana* (Kljuykov *et al.*, 2019; Lyskov *et al.*, 2022).

Zeravschania khorasanica, recently described from Khorasan Province (Binalud Mountains), varies from two closely related species i.e., *Z. regeliana* and *Z. scabrifolia* Pimenov, morphologically (Kljuykov *et al.*, 2019).

Several investigations have been carried out to explore the phytochemical compositions of *Zeravschania* (Mobarakeh *et al*., 2014; Saei-Dehkordi *et al*., 2014; Yassa *et al*., 2003; Yassa *et al*., 2005; Zahedi and Aboli, 2014). However, there is a lack of comprehensive research on the phytochemistry and potential antioxidant properties of *Zeravschania khorasanica*. Therefore, in this study, we aim to compare different extraction techniques to determine the chemical compounds of *Zeravschania khorasanica* and evaluate its antioxidant properties.

MATERIALS AND METHODS

Plant materials

The *Zeravschania khorasanica* fresh aerial parts were obtained in June 2022 from the Kang region of Khorasan

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Province in Eastern Iran, at an altitude of 1760 meters (36°180' 480" N, 59° 13' 254" E) during the full flowering stage (see fig. 1). It was recognized by M.R. Joharchi and deposited at FUMH (No. FUMH-36646). The plant material was meticulously dried at room temperature, and the resulting powder was extracted to be used for further analyses.

Extraction procedures

Plant extraction by maceration

For this aim, two different solvents i.e., water and 80% ethanol were used exclusively. For each solvent, a quantity of 1 gr of the milled raw material was added to 10 mL of the solvent. The admixture was shaken gently overnight (180 rpm) and was centrifuged at 1000 rpm (4 °C) for two minutes. The mixture passed through a filter (0.45 µm in diameter), and was condensed under a vacuum employing a rotary evaporator (Heidolph, Laborota, Germany) to remove the solvent at 45 ◦C for 1 hour. The obtained extracts were then placed in the dark vials and kept at refrigerator temperature until usage (Tofighi *et al.,* 2020).

Plant extraction by Soxhlet

The Soxhlet extraction process was performed following the protocols in the studies conducted by Galviz-Quezada *et al.* (2019) and Lopeda-Correa *et al.* (2022). To perform this process, a Soxhlet device with a capacity of 250 mL was loaded with 5 grams of milled raw material and 200 mL of 80% ethanolic solvent. The extraction was carried out at an atmospheric pressure of 95°C for six to eight hours. The extract was filtered through a 0.45 m filter and stored at -22°C for more analysis. The Soxhlet extraction process was carried out twice to ensure the accuracy of the results. After one hour of evaporation at 45°C and 100 millibars pressure using a Heidolf Laborota rotary evaporator, the volume of the ethanolic solvent was reduced to 50mL.

Plant extraction by ultrasound

To conduct the ultrasound-assisted extraction experiment, we followed the methodology described in the studies conducted by Torres *et al.* (2018) and Jaimez-Ordaz *et al*. (2021), with some modifications. We used an ultrasonic homogenizer (Branson, B42-162, USA). To prepare the sample, we mixed 1 gram of raw material with 10 mL of water and ethanol to maintain an S/F ratio of 10 for all extractions. The mixture was left to stand at room temperature. The extracted solution was then filtered through a 0.45 m diameter filter and stored in a 100 mL amber flask at -22 ◦C for further analysis.

Assessment of total phenolic content (TPC)

To determine TPC in the extracts, the Folin-Ciocalteu reagent assay was used (Pacifico *et al*., 2008). A mixture of 1mL of Folin-Ciocalteu reagent (Merck, Darmstadt, Germany) and 1mL of the crude extract were combined and incubated at room temperature for 5 minutes. Adding 5mL of 5% w/v Na2CO3 to the mix was followed by incubation at room temperature. The absorbance at 760 nm was measured employing the spectrophotometer apparatus and the prepared reagent blank (Thermo Fisher Scientific, Madison, WI, USA). The calibration curve was established using concentrations of 1 to 7mg/L, and the TPC was measured in gallic acid equivalents (µg GAE/mL) as a standard.

Assessment of antioxidant activity

To determine the antioxidant activity of plant extracts, a modified 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging method was used (Blois 1958; Yassa *et al.,* 2005). The method involved adding 300µL of each extract to 900µL of DPPH methanolic solution, followed by incubating 0.3ml of 1 mm DPPH in methanol in 96-well Microplates. A microplate reader was used to measure the absorbance at 517 nm after 20 minutes of incubation at room temperature in the dark (Bio-Rad, model 680). The formula used to calculate the percentage of inhibition was as follows: % Inhibition = (blank absorption - sample absorption) / blank absorption X 100. Methanol was added to all samples to obtain a 1.5 mL solution. The experiment was performed in triplicates, and the IC50 value represents the amount of extract required to scavenge 50% of DPPH free radical, was calculated accurately.

HPLC analysis

Agilent 1260 Series HPLC with UV detector was used for HPLC analysis of plant extracts (Agilent Technologies, Waldbronn, Germany). Eight flavonoid and phenolic compounds, namely catechin, salicylic acid, caffeic acid, gallic acid, chlorogenic acid, quercetin, rosmarinic acid, and rutin were employed as standards for the analysis (Sigma-Aldrich, St. Louis, MO, USA). Before conducting HPLC analysis, the samples underwent filtration using a 0.45 µm nitrocellulose membrane. An Ultisil XB- C-18 column (5 µm, 250 × 4.6 mm) and a variable wavelength detector system of 254, 280,300, and 330 nm were employed to analyze the samples by HPLC. A gradient mixture (mobile phase) was prepared weekly by mixing methanol as an eluent A and phosphate buffer (pH = 2.3) as an eluent B. The samples were then analyzed employing a flow rate of 1 mL/min at 30 ◦C.

GC/MS analysis of essential oil

The Agilent 7890A/5975C GC/MS system with DB-5 fused silica column analyzed the EO composition (30 m × 0.25 mm, i.d., film thickness 0.25 µm- ISO15303). The analysis involved comparing the GC retention indices and mass spectra of the EOs with those previously documented in the literature by Adams (2007). The oven temperature program used for the experiment was as follows: The initial temperature of 60°C was quickly raised to 220°C at a rate of 3°C per minute. It was then further increased to 260°C at a rate of 20°C per minute and held at this temperature for 5 minutes. Both the

temperature of the injector and transfer line were maintained constant at 260°C and 280°C respectively. The carrier gas used was helium with a linear velocity of 30.6 cm/s and a split ratio of 1:100. The ionization energy used was 70 eV, the scan time was set to 1 second, and the mass range was from 40 to 300 a.m.u.

STATISTICAL ANALYSES

The data normalization process was evaluated using the Kolmogorov-Smirnov test. To statistically analyze the results, we used one-way ANOVA and considered differences between means to be significant. To compare the mean values, we applied the least significant difference test (LSD). The analyses were carried out in triple. The correlation analysis of antioxidant activity and TPC is represented in Pearson correlation coefficients. All analyses and diagram depictions were performed employing SPSS ver. 24.0 software (SPSS, Chicago, IL, USA).

RESULTS

Total phenolic content and antioxidant activity of the extract

The analysis of variance (ANOVA) revealed a significant difference between different types of extraction for both *Zeravschania khorasanica* TPC and its antioxidant activity as shown in table 1. The results of the experiments conducted to determine TPC and antioxidant activity are presented in table 2 and fig. 2. Specifically, the TPC of the Soxhlet extract was found to be significantly different ($P < 0.01$) according to the results. This method produced the highest amount of phenolic content (88.19±1.99 gallic acid mg/100g), followed by ethanolic ultrasound-assisted extraction (71.09±3.28), while the water sonication extract (30.92±3.43) had the lowest level of phenolic content. The water maceration extraction method also showed significant difference in terms of mean inhibition percentages ($P<0.01$). The water maceration (68.1±5.48) followed by water ultrasonic extracts (64.46±3.34) was found to be the most efficient method in terms of DPPH antioxidant activity (table 2, fig. 1). The ethanolic maceration method showed the lowest antioxidant activity percentage (48.45±3.64) (table 2). The Pearson correlation coefficient between TPC and antioxidant activity is shown in table 3. As shown in the table, increasing TPC is correlated with decreasing the antioxidant activity of the plant. On the other hand, these two mentioned variables are conversely related to each other.

HPLC component determination

The chemical compounds of the *Z. khorasanica* extract were determined using HPLC analysis. As per table 4, the extract contained three components - chlorogenic acid, caffeic acid, and salicylic acid. The phenolic components present in the extract are known for their bioactivity. The results indicated that the Soxhlet method was the most effective in extracting phenolic compounds. Overall, ethanolic extraction was found to be more efficient than water extraction.

A

B

Fig. 1: The distribution, and morphology of *Zeravschania khorasanica* (A) Locality of the samples collected for this study (B) The whole plant

Essential oil composition

The EO derived from the areal parts of *Z. khorasanica* consisted of eleven constituents (table 5). The main

compound was elemicin followed by trans-α-bergamotene, germacrene D and myristicin.

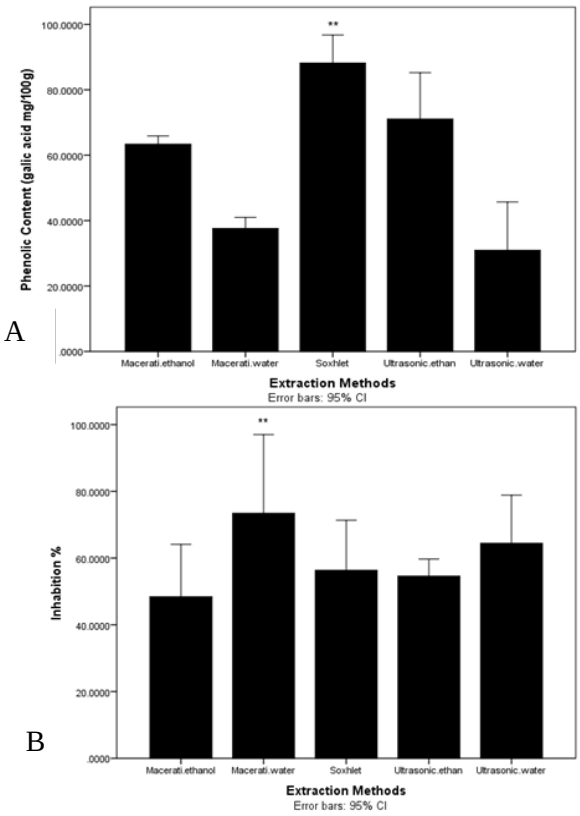

Fig. 2: TPC (A) and antioxidant activity (DPPH) (B) of *Zeravschania khorasanica* species obtained by different extraction techniques (**significantly different at P< 0.01)

DISCUSSION

In the present study, five different extraction techniques were compared in terms of TPC, DPPH, and effective compositions in an Iranian endemic species *Zeravschania khorasanica* (Apiaceae) for the first time. The results demonstrated the significant role of employing different extraction methods and applying varied solvents for TPC as well as its antioxidant activities. Since, the water solubility of polyphenols might be decreased due to their connections to the cell wall matrix, and considering the high solubility of the natural products in alcohols, some authors confirm the role of alcoholic substances, such as ethanol and methanol, as appropriate solvents for the extraction of phenolic constituents from the plants (Ignat *et al.,* 2011; Rasouli *et al.,* 2017). According to the present study, ethanolic solvent was much more influential in the extraction of total phenolics in contrast with water using different extraction methods.

Moreover, the antioxidant activity of medicinal plants with high amounts of phenols and flavonoids has been proved by several authors (Dehshiri *et al.,* 2013; Fallah *et al.,* 2011; Ksouri *et al.,* 2009; Lefahal *et al.,* 2018; Villalva *et al.,* 2021). According to previous studies ((Al-Hadhrami *et al.,* 2016; Chatatikun *et al.,* 2013; Ebrahimzadeh *et al.,* 2011; Lefahal *et al.,* 2018; Matejić *et al.,* 2012; Piluzza and Bulitta, 2011; Shah *et al.,* 2014)), there is a direct correlation between TPC and antioxidant activity in Apiaceae species. However, unlike the previous findings where the antioxidant activity was attributed to the presence of phenolic compounds (Piluzza and Bulitta, 2011), this study and some related ones (Terpinc *et al.,* 2012) found no positive association between TPC and antioxidant activity (table 3). It is intriguing that the extracts obtained through maceration and ultrasound-assisted extraction techniques using water solvent, which had lower phenolic content values (37.59±0.79%, 30.92±3.43%), exhibited the greatest antioxidant activity. This could be due to several reasons.

One possible explanation is that total phenolics might not be an appropriate predictor of the antioxidant capacity, which suggests that the Folin-Ciocalteu method This may not be sufficiently specific. It is possible that nonphenolic components, including citric acid, d-glucose, and ferrous sulfate, may have an impact on the association between TPC and the antioxidant capacity of different extracts (Magalhães *et al.,* 2006). Although polyphenols are often considered to be the primary plant components associated with antioxidant activity, they may not be the only ones (Moure *et al.,* 2001). Additionally, differences in phenolic compounds among plant extracts could explain variations in antioxidant activity. The genotype is important for the presence of phenolic compounds.

It can change the hydrophilic nature and the number of hydroxyl groups. This was observed by Balogh *et al.* (2010). Even if two different plants have the same TPC, their antioxidant capacity could differ. This is because of the synergistic and antagonistic interactions between antioxidants. Concerns were raised by Lefahal *et al.* (2018) regarding the significant role of phenolics and flavonoid compounds in the crude methanolic extract, and that the high phenolic and flavonoid content of the extract could account for its efficiency. According to Kozłowska *et al.* (2022) results, the phenolic contents of extracts are affected by both the solvent type and the part of the plant used.

Our results follow those previous works showing the type of solvent and extraction methods have a crucial role in the phenolic contents of the extract. They also mentioned chlorogenic acid as one of the important compounds, positively correlated with the antioxidant activity in the extracts (Kozłowska *et al.,* 2022). Indeed, one of the most abundant, and vastly efficient polyphenolics is chlorogenic acid with high antioxidant capacity found in everyday foods and beverages like green coffee extract and black tea (Lu *et al.,* 2020).

Table 1: Analysis of Variance (ANOVA) for total phenolic content and the antioxidant activity showing significant difference between different extraction technique

ANOVA											
			Sum of Squares		df	Mean Square		F-value		Sig.	
			TPC	AA		TPC	AA	TPC	AA	TPC	AA
Extraction Type	(Combined)		6783.337	1123.350	4	1695.834	280.838	102.636	6.884	.000	.006
	Linear Term	Contrast	295.714	52.398	1	295.714	52.398	17.897	1.284	.002	.284
		Deviation	6487.623	1070.952	3	2162.541	356.984	130.883	8.750	.000	.004
repetitions			165.227	407.962	10	16.523	40.796				
Total			6948.564	1531.312	14						

TPC: Total phenolic content; AA: Antioxidant activity

Table 2: Comparison of five different types of extraction and their effects on total phenolic contents and antioxidant activity percentages of *Zeravschania khorasanica*

Extraction Type/solvent	TPC (gallic acid mg/100g)	AA %
Maceration/ethanol	63.37±0.58	48.45±3.64
Maceration/water	37.59±0.79	68.1±5.48**
Soxhlet/ethanol	88.19±1.99**	58.7±3.48
Ultrasonic/ethanol	71.09±3.28	54.63±1.17
Ultrasonic/water	30.92±3.43	64.46±3.34

TPC: Total phenolic content; AA: Antioxidant activity. Each value is shown as a mean ± standard error (SE) (n=3) (**significantly different at $P< 0.01$)

Table 3: Pearson correlation coefficient between antioxidant activity and TPC showing negative linear correlation (-0.604) between two variables.

Correlations			
		AA	TPC
AA	Pearson Correlation	1	-.604*
	Sig. (2-tailed)		.017
	N	15	15
TPC	Pearson Correlation	-.604*	1
	Sig. (2-tailed)	.017	
	N	15	15
***.** Correlation is significant at the 0.05 level (2-tailed).			

AA: Antioxidant activity; TPC: Total phenolic content

Table 4: HPLC results of extraction of 1g of *Zeravschania khorasanica* leaves showing three effective compounds found in the extract using different extraction techniques

Extraction Type/Solvent	Quercetin (ppm)	Rutin (ppm)	Chlorogenic acid (ppm)	Caffeic acid (ppm)	Salicylic acid (ppm)	Catechin (ppm)	Gallic acid (ppm)	Rosmarinic acid (ppm)
Maceration-ethanol	-	-	32.4	0.9	10.2	-	-	-
Maceration-water	-	-	2.6	1.4	3.1	-	-	-
Soxhlet- ethanol	-	-	45.8	4.1	10.3	-	-	-
Ultrasonic-ethanol	-	-	35.7	1.0	10.3	-	-	-
Ultrasonic-water	-	-	2.1	1.5	2.5	-	-	-

Table 5: Essential oil composition of *Zeravschania khorasanica* leaves.

Constituent	RT (min)	RI	RL	Content in oil (%) (GC Peak Area)
α-pinene	8.52	940	932	0.78
myrcene	10.29	989	988	1.34
limonene	11.97	1036	1024	0.69
δ-terpinene	13.12	1064	1054	0.38
carvacrol methyl ether	20.46	1234	1241	0.61
linalyl acetate	21.22	1252	1254	0.48
trans-α-bergamotene	29.14	1439	1432	25.78
germacrene D	31.17	1488	1484	5.88
bicyclogermacrene	31.76	1502	1500	1.40
myristicin	32.74	1524	1517	4.47
elemicin	33.78	1550	1555	58.19

RT: Retention time; RI: Experimental retention index; RL: Literature retention index (According to Adams 2007).

It is known for several therapeutic roles such as antioxidant, anti-inflammatory, antipyretic, antibacterial, and so on. Chlorogenic acid is also important in glucose and lipid metabolism regulation and subsequently can be quite effective in treating many diseases (Lu *et al.,* 2020). The results of our investigation demonstrated the highest content of this component by using the Soxhlet method with ethanolic solvent (45.8 ppm) in the plant extract. Other techniques except for ultrasound-assisted extraction and maceration (by water solvent) were also quite influential in the extraction of this compound.

Caffeic acid is a polyphenol with potent antioxidant properties that may prevent cancer, diabetes, Alzheimer's, and viral/bacterial infections (Pavlikova, 2023).

Our findings indicate that the Soxhlet method using an ethanolic solvent yielded the highest amount of the compound in *Z. khorsanica* extract. Salicylic acid as a phenolic component is synthesized by plants. Additionally, aspirin and salicylic acid are nonsteroidal anti-inflammatory drugs that can induce apoptosis in human lung adenocarcinoma (Maddah *et al.,* 2021; Vejselova and Kutlu, 2015). *Z. khorasanica* extract contains salicylic acid, which was found to be present in higher amounts with both ethanolic Soxhlet and ultrasound-assisted extraction techniques. The HPLC analysis showed that ethanol was more effective in extracting the constituents required for the desired therapeutic effects.

Overall, the results suggest that ethanol can more efficiently extract the phenolic compounds and other beneficial components from the plant material possibly due to its ability to solubilize a wide range of compounds.

Thus, according to the above statements, the existence of such phenolic compounds indicates that Z. *khorasanica* extracts can be applied in the food and pharmaceutical industries.

In this context, we also worked on the EO profile of *Z. khorasanica* for the first time. Based on our results, elemicin (58.19%), trans-α-bergamotene (25.78%), germacrene D (5.88%), and myristicin (4.47%), constituted the major compounds (table 5). Two major and related compounds in the present study i.e., elemicin and myristicin, belonging to the alkenylbenzenes group, are regarded as secondary plant metabolites particularly found in the natural oils of Apiaceae and some related families (Götz *et al.,* 2022). The probable anticancer activity of these two constituents has been reported by several authors (Auerbach *et al.,* 2010, Hallstrom *et al.,* 1997, Ikeda *et al.,* 1998, NTP 2019). The EO of *Z. pastinacifolia* (Boiss. & Hohen.) Salimian & Akhani, contained 33 components. Elemicin was amongst one of the major compounds of the species (4.1%) (Yassa *et al.,* 2003). Moreover, in another species of the genus i.e., *Z. aucheri*, 32 compounds were detected among which Cis-Asarone and α-Terpinenyl acetate, constituted two major constituents (Zahedi and Aboli, 2014). It was found that the main compounds of *Z. membranacea* EO were δ-3-carene and linalyl acetate (Saei-Dehkordi *et al.,* 2014). However, Yassa *et al.* (2005) had previously discovered that the primary components of this species were Thymohydroquinone dimethyl ether and Trans-methyl iso Eugenol. They concluded that different habitats and climate changes could be important factors in the EO content.

CONCLUSION

The study findings indicate that the method of extraction significantly affects the content of total phenolics and antioxidant activities. The ethanolic Soxhlet extraction was found to be the most efficient method for extracting TPC and effective phenolic compounds. On the other hand, the water maceration method performed better in terms of antioxidant activity. Interestingly, there was no positive correlation between phenolic content and antioxidant activity, suggesting that other compounds might also be contributing significantly to the observed antioxidant activity, apart from phenolic compounds. Therefore, further investigation is needed to identify the underlying mechanisms responsible for the observed results.

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