SUPPLEMENTARY MATERIAL

Chemical composition, N-Nitrosamine inhibition, and antioxidant and antimicrobial Properties of essential Oil from *Coreopsis tinctoria* flowering tops

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Abstract *Coreopsis tinctoria* flowering tops (CTF) from the Kunlun Mountains in Xinjing (north-western China) have been used for tea production for about a century. This study was to assess antioxidant, nitrite-scavenging, and N-nitrosamine inhibitory and antimicrobial activities of the essential oil extracted from CTF. The essential oil was extracted through hydrodistillation, and its chemical compositions was analysed by GC–MS. Seventy compounds of the oil were identified, representing 81.87% of total oil. The antioxidant capacities of the oil with IC₅₀ values for scavenging DPPH and ABTS were 287.66 ± 12.60 and 1.251 ± 0.127 µg mL⁻¹, respectively. The nitrite-scavenging and N-nitrosamine inhibitory activities (IC₅₀) were 0.3912 ± 0.0127 and 0.6564 ± 0.036 µg mL⁻¹, respectively. The oil has a certain antimicrobial capacity, but its capacity was weaker than that of the penicillinG (24 µg mL⁻¹). The oil showed antioxidant and antimicrobial capacities and had a stronger nitrite-scavenging and N-nitrosamine inhibitory properties.

Keywords: Antimicrobial activity; Antioxidant activity; Chemical compositions; *Coreopsis tinctoria* Nutt.; Essential oil; N-nitrosamine inhibition

Experimental

Plant and Materials

C. tinctoria Nutt. (Compositae) was collected in October 2013 in Hetian County (Xijiang Uygur Autonomous Region, China) and was identified by Assistant Professor Peng Li (College of Pharmacy, Shihezi University). A voucher specimen (No. CTF-006) was kept in the herbarium of the Ministry of Education Key Laboratory of Xinjiang Endemic Phytomedicine Resources, Shihezi University.

Chemicals and reagents

2,2'-Azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and α, α -diphenyl- β -picrylhydrazyl (DPPH) were purchased from Fluka (Menlo Park, CA, USA) and Sigma-Aldrich Co. (St. Louis, USA), respectively. Ascorbic acid, butylated hydroxyanisole (BHA) and other chemicals were of analytical grade. All other reagents were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

Bacterial Strains Used

Four microorganisms were obtained from China Center of Industrial Culture Collection (CICC), China. The *Streptococcus uberis* [ATCC 9927] was purchased from Microbiologics Co. (USA). The essential oil was individually tested against *Shigella* CICC 10865 [ATCC12022], *S. uberis* [ATCC 9927], *Salmonella enteritidis* CICC 21482 [ATCC14028], *Escherichia coli* CICC 10300 [ATCC35150] and *E.coli* CICC 23657[ATCC25922]. The bacterial strain cultures were maintained at 4 °C.

Extraction of Essential Oil

Approximately dried CTF powder (800 g, <1.0 mm) was extracted by hydrodistillation in a Clevenger-type apparatus for 4 h. The oils were dried over anhydrous sodium sulfate then filtered and preserved in a sealed vial stored in dark conditions at 4 °C until further analysis.

GC–MS Analysis for Essential Oil

The chemical composition of the essential oil was analysed using GC–MS. The essential oil (1.0 μ L) was injected into a GC–MS (Agilent 7890A/5975C, Little Falls, CA, USA) and separated on a HP-5MS (85% phenyl methyl siloxane, J&W Scientific, USA), 0.25 mm i.d. $\times$

60 m, 0.25 micron film thickness, fused silica capillary column, and using split mode (split ratio, 1:50). Helium was used as the carrier gas at a flow rate of 1.00 mL min⁻¹. A linear gradient temperature process was programmed as follows: the GC column oven temperature was increased from 50 °C to 100 °C at a rate of 5 °C min⁻¹ with a final hold time of 6 min. The temperature was increased from 100 °C to 230 °C at the same rate with a final hold time of 20 min. Finally, the column temperature was increased from 230 °C to 250 °C at a rate of 10 °C min⁻¹ with a final hold time of 20 min. The injector and detector temperatures were set at 250 and 280 °C, respectively. The mass spectrometer operating conditions were as follows: ionization voltage of 70 eV, ion source of 230 °C, and mass range of 45 amu to 600 amu. The identification and quantification of the compounds were based on mass spectra with those stored in authentic compounds by means of GC–MS system (Agilent, J&W Scientific, USA). The composition was reported as a relative percentage of the total peak area.

DPPH, ABTS Radical Scavenging Assay

The DPPH, ABTS radical-scavenging effect of the essential oils with different concentrations (5 µg mL⁻¹ to 100 µg mL⁻¹ in ethanol) was determined using the previously described method (Ozgen et al, 2006). Ascorbic acid and BHA, at the same concentrations as the samples, were both set as the positive controls. The decrease in absorbance upon the addition of the test samples was used to calculate the antiradical activity. This activity was expressed as radical- scavenging assay (RSA), which was calculated as

$$\text{RSA (\%)} = [1 - (\text{sample ODs} - \text{sample OD0}) / \text{Control OD}] \times 100$$

where control OD, sample ODs, and sample OD0 are the absorbances of the control (containing all reagents without test sample), the test sample, and blank samples (containing test sample without DPPH solution) after 30 min, respectively. Basing on the plot of concentration (log C) vs. RSA (%), we conducted a linear regression analysis to determine the IC₅₀ (50% concentration of inhibition for RSA) value of each test sample.

****O₂⁻ scavenging assay***

The *O₂⁻ scavenging ability was assayed according to the method previously described method (Yao, Zhu, Chen, Tian, & Wang, 2013). Briefly, 0.5 mL of the essential oil (40 µg mL⁻¹ in 4%Tween-20 aqueous solution) was added to 4.5 mL of 50 mmol L⁻¹ Tris–HCl buffer

(pH 8.2). The mixture was incubated at 25 °C for 20 min, and then 10 µL of 45 mmol L⁻¹ pyrogallol (25 °C) was added. The absorbance of the reaction mixture was measured at 325 nm every 30 s until the reaction time reached 6 min. The same concentration of essential oil without pyrogallol was used as the blank. The autoxidation rate constant (K_b) of pyrogallol acid was calculated from the curve of $\lambda = 325$ nm vs. time. The negative control did not contain any test sample. A low K_b value suggests a high anti-oxidation activity. Ascorbic acid and BHA at the same concentrations as the samples were set as the positive controls.

Nitrite-Scavenging Activity

The nitrite-scavenging activity of the essential oil was determined using the previously described method (Choi et al, 2007) with a minor modification. Briefly, essential oil solutions (10 µg mL⁻¹ in 2% Tween-20 aqueous solution) measuring 0.1, 0.2, 0.3, 0.4, and 0.5 mL were placed in a 10.0 mL volumetric flask. Approximately 2.0 mL of citric acid–disodium hydrogen phosphate aqueous buffer (pH = 3.0) was added. After vigorous shaking, 1.0 mL of NaNO₂ (10 µg mL⁻¹) was added. The solution was placed in a 37 °C water bath for 1 h. Subsequently, 2.0 mL of p-aminobenzene sulfonic acid (0.4%, w/v) was added. The solution was blended for 5 min. Approximately 1.0 mL of naphthalene ethylenediamine hydrochloric acid solution (0.2%, w/v) was added, followed by the addition of distilled water to reach 10.0 mL. The solution was mixed and incubated for 15 min at room temperature. The solutions were scanned by UV-2401PC spectrophotometer (Shimadzu, Japan) at $\lambda = 538$ nm against the control. The control contained all the reagents except for the test sample. Ascorbic acid and BHA at the same concentrations as the samples were both set as positive controls. The assay was performed in triplicate. The decrease in absorbance upon the addition of the test samples was used to calculate the nitrite-scavenging activity. This activity was expressed as

$$\text{Nitrite scavenging activity (\%)} = [1 - (\text{sample ODs} - \text{sample OD}_0) / \text{Control OD}] \times 100$$

where control OD, sample ODs, and sample OD₀ are the absorbances of the control (containing all reagents without test sample), the test sample and the sample blank (containing test sample without NaNO₂ solution), respectively.

Inhibition of N-nitrosamine Formation Assay

The inhibition of N-nitrosamine formation of the essential oil was determined using a previously described method (Choi, Chung, & Sung, 2002) with a minor modification. Briefly, aliquots of the essential oil dissolved separately in 2% (v/v) Tween-20 aqueous solution were placed in a 10.0 mL volumetric flask. Subsequently, 3.0 mL of citric acid–disodium hydrogen phosphate aqueous buffer (pH = 3.0) was added. After vigorous shaking, 2.0 mL of NaNO₂ (5 µg mL⁻¹) and 1.0 mL of dimethylamine solution (1.0 mmol L⁻¹) were added. After a 37 °C water bath for 1 h, 1.0 mL of *p*-aminobenzene sulfonic acid (0.4%, w/v) was added. The solution was blended for 5 min. About 1.0 mL of naphthalene ethylenediamine hydrochloric acid solution (0.2%, w/v) was added, followed by the addition of distilled water to reach 10.0 mL. The solution was mixed and incubated for 15 min at room temperature. The solutions were scanned at $\lambda = 525$ nm against the control. The control contained all the reagents except for the test sample. The assay was performed in triplicate. This activity was expressed as

$$\text{Inhibition nitrosamine formation rate (\%)} = (1 - \text{sample ODs} / \text{Control OD}) \times 100$$

The IC₅₀ (50% concentration of inhibition nitrosamine formation rate) value of each test sample was calculated.

Antimicrobial Capacity of Essential Oil

The antibacterial activity of essential oil was tested on five bacteria, namely *Shigella* [ATCC12022], *S. uberis* [ATCC 9927], *S. enteritidis* [ATCC14028], *E. coli* [ATCC35150], and *E. coli* [ATCC25922]. The minimum inhibitory concentrations (MIC) and minimal bactericidal concentration (MBC) values of the essential oil were determined using microdilution method, and antibacterial activity was determined using disk diffusion method (Derwich, Manar, Benziane, & Boukir, 2010). The bacteria strains were first grown on Muller Hinton medium at 37 °C for 24 h prior to seeding on the nutrient agar.

Figures

Figure S1. α,α -Diphenyl- β -picrylhydrazyl (DPPH), 2,2'-azinobis(3-ethylbenzthiazoline-6- sulfonic acid (ABTS), and superoxide anion ($\text{O}_2^{\cdot -}$) radical-scavenging activity of essential oil extracted from *Coreopsis tinctoria* flowering tops. Data are presented as mean $\pm$ SD (n = 3). DPPH assay (A); ABTS assay (B); superoxide anion radical-scavenging assay (C).

Figure S2. Nitrite-scavenging and inhibition of N-nitrosamine formation activities of essential oil extracted from *Coreopsis tinctoria* flowering tops. Data are presented as mean $\pm$ SD (n = 3). Nitrite scavenging assay (A); N-nitrosamine formation inhibitory activity assay (B).

Tables

Table S1. Chemical composition of the essential oil extracted through hydrodistillation from *Coreopsis tinctoria* flowering tops.

No.	Retention time	Compounds	Peak area (%)
1	19.666	(1R)-(+) α -Pinene	3.35
2	20.361	Camphene	0.20
3	20.612	2,4(10)-Thujadiene	0.28
4	21.625	β -Phellandrene	0.26
5	21.901	β -Terpinene	0.15
6	22.454	β -Myrcene	0.18
7	23.551	α -Phellandrene	0.15
8	26.532	Limonene	33.13
9	27.059	Ocimene	0.12
10	27.729	3-Carene	0.18
11	28.499	Bicyclo[3.2.0]hept-6-ene	0.16
12	29.470	2,4-Dimethyl styrene	0.52

13	29.621	3-tert-Butylphenol	0.17
14	30.014	1-Nonanal	0.32
15	31.404	trans-p-Mentha-2,8-dienol	0.70
16	31.664	3-Cyclopentene-1-acetaldehyde	0.67
17	31.856	2-Methoxy-4-methylphenol	0.35
18	32.208	cis-p-Menth-2,8-diene-1-ol	0.92
19	32.568	trans-Pinocarveol	0.36
20	32.853	1(7),8-p-Menthadien-2-ol	0.52
21	32.970	1,3-Cycloheptadiene	0.50
22	33.439	trans-Sabinol	0.15
23	33.589	trans-3-Pinanone	0.14
24	34.008	1-Methylcyclohexa-1,3-diene	1.35
25	34.410	7-methyl-3-methylene-6-Octen-1-ol	0.82
26	34.778	2-Acetyltoluene	0.41
27	34.954	2-(4-Methylphenyl)propan-2-ol	0.61
28	35.105	2-Methylene-5-(1-methylethenyl)-cyclohexanol	0.27
29	35.230	2,5,6-Trimethyl-1,3,6-heptatriene	0.20
30	35.398	trans-Dihydrocarvone	0.94
31	35.708	2-Cyclopentylcyclopentanone	1.52
32	36.336	(1R,4S)-2,3-Dimethylbicyclo[2.2.1]hept-2-ene	0.95
33	36.863	cis-Carveol	3.31
34	37.457	trans-Carveol	0.97
35	38.069	L-Carvone	3.93
36	39.425	Perillyl aldehyde	0.45
37	39.835	Bornyl acetate	0.58
38	40.564	3-Methyl-4-isopropylphenol	0.41
39	43.544	α -Gurjunene	0.95
40	43.971	Eugenol	0.19
41	44.306	1,4-Dimethyl-2-cyclopentylbenzene	0.20
42	45.470	α -Cubebene	0.11
43	47.479	α -Selinene	0.11
44	47.923	1-Caryophyllene	1.62
45	48.400	2,6-dimethyl-Bicyclo[3.1.1]hept-2-ene	1.6
46	48.744	Geranylacetone	0.18
47	49.346	α -Caryophyllene	0.12
48	50.150	α -Curcumene	0.89
49	51.657	Camphene	0.51
50	52.009	α -Cedrene oxide	0.12
51	52.168	(Z)- α -trans-Bergamotol	0.17
52	52.737	Dodecanoic acid	2.26

53	53.189	3-isopropyltoluene	0.15
54	53.415	Espatulenol	0.5
55	53.566	Caryophyllene oxide	1.11
56	53.817	Cyclopent-2-ene-1-carboxylic acid	1.08
57	54.152	β -Ionone	1.17
58	54.537	Tricyclo[6.3.0.0(1,5)]undec-2-ene	1.87
59	54.856	γ -Cadinene	0.25
60	55.040	trans-Longipinocarveol	0.60
61	55.165	1,1,7-Trimethyl-4-methylenedecahydro-	0.37
62	55.458	n-Heptadecane	0.78
63	55.726	1H-Phenylene	0.29
64	55.927	7R,8R-8-Hydroxy-4-isopropylidene	1.37
65	57.510	Tetrahydroneophytadiene	0.31
66	58.246	Hexahydrofarnesylacetone	0.44
67	60.599	n-Hexadecanoic acid	1.35
68	62.500	(+)-Manoyl oxide	0.19
69	65.413	1,13-Tetradecadiene	0.34
70	68.277	Nonacosane	0.31

Table S2. Antioxidant activity of essential oil extracted from *Coreopsis tinctoria* flowering tops

Sample	antioxidant activities	
	DPPH (IC ₅₀)	ABTS (IC ₅₀)
essential oil	287.66 $\pm$ 12.60 ^a	1.251 $\pm$ 0.127 ^c
ascorbic acid(Vitamin C) [†]	86.23 $\pm$ 5.134 ^b	12.36 $\pm$ 0.615 ^a
BHA [†]	92.32 $\pm$ 4.763 ^b	8.62 $\pm$ 0.237 ^b

IC₅₀ values express the concentration in $\mu\text{g mL}^{-1}$ for each sample. Values are mean $\pm$ S.D. of triplicates.

Values that do not sharing the same letter are significantly different (LSD) at the $P < 0.05$ level in each column.

[†]Reference compounds.

BHA: Butylated hydroxyanisole.

Table S3. Inhibition of pyrogalllic acid auto-oxidation by essential oil extracted from *Coreopsis tinctoria* flowering tops

Sample	control	essential oil	ascorbic acid [†]	BHA [†]
Kb value	0.1778±0.018 ^a	0.1547±0.021 ^b	0.0851±0.011 ^c	0.0572±0.007 ^d

Values that do not sharing the same letter are significantly different (LSD) at the $P < 0.05$ level in each row. BHA, butylated hydroxyanisole.

[†]Reference compounds.

Table S4. Nitrite-scavenging and inhibition of N-nitrosamine formation activities of essential oil extracted from *Coreopsis tinctoria* flowering tops.

Sample	essential oil	ascorbic acid [†]	BHA [†]
Nitrite scavenging activity	0.3912±0.0127 ^a	1.0345±0.0648	1.2783±0.0592 ^c
IC ₅₀ (µg mL ⁻¹)		^b	
Inhibition N-nitrosamine formation IC ₅₀ (µg mL ⁻¹)	0.6564±0.036 ^a	15.76±0.061 ^b	14.39±0.029 ^b

Values that do not sharing the same letter are significantly different (LSD) at the $P < 0.05$ level in each row. BHA, butylated hydroxyanisole.

[†]Reference compounds.

Table S5. Antimicrobial capacity of essential oil extracted from *Coreopsis tinctoria* flowering tops

Bacteria strains	essential oil		penicillinG [†]		inhibitory zone diameter (mm)	
	(µg mL ⁻¹)		(µg mL ⁻¹)		)	
	MIC	MBC	MIC	MBC	essential oil	penicillinG [†]
<i>Shigella</i> [ATCC12022]	5.54	11.08	0.468	0.937	15.31±1.12 ^a	21.52±1.01 ^b
<i>Streptococcus uberis</i> [ATCC 9927]	11.08	22.18	0.468	0.937	13.77±0.46 ^a	21.51±0.64 ^b
<i>Salmonella</i> [ATCC14028]	5.54	11.08	0.468	0.937	15.40±1.01 ^a	21.46±1.13 ^b
<i>Escherichia coli</i> [ATCC35150]	5.54	11.08	0.937	1.875	15.16±1.36 ^a	18.69±1.72 ^b
<i>Escherichia coli</i> [ATCC25922]	2.77	5.54	0.937	1.875	17.62±0.61 ^a	18.07±0.57 ^a

Values that do not sharing the same letter are significantly different (LSD) at the $P < 0.05$ level in each row.

[†]Positive control drug.

MIC, the minimum inhibitory concentrations; MBC, the minimal bactericidal concentration.

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