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Chemical Composition, Some Allelopathic Aspects, Free-Radical-Scavenging Property and Antifungal Activity of the Volatile Oil of the Flowering Tops of *Leucanthemum vulgare* Lam.

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Abstract: Hydrodistillation of the ground flowering tops of *Leucanthemum vulgare* (Asteraceae), collected from Heyran (Ardabil Province, Iran), afforded a greenish yellow oil (yield 0.15%, v/w), which was analyzed by the GC-MS and the GC-FID. The volatile oil comprised 47 compounds representing 90.3% of the oil. Caryophyllene oxide (21.2%), aromadendrene oxide (13.7%), *cis*-β-farnesene (6.5%), 1-octen-3-yl-acetate (5.6%) and *trans*-caryophyllene (4.9%) were the major compounds. The volatile oil composition of *L. vulgare* collected from Iran (present study) was significantly different from that collected from elsewhere, indicating two possible chemotypes. The volatile oil showed free-radical-scavenging, antifungal and allelopathic effects.

Keywords: *Leucanthemum vulgare*; Asteraceae; volatile oil; caryophyllene oxide; DPPH; allelopathic effect.

1. Introduction

Leucanthemum vulgare Lam. (Asteraceae), commonly known as 'ox-eye daisy' is a perennial herb. This ornamental plant is indigenous to Europe, but also distributed widely in North America, Africa, East Asia and Australia [1]. In Iran, the plant distribution is limited to north-west provinces i.e. Ardabil and East Azerbaijan [2,3]. Extracts of this plant are valued and well known for their antispasmodic, diuretic and tonic properties [1]. Herbalists suggest that *L vulgare* tea can treat asthma, and bronchitis. It can also act as an antifungal, and an antibacterial agent, and its flowers possess active insecticide and flee-repellant potential [1-5]. There are a number of reports on the isolation of various secondary metabolites from this plant, e.g., alkaloids, flavonoids and other phenolics from the flowers [4, 6]. The composition of the volatile oil of *L vulgare* was previously studied on samples collected from Caucasia and Balkan regions, but none from Iran [4]. We now report, for the first time,

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on the composition of the volatile oil of *L. vulgare*, collected from Iran, and its allelopathic potential, antifungal and free-radical-scavenging properties.

2. Materials and Methods

2.1. Plant Material

The aerial parts of *Leucanthemum vulgare* Lam. were collected from Heyran (Ardabil, North-West Iran), at an altitude of 1600 m, in July 2012. The flowering tops were separated from other parts. All collected fresh samples were dried at room temperature. A voucher specimen (No. 1391-1) of this collection was deposited in the Department of Biology, Faculty of Science, University of Mohaghegh Ardabili, Ardabil, Iran.

2.2. Extraction

The dried and ground flowering tops (100 g) were Soxhlet-extracted sequentially with *n*-hexane, dichloromethane (DCM) and methanol (MeOH), 500 mL each. The extracts were dried using a rotary evaporator under vacuum keeping the temperature at 45^0C.

2.3. Hydrodistillation

The dried and ground flowering tops (100 g) were also subjected to hydrodistillation for 4h using a Clevenger-type apparatus. The resulting oil was subsequently dried over anhydrous sodium sulphate and dissolved in *n*-hexane (1 mL) for analysis.

2.4. GC-MS and GC-FID analyses

The volatile oil was analyzed using a ThermoQuest-Finnigan GC-MS fitted with a fused methyl silicon DB-5 column (60 m x 0.25 mm i.d., 0.25 μm film thickness). Helium was used as a carrier gas at a flow rate of 1.1 mL/min and a split ration of 1:100. The programmed temperature was increasing from 60 to 250°C at a rate of 5°C/min and finally held for 10 min. The injector temperature was 250°C. The mass spectral (MS) data were obtained at the following conditions: ionization potential 70 eV; ion source temperature 200°C; quadrupole temperature 100°C; solvent delay 3 min; scanning rate of 0.4sl mass range of 40-460 amu. EM voltage 3000 volts. Identification of compounds was based on direct comparison of the Kovats indices and MS data with those for standard compounds, and computer matching with the NIST NBS54K Library and Wiley Library [7,8]. Calculations were performed by using retention times of *n*-alkanes (C8-C20), which were injected after the oil at the same temperature and conditions.

For quantitation (area %), the GC analyses were also performed on an Agilent 6890 series apparatus fitted with a FID detector. The FID detector temperature was 300ºC. To obtain the same elution order as with GC-MS, simultaneous auto-injection was performed on a duplicate of the same column applying the same operational conditions. Relative percentage amounts of the separated compounds were calculated from FID chromatograms.

2.5. The DPPH assay

The free-radical-scavenging property of the volatile oil was evaluated using the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay as described in the literature [9-11]. DPPH was purchased from Fluka Chemie AG, Bucks. The essential oil was dissolved in $CHCl_3$ to obtain the stock concentration of 1 mg/mL. Dilutions were made to obtain concentrations of 5×10^{-1}, 2.5×10^{-1}, 1.25×10^{-1}, 6.25×10^{-2}, 3.13×10^{-2} and 1.56×10^{-2} mg/mL. Diluted solutions (5 mL each) were mixed with DPPH (5 mL; 0.08 mg/mL) and allowed to stand for 30 min for any reaction to occur. The UV absorbance was recorded at 517 nm. The experiment was performed in triplicate and the average absorption was noted for each concentration. The same procedure was followed for the positive controls, quercetin and vitamin C.

2.6. Phytotoxic and herbicidal assays

Phytotoxic and herbicidal activities of the *L. vulgare* volatile oil and extracts were evaluated on lettuce, *Lactuca satva* L. (*Romaine Siahoo* Cultivar) and pigweed, *Amaranthus retroflexus* L. The evaluation involved observation of responses of seed germination, root growth and shoot growth of tested plants to different concentrations of the *L. vulgare* volatile oil and extracts. The volatile oil, *n*-hexane and DCM extracts were dispersed as an emulsion in water using Tween20. Five concentrations, 0.001, 0.01, 0.1, 1, 10 mg/mL, were obtained by dilution of the emulsions with deionized water. The same concentrations were prepared for the methanol extract by dissolving the extract in water. A control test was conducted using distillated water alone. All seeds were surface sterilized with sodium hypochlorite (5%). Four replicates, each of 25 seed, were prepared for each treatment using sterile Petri dishes (90 mm) lined with one sterile filter paper (Whatman, number 2). Different concentrations of the oil and extracts (5 mL each) were added to each Petri dish. Prepared plates were then placed in a germination cabinet at 25°C in the dark. After one week, in the each treatment, germination percentage was determined and root and shoot length was measured. IC_{50} value, which is the concentration of volatile oil or extracts that inhibits 50% of measured parameters, was calculated [12].

2.7. Antifungal assay

The antifungal activity assay was performed on *Sclerotinia sclerotiorum* (Lib.) de Bary fungus that causes stem rot in many plants such as rapeseed, sunflower and lettuce, as the most prevalent plant pathogens. In this study, an isolate of *Sclerotinia sclerotiorum* from rapeseed was used. The stock solution of the volatile oil and extracts were prepared. Four concentrations, 1, 0.1, 0.01 and 0.001 mg/mL, were obtained by dilution with deionized water. Potato dextrose agar was used as the growth medium in this assay. The medium was poured into Petri dishes and then inoculated with 4 mm plugs from 7 days old cultures. The control experiments had distilled water in place of volatile oil and extracts. The cultures were incubated at 25ºC for 7 days. The diameter of the radial growth of the fungi was measured at the end of incubation period [12].

2.8. Statistical analysis

In all assays, SPSS 16 software was used for statistical analysis. Analysis of variance (ANOVA) followed by a Duncan test was used to identify the differences among various groups. The significance level was set at $p \leq 50$.

3. Results and Discussion

Hydrodistillation of the flowering tops of *L. vulgare* afforded greenish yellow oil with a yield of 0.15% (v/w). After GC-MS and GC-FID analyses, 48 compounds were identified and quantified from the oil representing 90.3% of the oil. The identified components and their percentages are listed in Table 1. The oil was dominated by caryophyllene oxide (21.2%), aromadendrene oxide (13.7%), *Z*-β-farnesene (6.5%), 1-octen-3-ylacetate (5.6%) and *trans*-caryophyllene (4.9%) as major compounds. The chemical contents of *L. vulgare* volatile oil could be classified in eight groups (Table 2). The most of these components belong to sesquiterpenes with 64.6% of the oil.

Leucanthemum vulgare extracts and volatile oil showed high level of fungitoxic activity against *Sclerotinia sclerotiorum*, a common plant pathogen, and considerably reduced mycelia growth of the fungus. Among the extracts *n*-hexane extract displayed potent fungitoxic effects (IC_{50}= 290 µg/mL), but the methanol extract was also active (IC_{50}= 480 µg/mL). However the volatile oil was found to have the most potent fungitoxic property IC_{50}= 1.60 mg/mL) (Figure 1). As the DCM extract did not show any activity, it has not been included in Figure 1.

Table 1. GC-MS and GC-FID data of the components of the volatile oils of the flowering tops of *L. vulgare* from Iran.

Compounds	Retention time (RT) in min	Kovats indices (KI)	Real % area	Compounds	Retention time (RT) in min	Kovats indices (KI)	Real % area
Pyridine	5.81	-	0.2	*Z*-β-Farnesene	23.50	1458	6.5
5-Hexenal	6.25	-	0.1	α-Humulene	23.85	1472	0.4
Z-4-Heptanal	8.24	888.4	0.1	*Trans*-β-Ionone	24.39	1492	0.1
α-Pinene	9.43	938.4	0.1	Germacrene D	24.51	1498	0.4
1-Octen-3-ol	10.32	974.0	0.5	δ-Amorphene	25.61	1536	0.2
β-Pinene	10.55	983.2	0.1	*Trans*-Nerolidol	26.17	1567	0.6
2-Pentylfuran	10.71	989.6	0.3	δ-Gurjunene peroxide	26.28	1572	2.3
p-Cymene	11.72	1027	0.1	Spathulenol	26.91	1598	3.0
Limonene	11.86	1032	0.3	Caryophyllene oxide	27.10	1604	21.2
1,8-Cineol	11.96	1036	0.2	Salvia-4-(14)-en-1-one	27.3	1615	0.6
Cis-Sabinene hydrate	12.93	1071	0.1	Humulene epoxide II	27.73	1633	3.7
Terpinolene	13.53	1092	0.2	δ-Cadinol	28.31	1659	2.0
Linalool	13.70	1099	0.3	β-Eudesmol	28.56	1670	3.1
1-Octen-3-acetate	13.91	1106	5.6	14-Hydroxy-9-*epi*-*(E)*-caryophyllene	28.87	1679	1.5
Menthone	15.42	1159	1.5	Aromadendrene oxide	29.03	1690	13.7
Menthol	15.94	1177	3.6	Benzyl benzoate	30.96	1779	0.6
4-Terpinol	16.11	1183	0.5	Cyclohexanone	33.00	1876	4.0
α-Terpineol	16.45	1195	0.3	Methyl palmitate	34.03	1926	0.2
Safranol	16.75	1206	0.2	Eicosa-5,11,14-tetrayonic acid	34.22	1965	2.9
Cis-3-Hexenyl valerate	17.50	1233	0.1	Palmitic acid	34.75	1961	0.7
Thymol	19.13	1292	0.1	Di-butylphthalate	34.90	1969	0.4
Methyl acetate	13.25	1296	0.1	Ambrettolide	39.07	2088	1.0
Decanoic acid	21.00	1362	0.9	Tricosane	41.93	2300	0.8
Trans-Caryophyllene	23.00	1438	4.9	Total			90.3

The volatile oil of the flowering tops of *L. vulgare* exhibited considerable phytotoxic and herbicidal effects (Table 3). The oil inhibited seed germination, root and shoot growth of the tested herbs in a dose-dependent manner. It showed inhibitory effects on seed germination of lettuce and pigweed with IC_{50} values of 2.73 and 3.90 mg/mL, respectively. At the concentration of 10 mg/mL, the oil entirely stunted the seed germination, root and shoot growth of tested plants. All extracts of *L. vulgare* also exhibited considerable phytotoxic and herbicidal effects, and reduced seed germination shoot and root growth of lettuce and pigweed at all test concentrations (Tables 4 and 5). The *n*-hexane extract displayed stronger effects than that of the methanol extract with IC_{50} values of 2.62, 1.19 and 2.45 mg/mL for seed germination, root and shoot elongation of lettuce, respectively. Growth of pigweed was more influenced by the DCM extract than by the others. Methanol extract had weak effect on seed germination of pigweed, as well as on shoot germination.

The DPPH assay indicated that the volatile oil of *L.vulgare* flowering tops had some free-radical-scavenging activity (RC_{50} value of 0.4 mg/mL).

Various reports on the volatile oil compositions of *L. vulgare* flowering tops collected from different parts of the world are available to date. The main components of the volatile oil of *L. vulgare* growing in Georgia was reported as farnesene (38.3%) and α-bisabalol (15.5%) [4]. The specimen from North Estonia was dominated by (*E*)-β-farnesene (7.3%) and caryophyllene oxide (5%) as major compounds [13]. In the present study, the profile of *L. vulgare* oil collected from Iran was significantly different from that of Georgia. It could be assumed that the sample from Iran might be a different chemo-type.

Table 2. Chemical classes of the components of the volatile oil of the flowering tops of *L. vulgare* from Iran

Chemical classes	Percentage
Sesquiterpenes	64.6
Monoterpenes	7.5
Esters	7.0
Hydrocarbons	4.8
Fatty acids	4.2
Aromatics	1.4
Alcohols	0.5
Aldehydes	0.3

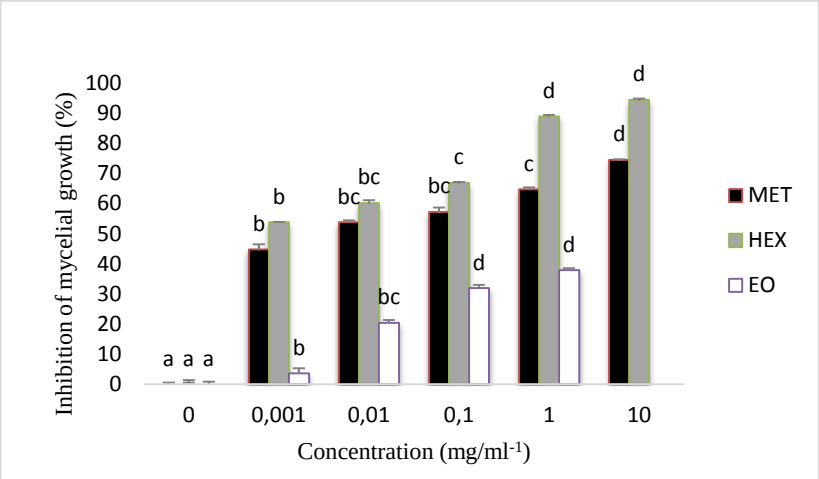

Figure 1. Effects of different doses of the volatile oil, *n*-hexane (Hex), and methanol (Met) extracts of the flowering tops of *L.vulgare* on mycelial growth of *Sclerotinia sclerotiorum.*

It is well known that *L. vulgare* is an invasive species, widely distributed in different rangelands and farms. The mechanism by which this invasive species affects native communities is not well resolved [14]. However, it has been suggested that *L. vulgare* may control plant community composition through negative feedback with the soil. The invasive effects of the plant are limited to closely related species, for example, to species within the Asteraceae, such as *Taraxacum* and *Erigeron* [15]. *L. vulgare* is a prolific seed producer, and it has been reported to produce about 26000 seeds and seeds can survive in the soil up to 39 years. These characteristics may enable it to form dense colonies and make an invasive species [3]. The ability of this plant to produce allelochemicals may be another reason for the plant to invade surrounding plants and form dense colonies.

Present results demonstrated that caryophyllene oxide (CPO) was the main constituent of the volatile oil of *L. vulgare* collected from Iran. Caryophyllene oxide exhibits wide variety of pharmacological effects. For example, it inhibits the mitochondrial electron transport chain. Complex I is the major target for inhibition by caryophyllene oxide in mitochondria in the absence of transition metal. It was shown that caryophyllene oxide modulates certain Ca^{2+} permeable transient receptor potential (TRP) channel and inhibits sarcoplasmic reticulum and Ca^{2+} ATPase and activates ryanodine receptors in skeletal muscle, Thus it might influence intracellular calcium homeostasis [16] and be responsible for some medicinal activity of the oil, e.g., in the treatment of asthma, cough and nervous excitability [1]. There are a number of reports on antimicrobial effects of this compound. For CPO, antifungal, antibacterial and butylcholinesterase-inhibiting effects have recently been described. Extracts of *L. vulgare* are also valued for their antifungal and antibacterial effect. So it is reasonable to suggest that the effects of this plant as outlined above might be due to the presence of CPO [17].

Table 3. Phytotoxic and herbicidal activity of the volatile oil of the flowering tops of *L.vulgare*.

Concentration (mg/ml)	Germination (%)		Root length (mm)		Shoot length (mm)	
	Lettuce	Pigweed	Lettuce	Pigweed	Lettuce	Pigweed
0	**100^{a} ±0**	**100^{a} ±0**	**33.35^{a} ±2.31**	**38.76^{a} ±0.27**	**43.75^{a} ±2.07**	**35.86^{a} ±0.5**
0.001	**80^{b} ±2.39**	**85^{b} ±1**	**27.10^{b} ±2.16**	**36.02^{b} ±0.69**	**38.09^{ab}** ±0	**30.42^{b} ±1.05**
0.01	**71^{c} ±1.91**	**82^{bc} ±1.15**	**27.10^{b} ±2.57**	**35.11^{b} ±0.55**	**36.95^{ab} ±2.40**	**30.33^{b} ±1.21**
0.1	**65^{d} ±1**	**79^{c} ±1.91**	**21.62^{bc} ±1**	**34.79^{b} ±1.03**	**30.80^{bc} ±2.17**	**28.62^{b} ±2.31**
1	**52^{e} ±1.63**	**73^{d} ±3**	**18.92^{c} ±1.30**	**34.65^{c} ±0.84**	**28.72^{c} ±3.33**	**22.21^{c} ± 0.69**
10	**0^{f}**	**0^{e}**	**0^{d}**	**0^{d}**	**0^{d}**	**0^{e}**
IC_{50}	**2.73**	**3.90**	**3.13**	**4.18**	**3.66**	**3.72**

Mean values in the same column followed by the same letter are not significantly different at the 0.05 level according to the Duncan test

Table 4. Herbicidal activity of the *n*-hexane (Hex), dichloromethane (DCM) and methanol (Met) extracts of the flowering tops of *L.vulgare* in lettuce.

Concentration (mg/ml)	Germination (%)			Root length (mm)			Shoot length (mm)		
	HEX	DCM	MET	HEX	DCM	MET	HEX	DCM	MET
0	**100^{a} ±1**	**100^{a} ±1**	**100^{a} ±1**	**23.75^{a} ±0.85**	**33.75^{a} ±0.85**	**33.75^{a} ±0.85**	**40.72^{a} ±0.65**	**40.72^{a} ±0.65**	**40.72^{a} ±0.65**
0.001	**96^{a} ±1.63**	**84^{b} ±1.63**	**98^{b} ±0**	**31.16^{b} ±0.98**	**28.73^{b} ±0.98**	**32.25^{a} ±1.75**	**38.83^{ab} ±4.00**	**28.26^{ab} ±0.31**	**32.75^{ab} ±1.10**
0.01	**93^{a} ±1.91**	**82^{b} ±1.15**	**97^{b} ±1.15**	**29.73^{b} ±0.84**	**27.34^{b} ±0.75**	**32.16^{a} ±1.43**	**37.01^{bc} ±3.57**	**27.7^{ab} ±0.46**	**32.25^{ab} ±0.47**
0.1	**92^{a} ±1.63**	**78^{c} ±1.15**	**96^{b} ±0**	**28.9^{b} ±1.06**	**26.4^{b} ±0.18**	**31.25^{b} ±0.25**	**35.41^{c} ±1.72**	**26.85^{b} ±0.36**	**30.25^{bc} ±0.47**
1	**82^{b} ±2.58**	**76^{c} ±0**	**94^{c} ±1**	**21.73^{c} ±1.54**	**21.85^{c} ±0.84**	**29.75^{b} ±1.31**	**25.63^{d} ±4.15**	**24.86^{c} ±0.79**	**28.5^{bc} ±0.25**
10	**0^{c}**	**0^{d}**	**86^{d} ±4.16**	**0^{d}**	**0^{d}**	**10.5^{c} ±1.04**	**0^{e}**	**0^{d}**	**21^{c} ±3.76**
IC_{50}	4.51	3.92	4.63	4.08	3.67	7.02	4.24	2.64	10.44

Mean values in the same column followed by the same letter are not significantly different at the 0.05 level according to the Duncan test

The current study showed that the volatile of *L. vulgare* flowering tops had some free-radical-scavenging activity. It has been reported that 1,8-cineol, α–humulene, germacrene D and a few other components found in the volatile oil of this plant possess antioxidant activity [18]. Thus it is assumed that antioxidant potential of the *L. vulgare* oil may be related to the presences of those compounds. Many plant-derived essential/volatile oils and their constituents have been reported to possess potent antifungal and antioxidant activities, and *L. vulgare* is of no exception.

The flowering tops of *L.vulgare* exhibited considerable phytotoxic and herbicidal effects that might be primarily due to the presence of caryophyllene oxide and aromadendranes in the plant inflorescenses. Thus, this plant might have some allelopathic potential. This characteristic may influence the density and the composition of individual plant communities. Elucidating allelopathic interactions has been of interest for practical reasons, e.g., understanding this phenomenon could lead to the development of new herbicides. In spite of the successful weed control achieved with synthetic herbicides, certain weed species ultimately have developed resistance to a specific herbicide rapidly

(2-3 year) and has led to cross resistance to entire chemical classes. The use of synthetic herbicides is also claimed to negatively affect the environment, underlining the constant need for natural herbicides [19]. Therefore, the herbicide effect of the flowering tops of *L. vulgare* might have some potential commercial implications.

Table 5. Phytotoxic activity of the *n*-hexane (Hex), dichloromethane (DCM) and methanol (Met) extracts of the flowering tops of *L.vulgare* in pigweed.

Conc. (mg/mL)	Germination (%)			Root length (mm)			Shoot length (mm)		
	HEX	DCM	MET	HEX	DCM	MET	HEX	DCM	MET
0	**100^{a} ±0**	**100^{a} ±0**	**100^{a} ±0**	**54.25^{a} ±1.89**	**54.22^{a} ±1.82**	**54.25^{a} ±1.89**	**34.71^{a} ±0.64**	**34.71^{a} ±0.64**	**34.71^{a} ±0.64**
0.001	**87^{b} ±1.91**	**86^{b} ±1.81**	**86^{a} ±3.83**	**39.2^{a} ±2.51**	**40.25^{b} ±1.11**	**48.25^{b} ±0.94**	**30.73^{ab} ±0.80**	**31.33^{ab} ±0.90**	**32.75^{b} ±1.03**
0.01	**86^{b} ±1.15**	**89^{b} ±1.12**	**90^{a} ±3.46**	**32.23^{b} ±2.74**	**42.25^{b} ±1.03**	**47.25^{b} ±1.03**	**29.91^{b} ±3.18**	**30.91^{b} ±3.18**	**32.75^{b} ±0.75**
0.1	**81^{b} ±0.95**	**92^{b} ±0.80**	**94^{b} ±3.46**	**31.67^{b} ±2.17**	**43.00^{b} ±1.33**	**47.25^{b} ±2.83**	**28.06^{b} ±2.41**	**29.06^{b} ±2.41**	**32.25^{b} ±0.47**
1	**76^{c} ±0**	**88^{c} ±1.1**	**90^{c} ±2.56**	**31.63^{c} ±2.39**	**34.25^{c} ±1.13**	**39.25^{c} ±1.03**	**25.61^{bc} ±2.12**	**26.61^{bc} ±2.12**	**29.5^{b} ± 0.95**
10	**15^{d} ±3.73**	**85^{d} ±1.23**	**86^{d} ±2.58**	**24.26^{d} ±1.51**	**15^{d} ±0.80**	**15^{d} ±0.91**	**20.23^{c} ±0.73**	**22.23^{c} ±0.73**	**29.25^{c} ±1.79**
IC_{50}	2.62	3.43	4.94	1.19	5.02	6.07	2.45	3.20	7.06

Mean values in the same column followed by the same letter are not significantly different at the 0.05 level according to the Duncan test

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