Green extraction, chemical composition, and in vitro antioxidant activity of theabrowns from Kangzhuan dark tea

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1. Introduction

Chinese dark tea is mainly classified into Kangzhuan dark tea (KZDT), Liubao tea, Fubrick tea, Pu-erh tea, and Qingzhuan tea according to their manufacturing procedures and source of raw materials (Lin et al., 2021; Liu Y.T. et al., 2022). Among above tea, KZDT is also known as “Ya’an Tibetan tea” from China’s Sichuan Province (Zheng et al., 2020; Zheng et al., 2021; Liu Y.T. et al., 2022). The processing procedures of tea influence its phytochemical compositions and bioactivities (Ding et al., 2022). The manufacturing procedures of KZDT include withering, rolling, piling, brick-pressing, drying, and storage (Xie et al., 2018). Theabrowns (TBs), one of the most active and abundant pigments found in dark tea, have the highest water solubility (Liu Y.T. et al., 2022). They are considered superior to theaflavins (TFs) or thearubigins (TRs) in physicochemical and medicinal properties (Jin et al., 2018; Chen et al., 2022). The health-promoting benefits of TBs, such as antioxidation, anti-inflammatory, and hypolipidemic activities (Ma et al., 2022), have been reported, but the development and utilization of them are insufficient. The chemical composition of TBs directly determines their bioactivities. TBs are a family of macromolecules transformed from a complex of heterogeneous components, namely, polyphenols, caffeine, proteins, carbohydrates, and amino acids, but only a handful of studies described their chemical makeup (Kuang et al., 2020; Chen et al., 2022). Therefore, it is of great importance to clarify the chemical composition of TBs from KZDT.

Organic solvents (i.e., ethanol, chloroform, methanol, and ethyl acetate) are mainly used to extract TBs in dark tea (Zou et al., 2016; Huang et al., 2019). The organic solvents exhibit many drawbacks, such as environmentally unfriendly, and hard to degrade (Liu et al., 2022a). So,
it is needful to develop green and effective extraction technologies to obtain TBs from KZDT. Compared to organic solvents, deep eutectic solvents (DES) possess some obvious advantages such as easy preparation, stability, low cost, and safety, which fully follow the rules of green extraction (Cai et al., 2019). Choline chloride (ChCl) is a widely used hydrogen bond acceptor (HBA), and ChCl-DES has been applied in the extraction of bioactive compounds from plants, such as Cannabis sativa (Liu et al., 2022b) and green tea (Camellia sinensis) (Luo et al., 2020). The ultrasound assisted extraction with DES (UAE-DES) can enhance the yield of bioactive compounds via acoustic cavitation (Wang et al., 2021). This process follows another rules of green extraction, that is, to degrade energy consumption using innovative technologies (Luo et al., 2020). Thus, UAE-DES combined with silica gel is speculated as an effective method to extract TBs from KZDT, and theabrownine fractions (TBFs) eluded by silica gel from TBs, which could help understand their health functions and promote their application.

2. Materials and methods

2.1. Chemicals and reagents

Glycerol (Gly), ethylene glycol (EG), sucrose (Suc), xylitol (Xyl), citric acid (CA), malic acid (MA), and gallic acid were bought from Macklin Biochemical Co., Ltd., Shanghai, China. Silica powder, Polin–Gocalite’s phenol, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-tri(2-pyridyl)-S-triazine (TPTZ), and 2,2’-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) were purchased from Yishan Huitong Technology Co., Ltd., Beijing, China. Analytically-grade or chromatographic-grade methanol, formic acid, acetonitrile, hydrochloric acid, and ethanol were bought from Kelon Chemical Reagent Factory, Chengdu, China. Deionized water was made from a water purification system, ELGA, China.

2.2. Preparation of sample

Kangzhuan tea (KZDT) was purchased from Sichuan Kangrun Tea Co., Ltd. (Ya’an, China). The tea was run into powders using a pulverizer. The final powders were stored at 4 °C for two weeks.

2.3. Preparation of DES

DES was synthesized based on our prior study (Luo et al., 2020). The ChCl/hydrogen bond donors (ChCl/HBD), abbreviations of the prepared DES solutions, and ChCl/HBD molar ratios were listed in Table S1. Briefly, the molar ratio of ChCl/HBD of DES was mixed at 1:2. Deionized water of 20% (m/V) was added with gentle stirring and heated at 80 °C until uniform DES solutions were obtained. DES solutions were cooled down and stored at 4 °C.

2.4. Extraction of KZDT

2.4.1. UAE-DES

KZDT powder was extracted by UAE-DES according to our prior study (Luo et al., 2020). Briefly, KZDT powder (1.0 g) was admixed into a 100 mL glass beaker, and then added 30 mL DES solutions to mix. The UAE was carried out using a XH-300UP Multifunctional extractor (Beijing, China), and the UAE-DES extraction conditions were the liquid to solid ratio at 10:1–50:1, the ultrasonic power at 330–850 W, the ultrasonic time at 2–34 min, and the water content in ChCl/MA at 10%–50%. In the course of the extraction, samples were always kept in ice water to avoid the degrading of TBs. The supernatant was collected after the extracts were centrifuged using a 5424R Eppendorf centrifuge (Shanghai, China) at 12000×g for 5 min.

2.4.2. UAE with ethanol extraction

KZDT powder was extracted by UAE with ethanol depending on a precedent study (Liu et al., 2021). Briefly, KZDT powder (1.0 g) was admixed with anhydrous ethanol (20 mL). The extraction process was then acted at ultrasonic power of 577 W for 25 min. In the course of the extraction, the sample was always kept in ice water. The supernatant was collected after the extracts were centrifuged using a 5424R Eppendorf centrifuge (Shanghai, China) at 12000×g for 5 min.

2.4.3. DES-maceration extraction (ME)

KZDT powder was extracted by ME as described previously (Liu et al., 2021). Briefly, KZDT powder (1.0 g) was admixed with 30% ChCl/MA aqueous solution (20 mL). The extraction was then acted in a shaking water bath at 25 °C for 24 h. The supernatant was collected after the extracts were centrifuged using a 5424R Eppendorf centrifuge (Shanghai, China) at 12000×g for 5 min.

2.5. Optimization of UAE-DES

Initially, TBs were extracted by UAE-DES, and the effects of five factors, including liquid to solid ratio (10:1–50:1 mL/g), ultrasonic power (330–850 W), ultrasonic time (2–34 min), and water content in ChCl/MA (10%–50%) were researched by a single-factor experiment based on our previous study (Luo et al., 2020). Response surface methodology (RSM) with a four-level-three-factor Box-Behnken Design (BBD) was used to optimize the extraction parameters, like liquid to solid ratio (X1), ultrasonic power (X2), ultrasonic time (X3), and water content in ChCl/MA (X4), to obtain the highest TBs. According to the single-factor-experiment results, the actual levels and coded of 4 parameters in the extraction process are shown in Table S2. The analysis of variance (ANOVA) was used to determine significant differences among the treatments. 3D-surface plots were applied to observe the interactive effects of the significant variables on the TBs yields.

2.6. Preparation of TBs from KZDT

TBs from KZDT were prepared as described previously (Wu et al., 2020). After the extraction of KZDT under the optimal conditions of UAE-DES, the extracts were collected and mixed, filtered, and concentrated. The above concentrated solution was extracted by the ethyl acetate for 3 times successively. The aqueous phases were collected and...
poured into a conical flask, and anhydrous ethanol was slowly added to make the volume fraction of ethanol 80% before standing for 8 h. Centrifugation was performed at 4200 \( \times g \) for 20 min to collect the precipitates, which were TBs in KZDT. The TBs were fully dissolved with distilled water before freeze-drying. The lyophilized TBs powder was collected and stored at 4 °C for next use.

2.7. Quantification of theabrownins (TBs)

The content of TBs was measured using a system approach as previously described \( \text{Wang et al., 2018} \). TBs solution was prepared by dissolving TBs (0.1 g) in distilled water (20 mL). TBs solution/KZDT extracts (25 mL) and 25 mL n-butanol were mixed into a funnel and shaken for 3 min. After stratification, 2 mL sample of the aqueous phases was mixed with 2 mL of saturated oxalic acid solution, 6 mL of distilled water, and 15 mL 95% ethanol (solution B) to 25 mL. The \( E_0 \) of solution B was measured with a UV–S500 spectrophotometer (Metash Instruments Co., Ltd., Shanghai, China) at 380 nm. The TBs yield (%) was calculated as the following:

\[
TBs \% = \frac{7.06 \times 2 \times E_0}{\text{dried weight} \%} \times 100
\]

where \( E_0 \) is the corresponding spectrophotometer reading of all the samples. Dried weight is the weight of a sample without water.

2.8. Preparation of theabrownine fractions (TBFs)

Based on a previous method, TBs from KZDT were dispersed by using silica gel as the stationary phase and methanol aqueous solution as the mobile phase \( \text{Xie et al., 2019} \). First, the 100–200 mesh silica gel powder was washed with distilled water to neutral pH before drying at 110 °C for 24 h. It was required to be activated at 100 °C for 2–3 h before the experiment. The pre-treated silica gel powder (500 g) was sequentially slurry-packed in a 60.6 mm × 218.5 mm column with the methanol, and 2–3 volume of methanol was used for equilibration. The TBs (50 mg/mL) were then loaded onto this column. Preparation of theabrownine fractions (TBFs) was carried out by sequentially eluting with 100% methanol (0–20 min), 100–80% methanol (21–40 min), 80–60% methanol (41–60 min), 60–40% methanol (61–80 min), 40–20% methanol (81–100 min), and 20–0% methanol (101–120 min) with a flow of 20 mL/min. Six TBFs were collected, namely, TBF1 (100% methanol), TBF2s (200–80% methanol), TBF3s (80–60% methanol), TBF4s (60–40% methanol), TBF5s (40–20% methanol), and TBF6s (20–0% methanol). TBFs were rotary-evaporated at 50 °C, and then stored at 4 °C for further identification and antioxidant activity assay.

2.9. LC-MS/MS analysis of TBFs

The chemical components in TBFs were detected by LC-MS/MS based on our precedent study \( \text{Mai et al., 2022} \). It was separated on a LC System (Thermo Scientific, San Jose, USA) with a Hypersil Gold C18 column (2.1 × 100 mm, 1.9 μm). The mobile phase consisted of solvent A (0.1% formic acid aqueous solution) and solvent B (acetonitrile). The column was eluted at a flow rate of 0.3 mL/min with a gradient of 1% solvent B (0–4 min), 1–10% solvent B (4–15 min), 10–15% solvent B (15–25 min), 15–25% solvent B (25–30 min), 25–40% solvent B (30–35 min), 40–80% solvent B (35–45 min), and 80–100% solvent B (45–50 min). The injection volume is 5 μL, and column temperature is 30 °C. ESI–MS (orbitrap) analysis was performed in the m/z range of 100–1000, and the enhanced product ion (EPI) scan was performed to identify their fragment ions. The ion source parameters were capillary voltage with −12 V, spray voltage with 4.5 kV, resolution at 70,000, and heated capillary temperature at 275 °C. The chemical components were analyzed by comparing the parent and fragment ions with the MassBank database (https://massbank.eu/MassBank/).

Fig. 1. Theabrownins (TBs) yields of the Kangzhuan dark tea extracts based on 6 selected DESs. Data are expressed as means ± SD (n = 3). Values with superscript letters (a–c) are significantly different across columns (P < 0.05).

2.10. Determination of antioxidant capacity in vitro

2.10.1. DPPH radical scavenging activity

The DPPH antioxidant activity of TBs was determined based on a prior study with a minor modification \( \text{Liu et al., 2021; Zou et al., 2021; Zhu et al., 2021; Ding et al., 2022} \). The DPPH methanol solution (0.05 mg/mL) was adjusted with methanol to reach an absorbance of 0.789 ± 0.02 at 517 nm. 100 μL of sample solutions was mixed with 1.9 mL of the DPPH working solution at room temperature for 10 min in the dark. Then the spectrophotometer reading at 517 nm of the mixture was determined. The results were calculated using a calibration curve of ascorbic acid (5–50 μM) and expressed as μM Ascorbic acid/g dry weight (DW) of TBs.

2.10.2. ABTS radical scavenging activity

The ABTS capacity of TBs was determined based on our prior study with a minor modification \( \text{Mai et al., 2022} \). The ABTS+ solution (7 mM) was mixed with potassium persulfate solution (2.45 mM) (1:1, v/v) and incubated for 17 h in the dark to prepare the ABTS+ stock solution. The ABTS+ stock solution was adjusted with deionized water to reach an absorbance of 0.758 ± 0.03 at 734 nm. 100 μL of samples solutions were mixed with 1.9 mL of ABTS+ working solution at room temperature for 6 min in the dark, then the absorbance of the mixture at 734 nm was measured. The standard curve was carried out with standard Trolox solution (50–800 μM), and the results were expressed as μM Trolox/g dry weight (DW) of TBs.

2.10.3. FRAP assay

The FRAP capacity of TBs was determined based on our prior study with a minor modification \( \text{Gan et al., 2017} \). The FRAP working solution was prepared by incubating 10 mM TPTZ solution with 20 mM ferric chloride solution in 300 mM sodium acetate buffer at a volume ratio of 10:1:1 (v/v/v). 100 μL of sample solutions was mixed with 1.9 mL of the FRAP working solution for 4 min at room temperature. Then the absorbance of the mixture at 593 nm was measured. The ferrous sulfate (20–1000 μM) was applied to make a standard curve, and the results were shown as μM Fe(II)/g DW of TBs.

2.11. Statistical analysis

The data among treatments were compared by the ANOVA with post
3. Results and discussion

3.1. Selection of DES

DES is an emerging solvent with high extraction rate of phytochemicals. The screening of different DES is the foremost work that should be done for TBs extraction from KZDT. In Fig. 1, the prepared ChCl-based DES, including ChCl/MA, ChCl/Gly, ChCl/Suc, ChCl/Xyl, ChCl/EG, and ChCl/CA, were further tested. It was significant that the yield of TBs in the ChCl/MA was the highest. A prior study reported that the ChCl/MA presented a high protective effect against the thermal degradation of extracts, which was mainly related to the low pH value of the DES and the molecular interactions (Benvenuti et al., 2022). Thus, ChCl/MA was selected as the DES for TBs extraction for further study.

3.2. Extraction optimization of TBs using UAE-DES

3.2.1. Single-factor experiments results

There has been no research to optimize the fermentation parameters that affect the yield of TBs in KZDT using single-factor experiments and RSM. The impacting factors for TBs content in UAE-DES, including solid to liquid ratio, water content in ChCl/MA, ultrasonic power, and ultrasonic time, were studied through single-factor experiment.

As the liquid to solid ratio (Fig. 2A), the TBs yield elevated as the liquid to solid ratio elevated from 10:1 to 20:1 mL/g, but then the TBs yield dropped, as the liquid to solid ratio was further raised from 20:1 mL/g. The decreasing trend of TBs yield after the point “20:1 mL/g” may be explained as the volume of the DES increased, the dissolution of TBs was inhibited by other components of tea, which was not conducive to extraction (Durak and Gawlik-Dziki, 2014). So, the most fitted liquid to solid ratio range from 10:1 to 30:1 mL/g was recommended for the highest TBs. For the ultrasonic power (Fig. 2B), the TBs yield elevated as the ultrasonic power heightened from 330 to 720 W, but the TBs yield then decreased when the ultrasonic power was further up from 720 W. Consequently, ultrasonic power in the range of 590–850 W was fitted mostly for the highest TBs.

As shown in Fig. 2C, the TBs yield was elevated as the ultrasonic time elevated from 2 to 26 min, but the raise of the ultrasonic time did not provide a higher TBs yield. Hence, the most fitted ultrasonic time was in the range of 18–34 min to obtain the highest TBs.

Similar to other factors, the water content in ChCl/MA also significantly influenced TBs yield, where an increase of the water content in ChCl/MA from 10 to 30% heightened the TBs yield (Fig. 2D). Howbeit, further raise of the water content in ChCl/MA did not good for the extraction of TBs as the yield dropped. Thus, the water content in ChCl/MA with 20–40% was recommended for the highest TBs.

3.2.2. Results of the BBD

Based on the above single-factor experiment results, the solid to liquid ratio, water content in ChCl/MA, ultrasonic power, and ultrasonic time were then tested by BBD to obtain the optimal UAE-DES conditions.
with a maximum TBs yield from the KZDT. The BBD design is shown in Table S3. The final model equation of the maximum TBs yield is given as below:

\[
Y = + 121.13 + 0.5918X_1 - 8.44X_2 - 1.29 X_3 - 0.0712 X_4 - 1.59X_1X_2 - 1.48X_1X_3 - 1.25 \times 1 X_4 + 1.15X_2X_3 + 1.91X_2X_4 + 3.64X_3X_4 - 11.83X_1^2 - 43.84X_2^2 - 12.93X_3^2 - 12.71X_4^2
\]

Where, \( Y \) is the TBs yield, \( X_1 \) is the liquid to solid, \( X_2 \) is the ultrasonic power, \( X_3 \) is the ultrasonic time, and \( X_4 \) is the water content in ChCl/MA.

The results indicated that the predicted TBs value of 12.16% KZDT powder could be obtained under the optimal UAE-DES conditions as follows: liquid to solid ratio of 20.37:1 (v/w), ultrasonic power of 577.26 W, ultrasonic time of 25 min, and water content of 29.80%.

For practical purposes, the optimal UAE-DES conditions were predicted as follows: liquid to solid ratio of 20.1 (v/w), ultrasonic power of 577 W, ultrasonic time of 25 min, and water content of 30%.

ANOVA was applied to analyze the model equation significance, which is shown in Table S4. The \( p \)-value (\( < 0.0001 \)) indicated that the quadratic model was significant. The coefficient of determination \( R^2 = 0.9123 \) indicated that the predicted results met well with the experimental results. The difference between the predicted determination coefficient \( (R^2 \text{ pred } = 0.8374) \) and \( R^2 \) was \( < 0.2 \). The adjusted coefficient of determination \( (R^2 \text{ Adj } = 0.8246) \) was commensurate to \( R^2 \), indicating the predicted and observed values have a high correlation. The “Lack of Fit F-value” was 0.033 (\( p > 0.05 \)), which indicated the model was effective. The coefficient of variation (C.V. = 11.10\%) suggested the experimental values have a high reliability and precision. Consequently, we concluded that the regression model was reasonable.

As shown in Table S4, the effect of ultrasonic power (\( p < 0.0001 \)) on TBs yield > ultrasonic time (\( p = 0.0044 \)) > the water content in ChCl/MA > liquid to solid ratio (\( p = 0.0078 \)). The response surface plots are shown in Fig. 3 to clarify the interactive effects of four factors on the TBs yield. The interactive effect between the liquid to solid ratio and ultrasonic power (Fig. 3A), ultrasonic power and water content in ChCl/MA (Fig. 3B), were obvious on the TBs yield. The interactive effect between the liquid to solid ratio and ultrasonic time (Fig. 3D), liquid to solid ratio and water content in ChCl/MA (Fig. 3C), ultrasonic power and the ultrasonic time (Fig. 3D), water content in ChCl/MA and ultrasonic time (Fig. 3F), were not significant for the TBs yield.

3.2.3. Model verification

The RSM result was verified to corroborate the suitability of this model. The actual max TBs yield (12.59% KZDT powder) was commonly met well with the predicted results (12.16% KZDT powder) under the optimal UAE-DES conditions as follows: water content in ChCl/MA of 30%, liquid to solid ratio of 20:1 mL/g, ultrasonic power of 577 W, and ultrasonic time of 25 min. So, the model was suitable for the extraction of TBs from KZDT. The actual maximal TBs yield was about 11.29% higher than that obtained by UAE-ethanol, suggesting DES has better extraction ability than ethanol. Besides, the TBs yield obtained by ME was 22.68% lower than the TBs value obtained by UAE-DES. Obviously, the application of UAE-DES provided an efficient and green extraction of plant bioactive compounds than UAE-ethanol and ME (Cai et al., 2019; Luo et al., 2020). In a previous study, 29.3% TBs of the instant dark tea could be achieved via submerged fermentation using Aspergillus niger (Wang et al., 2017). However, the cost of fermentation is too great for industrial use of TBs, hindering the industrialization of TBs. In addition, lower intermolecular interaction energy, larger accessible solvent surface area, and more hydrogen bonds between ChCl/MA and extracts explain for the efficient extraction ability of ChCl/MA (Fu et al., 2022). Therefore, the customized DES is an efficient and green alternative to...
### Table 1
Identification of the main chemical profile of TBFs by LC-MS/MS.

| NO. | RT [min] | m/z | MW | Formula | Identification | Classification | TBFs       | Area (Max.) | Reference Ion |
|-----|----------|-----|----|---------|---------------|----------------|------------|-------------|---------------|
| 1   | 1.12     | 112.0508 | 111.04354 | C4H5N3O | Cytosine       | Nucleotides and their derivatives | TBFs2, TBFs3, TBFs4 | 9.42E-07    | [M-H]-1       |
| 2   | 5.07     | 114.0916 | 113.08436 | C6H11NO | Caprolactam     | Others         | TBFs3, TBFs4 | 4.85E-08    | [M-H]-1       |
| 3   | 1.52     | 115.0025 | 116.00981 | C4H4O4 | Fumaric acid   | Phenolic acids | TBFs3, TBFs4 | 3.39E-08    | [M-H]-1       |
| 4   | 1.45     | 128.0343 | 129.04152 | C5H7O3N | 4-Oxoproline   | Amino acids and their derivatives | TBFs1, TBFs2, TBFs3, TBFs4 | 1.09E-08    | [M-H]-1       |
| 5   | 1.45     | 130.05   | 129.04268 | C5H7N3O | D(-)-Pyroglycamic acid | Phenolic acids | TBFs1, TBFs2, TBFs3, TBFs4, TBFs5 | 6.86E-07    | [M-H]-1       |
| 6   | 1.11     | 136.0618 | 135.05453 | C5H5N5 | Adenine        | Nucleotides and their derivatives | TBFs1, TBFs2, TBFs3, TBFs4, TBFs5 | 3.34E-08    | [M-H]-1       |
| 7   | 0.06     | 141.1135 | 140.10621 | C6H12N4 | Methenamine    | Others         | TBFs1, TBFs2, TBFs3, TBFs4, TBFs5 | 1.13E-08    | [M-H]-1       |
| 8   | 2.23     | 152.0567 | 151.04947 | C5H5N5O | Guanine        | Nucleotides and their derivatives | TBFs3, TBFs4 | 1.12E-08    | [M-H]-1       |
| 9   | 2.20     | 169.0134 | 170.02055 | C7H6O5 | Gallic acid    | Phenolic acids | TBFs3, TBFs4, TBFs5 | 5.65E-08    | [M-H]-1       |
| 10  | 4.25     | 181.0719 | 180.06466 | C7H8N4O2 | Theobromine    | Alkaloids      | TBFs3, TBFs4, TBFs5 | 7.44E-07    | [M-H]-1       |
| 11  | 4.30     | 185.0442 | 184.03743 | C5H11N4O4P | Phosphocholine | Alkaloids    | TBFs3, TBFs4, TBFs5 | 7.73E-08    | [M-H]-1       |
| 12  | 0.93     | 191.0555 | 192.06276 | C7H12O6 | D(-)-Quinic acid | Phenolic acids | TBFs3, TBFs4, TBFs5 | 4.08E-08    | [M-H]-1       |
| 13  | 4.44     | 192.1338 | 191.13097 | C12H17NO | Diethyloctamide | Others | TBFs3, TBFs4, TBFs5, TBFs6 | 3.42E-08    | [M-H]-1       |
| 14  | 3.83     | 192.1338 | 191.13103 | C12H17NO | Diethyl-2-phenylacetamide | Others | TBFs3, TBFs4, TBFs5, TBFs6 | 2.71E-08    | [M-H]-1       |
| 15  | 6.60     | 195.0876 | 194.0803 | C8H10N4O2 | Caffeine       | Alkaloids      | TBFs3, TBFs4, TBFs5 | 6.90E-09    | [M-H]-1       |
| 16  | 6.96     | 199.0576 | 176.06838 | C7H12O5 | 2-isopropylmalic acid | Phenolic acids | TBFs1 | 9.61E-08    | [M-Na]-1      |
| 17  | 35.48    | 204.1383 | 203.13102 | C13H17NO | Crotamiton     | Phenolic acids and their derivatives | TBFs3, TBFs4, TBFs5, TBFs6 | 1.12E-08    | [M-H]-1       |
| 18  | 33.78    | 210.0769 | 211.08414 | C11H9N5 | 1-Phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine | Others | TBFs3, TBFs4, TBFs5, TBFs6 | 1.69E-07    | [M-H]-1       |
| 19  | 33.59    | 214.2529 | 213.24559 | C14H31N | 1-Tetradecylamine | Others | TBFs3, TBFs4, TBFs5, TBFs6 | 4.44E-08    | [M-H]-1       |
| 20  | 4.54     | 217.1046 | 216.09734 | C11H12N4O | 6(-4-methoxyphenyl)pyrimidine-2,4-diamine | Others | TBFs3, TBFs4, TBFs5, TBFs6 | 5.42E-08    | [M-H]-1       |
| 21  | 35.72    | 221.1545 | 222.16182 | C14H22O2 | 2,5-di-tet-butylhydroquinone | Anthraquinones | TBFs3, TBFs4, TBFs5, TBFs6 | 2.51E-07    | [M-H]-1       |
| 22  | 35.56    | 233.1547 | 234.16195 | C15H24O3 | Ilicic acid    | Others         | TBFs3, TBFs4, TBFs5, TBFs6 | 4.45E-07    | [M-H]-1       |
| 23  | 35.34    | 239.0674 | 240.0747 | C10H12N2O5 | Dinonterb     | Others         | TBFs3, TBFs4, TBFs5, TBFs6 | 2.75E-07    | [M-H]-1       |
| 14  | 5.31     | 239.1488 | 238.14146 | C10H22O6 | Pentaethylene glycol | Others | TBFs3, TBFs4, TBFs5, TBFs6 | 7.11E-09    | [M-H]-1       |
| 25  | 33.64    | 256.2966 | 255.29236 | C17H37N | N-Methylisocarbamylamine | Others | TBFs3, TBFs4, TBFs5, TBFs6 | 1.04E-08    | [M-H]-1       |
| 26  | 35.96    | 265.1482 | 266.15552 | C12H26O4S | (Dodecylxoy) sulfonic acid | Phenolic acids | TBFs3, TBFs4, TBFs5, TBFs6 | 4.13E-08    | [M-H]-1       |
| 27  | 1.85     | 268.1039 | 267.09658 | C10H11N5O4 | 2’-Deoxyguanosine | Nucleotides and their derivatives | TBFs3, TBFs4, TBFs5, TBFs6 | 2.68E-08    | [M-H]-1       |
| 28  | 1.21     | 273.0215 | 274.21424 | C14H9CIN2S | (--)Epigallocatechin | Flavonoids | TBFs3, TBFs4, TBFs5, TBFs6 | 8.93E-07    | [M-H]-1       |
| 29  | 35.27    | 277.1449 | 278.15215 | C16H22O4 | Alpha-Linolenic acid | Lipids | TBFs3, TBFs4, TBFs5, TBFs6 | 6.32E-07    | [M-H]-1       |
| 30  | 36.26    | 279.1589 | 278.15812 | C16H22O4 | Gamma-Linolenic acid | Lipids | TBFs3, TBFs4, TBFs5, TBFs6 | 2.44E-08    | [M-H]-1       |
| 31  | 6.21     | 283.1749 | 282.16758 | C17H14O4 | 3,5-Dimethoxyflavone | Flavonoids | TBFs3, TBFs4, TBFs5, TBFs6 | 6.79E-09    | [M-H]-1       |

(continued on next page)
Table 1 (continued)

| NO. | RT [min] | m/z   | MW     | Formula            | Identification                           | Classification                          | TBFs     | Area (Max.) | Reference Ion  |
|-----|----------|-------|--------|--------------------|------------------------------------------|-----------------------------------------|----------|-------------|---------------|
| 32  | 40.16    | 284.2945 | 283.2872 | C10H13N5O5 | Guanosine                                | Nucleotides and their derivatives       | TBFs1    | 5.57E-08    | [M + H]^+ 1   |
| 33  | 35.56    | 284.3309 | 283.3265 | C10H13N5O5 | Isoguanosine                              | Nucleotides and their derivatives       | TBFs1, TBFs2, TBFs3, TBFs4, TBFs5, TBFs6 | 2.66E-08    | [M + H]^+ 1   |
| 34  | 40.21    | 293.1796 | 294.1867 | C17H14N2O3 | Cyclocreatine                             | Others                                  | TBFs1    | 2.66E-08    | [M + H]^+ 1   |
| 35  | 28.94    | 301.0721 | 302.0794 | C15H10O7   | Quercetin                                | Flavonoids                              | TBFs5    | 4.37E-07    | [M + H]^+ 1   |
| 36  | 20.35    | 303.0862 | 302.0782 | C16H14O6   | Hesperetin                               | Flavonoids                              | TBFs1, TBFs2 | 3.30E-08    | [M + H]^+ 1   |
| 37  | 40.70    | 325.1846 | 326.1919 | C18H3O3S   | 4-Dodecylbenzenesulfonylic acid          | Organic acid                            | TBFs1    | 2.11E-08    | [M + H]^+ 1   |
| 38  | 42.48    | 338.3416 | 337.3328 | C22H43NO   | Erucamide                                | Derivatives of unsaturated fatty acids  | TBFs1, TBFs2, TBFs3, TBFs4, TBFs5, TBFs6 | 4.16E-09    | [M + H]^+ 1   |
| 39  | 38.01    | 339.2331 | 340.2403 | C15H16O9   | 6,7-Dihydroxycoumarin-6-glucoside       | Flavonoids                              | TBFs1    | 2.90E-09    | [M + H]^+ 1   |
| 40  | 32.62    | 343.1175 | 342.1102 | C19H18O6   | Zapotin                                  | Flavonoids                              | TBFs1, TBFs2, TBFs3, TBFs4, TBFs5, TBFs6 | 1.11E-08    | [M + H]^+ 1   |
| 41  | 36.24    | 383.2039 | 360.2146 | C22H26N2O4 | Tofisopam                                | Others                                  | TBFs1, TBFs2, TBFs3, TBFs4, TBFs5, TBFs6 | 1.41E-08    | [M + Na]^+ 1  |
| 42  | 37.01    | 425.2144 | 402.2251 | C24H31FO4  | Citroflex A-4                            | Others                                  | TBFs1, TBFs2, TBFs3, TBFs4, TBFs5, TBFs6 | 3.59E-08    | [M + Na]^+ 1  |
| 43  | 11.26    | 473.3603 | 475.3512 | C22H43NO   | LysoPE(0:0/18:3(6Z,9Z,12Z))              | Lipids                                  | TBFs1    | 1.29E-10    | [M + H]^+ 1   |
| 44  | 12.95    | 520.3372 | 520.2983 | C22H46O12  | Galloclatechin gallate derivative         | Flavonoids derivatives                  | TBFs1, TBFs2, TBFs3, TBFs4, TBFs5, TBFs6 | 8.86E-09    | [M + H]^+ 1   |
| 45  | 14.66    | 564.3588 | 564.3249 | C26H28O14  | Apigenin 6-C-glucoside 8-C-arabinoside   | Flavonoids                              | TBFs1, TBFs2, TBFs3, TBFs4, TBFs5, TBFs6 | 5.42E-09    | [M + H]^+ 1   |
| 46  | 18.19    | 579.1731 | 580.1797 | C27H32O14 | Naringin                                  | Flavonoids                              | TBFs1, TBFs2, TBFs3, TBFs4, TBFs5, TBFs6 | 5.71E-07    | [M + H]^+ 1   |
| 47  | 20.36    | 609.1837 | 610.1904 | C28H34O15 | Neohesperidin                            | Flavonoids                              | TBFs1, TBFs2, TBFs3, TBFs4, TBFs5, TBFs6 | 1.30E-09    | [M + H]^+ 1   |
| 48  | 16.35    | 613.3401 | 590.3512 | C26H54O14 | PEG n13                                  | Others                                  | TBFs2, TBFs3, TBFs4, TBFs5, TBFs6       | 4.69E-07    | [M + Na]^+ 1  |
| 49  | 17.94    | 652.4113 | 651.4038 | C28H58O15  | PEG n14                                  | Others                                  | TBFs5, TBFs6                               | 7.90E-08    | [M + H]^+ 1   |

Note: All the chemicals were screened by the following criteria: mzCloud Best Match is equal or greater than to 85, Area (Max.) is equal or greater than to 10<sup>5</sup>.
detected among the six TBFs, a principal component analysis (PCA) was performed on LC-MS/MS data. As shown in Fig. 5A, in the PCA of all the LC-MS/MS data, the PC1 and PC2 are 68.9% and 28.4%, respectively, they explained 97.3% of the total variance across all samples. The PCA score plot showed that TBFs3, TBFs4, TBFs5, and TBFs6 were closely clustered in the PCA, and a significant separation was observed between TBFs1 and TBFs2, indicating that the chemical composition of TBFs3, TBFs4, TBFs5, and TBFs6 elution was the closest. A similar result was obtained in Fig. 5B, TBFs1, TBFs2, TBFs3, TBFs4, TBFs5, and TBFs6 can be distinguished as three groups on the hierarchical clustering analysis. Collectively, these results, for the first time, indicated that different chemical compounds in TBs were eluted by silica gel powder combined with different concentrations of methanol solutions.

3.5. Analysis of antioxidant capacities of TBFs

Oxidative stress is considered an important factor leading to aging and disease (Ma et al., 2022). Some studies have proved that dark tea...
exhibited strong antioxidant activity (Lin et al., 2021). It is widely known that TBs are crucial for the antioxidant activity of dark teas. To compare the in vitro antioxidant activities of all TBFs, DPPH free radical scavenging, ABTS free radical scavenging, and FRAP assay were carried. The results are shown in Fig. 6. Generally, the antioxidant capacities of six TBFs were verified by the DPPH radical scavenging assay in the order of TBFs4 > TBFs5 > TBF3 > TBFs1 > TBFs2 > TBFs6. The antioxidant capacities of six TBFs were also verified by the FRAP assay in the order of TBFs4 > TBFs1 > TBFs5 > TBFs3 = TBFs2 > TBFs6. As the results of DPPH, ABTS, and FRAP, the TBFs4 showed the highest antioxidant activity among all the TBFs, indicating that 60–40% methanol aqueous solution is most suitable for the elution of antioxidants in TBs. Meanwhile, the DPPH, ABTS, and FRAP values of TBFs4 were 45.08 ± 0.42 μM Ascorbic acid/g DW, 178.52 ± 0.29 μM Trolox/g DW, and 370.85 ± 6.00 μM Fe(II)/g DW, respectively. This result indicated that the TBFs from Tibetan tea had different DPPH free radical scavenging, ABTS free radical scavenging, and FRAP effects, which was accordant with the conclusion of a prior study, which reported that extracts from Tibetan tea had strong DPPH and ABTS radical scavenging capacities (Liu Y.T. et al., 2022).

4. Conclusions

This study developed a UAE-DES based extraction method to produce TBs from KZDT, and revealed the chemical composition of TFBs eluded by silica gel from TBs using LC-MS/MS. Under the optimal conditions of UAE-ChCl/MA, the highest yield (12.59%) of TBs from KZDT was achieved. A total of 49 chemicals were identified in TFBs, and 6,7-dihydroxycoumarin-6-glucoside, erucamide, caffeine, and neohesperidin were among the leading compounds in the TBFs. Results from in vitro antioxidant capacity showed that all TBFs exhibited antioxidant activities, while TBFs4 displayed the highest antioxidant activity. Consequently, it is necessary to further clarify specific antioxidants in TBs and delve into the antioxidant activity of TBs using in vivo models in the future. Overall, our findings not only furnish a theoretical basis for the extraction of KZDT with high TBs, but also provide a reference to understand the chemical composition of TBs in KZDT. These results have the potential to guide the further development of KZDT products.
Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cjrf.2022.10.019.

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