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Research Article

Standardization Study of Khurtsiin deed-6 Traditional Medicine

Myadagbadam U[1,2], Purevsuren S[1,*], Chimedragchaa Ch[2], Tserenkhand G[3], Norovnyam R[1]

ABSTRACT

Introduction: Although traditional medicines are widely used, quality and safety of some of them are not ensured due to lack of suitable quality controls and inadequate labeling. he diagnostic cellular structures and cell content for all powdered ingredients in Khurtsiin deed-6 traditional medicine were defined by "Novel" light microscopy. Biologically active compounds were identified by using TLC and HPLC. Main biologically active compounds contents were determined by UV spectrophotometer methods. Some quality and safety parameters of Khurtsiin deed-6 traditional medicine were determined by Mongolian National First Pharmacopoeia methods. **Results:** Some quality and safety parameters of the traditional medicine were determined as: moisture 9.63±0.09%, total ash 4.725±0.22%, and water-soluble extractive 21.28±0.11%, Alcohol soluble extractive total 46.30±0.09%, aerobic microbial count $5x10^3$, total yeast and mold count $3x10^2$. The fingerprints of TLC and HPLC to reveal gallic acid, apigenin, costunolide, E-guggulsterone were defined. The total contents were measured for phenolic compounds as 65.95±1.05 mg/g and for flavonoids as 10.55 ±0.16 mg/g. **Conclusion:** The standardization criteria for Khurtsiin deed-6 traditional medicine were defined and Mongolian National Pharmacopeia Monograph's draft for Khurtsiin deed-6 traditional medicine was developed.

Key words: Gallic acid, High-Pressure Liquid Chromatography, Herbal medicine, Thin layer chromatography.

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INTRODUCTION

Traditional medicines have been used in many countries throughout the world over many centuries and there is a growing trend to introduce traditional medicines into today's medical practice. Although traditional medicines are widely used, quality and safety of some of them are not ensured due to lack of suitable quality controls and inadequate labeling. One of the four objectives of WHO Traditional medicine strategy 2014-2023 is "safety, efficacy and quality - promote the safety, efficacy and quality of TM by expanding the knowledge base, and providing guidance on regulatory and quality assurance standards".[1] Therefore, it is important to improve the basic requirements for traditional medicines, to conduct standardization studies of traditional medicines, especially of multicomponent traditional medicines, to establish quality parameters, and to improve quality control to ensure the safety and effectiveness of traditional medicines.

Mongolia has a rich tradition to use herbal medicines over many centuries. Mongolian Traditional Medicine was prohibited for many years till 1990's in our country and since 1990 the studies and production of traditional medicines have been significantly expanded in Mongolia.[2] Although many studies on traditional medicines have been performed for last three decades, the standardization study is still important. There are some non-standardized traditional medicines and one of them is Khurtsiin Deed-6, a traditional medicine consists of fruit of *Terminalia chebula*, flower of *Carthamus tinctorius* L., rhizome of *Saussurea lappa* L., herba of *Odontites vulgaris*, resin of *Commiphora wightii* and musk of Musk deer. In Mongolian Traditional Medicine, it is used for the treatment of bacterial fever headaches, bloody yellow headaches, and headaches caused by yam disease, and also for some diseases such as cataracts.[3]

The main bioactive components of *O.vulgaris* are phenolcarboxylic acids, flavonoids, iridiod and phenolic acid. 35 compounds were identified from the n-butanol and ethyl acetate fractions of this plant's extract, of which 16 were identified by the reference substances using UPLC–MS. The compounds are mainly divided into four categories: phenylethanoid glycosides (verbascoside, isoacteoside, arenarioside, salidroside), flavonoids (kaempferol, cynaroside, luteolin, luteolin-7-O-glucuronide, apigenin, apigenin-7-O-glucoside, tricin, chrysoeriol, quercetin, diosmetin, hydroxygenkwanin), iridoids (melampyroside, shanzhiside methyl ester, aucubin, geniposide, geniposidic acid, shanzhiside methyl ester), phenolic acids (caffeic acid, trans-cinnamic acid, 2-hydroxycinnamic acid, salicylic acid, ferulic acid, 7,8-dihydroxycoumarin) and others (adenosine, syringaresinol, D-Mannitol, esculetin).[4]

Numerous researches conducted on *T.chebula* have confirmed the presence of wide range of the phytochemicals such as flavonoids, tannins, phenolic acids and other bioactive compounds. Juang *et al.* (2004) developed an reverse-phase HPLC method for the determination of 14 phenolic components of hydrolyzable tannins (gallic acid, chebulic acid, chebulanin, corilagin, punicalagin, neochebulinic acid, ellagic acid, chebulegic acid, chebulinic acid,1,2,3,4,6-Penta-O-galloyl-β-D-glucose, 1,6,-di-O-galloyl-D-glucose, casuarinin, 3,4,6-tri-O-galloyl-D-glucose, terchebulin) in the fruits of *T.chebula*.[5]

Phytochemical analysis of *S. lappa* roots showed the presence of monoterpenes, sesquiterpenoids, flavonoids, lignans, triterpenes, steroids, glycosides, and etc. *S. lappa* roots are rich source of sesquiterpenoids, especially sesquiterpene lactones.

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Essential oil of *S. lappa* roots obtained by hydrodistillation showed higher content of sesquiterpenoids (79.80%) than monoterpenoids (13.25%). The principal compounds in *S. lappa* essential oil were dehydrocostus lactone (46.75%), costunolide (9.26%), 8-cedren-13-ol (5.06%) and α-curcumene (4.33%). Based on bioactivity-directed fractionation, dehydrocostus lactone and costunolide were isolated from *S. lappa* essential oil.[6]

Guggul contained many phytochemicals constituents including quercetin, and guggulsterones E and Z. These phytochemical constituents have a wide range of pharmacological activities such as antioxidant, antibacterial, antimutagenic, antivenom and antitumor.[7]

The chemical composition of flowers of *Carthamus tinctorius* is interesting and rich, with 200 substances identified thus far. The chemical groups isolated from *Carthamus tinctorius* are oils, proteins, minerals, phenolics, flavonoids, alkaloids, lignans, carboxylic acids, steroids, polysaccharides, quinochalcone C-glycosides and quinone-containing chalcones.[8]

Studies found that musk of Musk deer mainly contains macrocyclic ketones, pyridine, steroids, fatty acids, amino acids and proteins, whilst the main active ingredient is muscone. Modern pharmacological studies have proven that musk and muscone, the main active ingredient possess potent anti-inflammatory, neuroprotective, anticancer, and antioxidant effects and other pharmacological effects.[9]

Although biologically active substances in each plant of Khurtsiin Deed-6 traditional medicine were identified by other studies, we are not aware of any study of the biologically active substances content of Khurtsiin Deed-6, a 6-ingredient traditional medicine. Therefore, there is a need to determine the optimal condition to reveal biological active compounds of ingredients of traditional medicine, the content of compounds, and quality and safety parameters of Khurtsiin Deed-6 traditional medicine. This study aimed to conduct a standardization study and determine some quality and safety parameters of Khurtsiin deed-6 traditional medicine to ensure the quality.

MATERIALS AND METHODS

Plants material

The crude herbal medicines from *Saussurea lappa* L., *Terminalia chebula* Retz, *Commiphora wightii* were purchased from Traditional Drug Factory at the Institute of Traditional Medicine and Technology (ITMT), Mongolia. *Odontites vulgaris* Moench and *Carthamus tinctorius* L. were collected from Undur-Khutag and Dashinchilen soums, Bulgan province. The musk extracted from the musk deer domesticated in the "Musk deer breeding center of ITMT" was used for this study**.** Each medicinal plant was identified by Munkh-Erdene T (Botanical Garden and Research Institute, Mongolia). Voucher specimens have been deposited in the Herbarium of the ITMT.

Standards and chemicals

Folin-Ciocalteu reagent from Sangon (China), reference gallic acid, rutin, apigenin, E-guggulsterone, costunolide, hydroxysafflor yellow A, and muscone from Sigma Aldrich (USA) were used for the study. All other reagents and solvents were of analytical grade.

Powder microscopy

Plant material

Each plant raw material taken from the middle part of the vegetative organs (leaves, stems, roots) was softened in hot water for 10 minutes and fixed in VCM-202III freezer microtome and cut a section about 14-20 micrometers wide. The translucent section was placed on a microscope slide and stained with *alcian blue dye* and dye saffron, and the microstructure was determined using a NOVEL light microscopy equipped with a digital camera.

Traditional powder medicine

The powder medicine was softened in hot water for 10 minutes. The softened sample was placed in chloral hydrate ($C_2H_3Cl_3O_2$) and different concentration (5-15%) solution of sodium hydroxide for 15 minutes and rinsed with distilled water. The small amount of powder was placed on a microscope slide with a drop of choral hydrate and covered with cover glass. After slight heating to remove air, the sample was sequentially stained with *alcian blue dye,* methyl blue, and dye saffron. The microstructure was determined using NOVEL light microscopy equipped with a digital camera.[10]

Chemical analysis

TLC identification of biologically active substances

Sample preparation

Khurtsiin deed-6 traditional medicine (20 g) was extracted 4 times with 100 mL of 70% EtOH using the Soxhlet apparatus, and the combined extract was concentrated under reduced pressure. The residue was suspended in H_2O and then successively partitioned with hexane, chloroform, ethyl acetate, and butanol. The chloroform and ethyl acetate fractions were evaporated and the dry residues were dissolved in methanol and used for the Thin-layer chromatography (TLC) as sample solutions. The sample solutions were applied to TLC plates (Merck Silicagel 60 GF 254) in 5μL at a 1.5 cm distance from the lower border of the plate. The development of the plate has been performed with a mobile phase of hexane-ethyl acetate-formic acid (10:5:1, v/v). After drying at room temperature, the plate was visualized under both 254 and 366 nm UV lights.[11]

HPLC identification of biologically active substances

Preparation of sample solution

1 g Khurtsiin deed-6 traditional medicine was precisely weighed to an accuracy of 0.001 g and placed in a 50 mL volumetric flask and extracted using ultrasonic bath with 25 mL methanol for 60 min at a temperature of 60°C. After cooling, the extract was filtered through 0.45 μm filter membranes before being injected into the HPLC instrument.

Standard solution preparation

2 mg of gallic acid, apigenin, luteolin and 1 mg of costunolide were precisely weighed to an accuracy of 0.001 g and placed in 25 mL volumetric flask. The mixed stock solution was prepared in 25 mL of methanol and filtered through a 0.45 μL filter.

Chromatographic procedures

C18 column (250 mm x 4.6 mm x 5 μm, Alltima) was used as stationary phase. Acetonitrile (A) and doubly distilled water (B) adjusted to pH 2.0 with 85% orthophosphoric acid were used as mobile phase with gradient system: 0-15 min, 95-65% B; 15-20 min 65%B; 20-30 min, 65-60% B; 30-35 min, 60% B; 35-40 min, 60-50% B; 40-52 min, 50-30% B; 52-60 min, 30-95% B; 60-65 min, 95% B. The total runtime: 65 min; flow rate: 0.5 mL/min; column temperature: room temperature; injection volume: 20 μL; UV detection wavelength: 210 nm.[12-14]

HPLC identification of muscone

Muscone standard solution

10 mg of a muscone reference substance was accurately weighed and put it in a 10.0 mL volumetric flask and dissolved in a small amount of absolute ethanol. Absolute ethanol was added to the nominated

volume to prepare the solution with the concentration of 1 mg/mL of muscone. 0.1 mL of the prepared solution of muscone was precisely measured and placed in a 5 mL volumetric flask and added with 4 mL of 0.2% DNPH and put in a constant temperature water bath at 65°C for 30 min to allow the reaction. The solution was taken out, cooled at room temperature, added DNPH solution to the nominated volume and shaken well and filtered through a 0.45 μL filter.

Derivatization reagents

200 mg of 2-dinitrophenylhydrazine was accurately weighed, and placed in a 100 mL brown volumetric flask. 100 mL of anhydrous ethanol solution (containing 1.25% hydrochloric acid, V/V) was added and the mixture was well shaken, and placed at 8°C for 24 hours in the dark, and filtered with 0.45 μm microporous membrane for a final concentration of about 2 mg/mL.

Preparation of sample solution

100 mg Khurtsiin deed-6 traditional medicine was precisely weighed to an accuracy of 0.001 g and placed in a 5 mL volumetric flask and added with 4 mL of 0.2% DNPH and put in a constant temperature water bath at 65°C for 30 min for the reaction, and taken out and cooled at room temperature. DNPH solution was added to the nominated volume and shaken well. The appropriate amount was taken from it and centrifuged at 13000 r/min for 10 min. The supernatant was taken and filtered through 0.45 μm filter membranes before being injected into the HPLC instrument.

Chromatographic conditions

Column: Alltima C18 5 μm; 4.6×250 mm (Alltech); mobile phase: acetonitrile-water (90:10); flow rate: 1 mL/min; column temperature: 30°C; detection wavelength: 365 nm; injection volume: 20 μL. The theoretical number of plates of muscone was 4,000 or more, and the degree of separation was greater than 4.[15]

HPLC identification of Hydroxysafflor yellow A

Chromatographic conditions

Column: Shimpack C18 5 μm; 4.6×250 mm; mobile phase: methanol, acetonitrile, and 0.7 % phosphate acid solution (26:2:72); flow rate: 1 mL/min; detection wavelength: 403 nm; injection volume: 20 μL.

Reference solution

A quantity of hydroxysafflor yellow A CRS was accurately weighed, dissolved in 25% methanol to produce a solution of 0.13 mg per mL.

Preparation of sample solution

0.4 g of Khurtsiin deed-6 traditional medicine was accurately weighed in a conical flask with a stopper and 50 mL of 25% methanol was added. The mixture was accurately weighed, put in ultrasonic bath with 40°C temperature for 40 minutes, and allowed it to cool and weighed again, replenished the loss of the weigh with 25% methanol, and mixed well. After filtering, the successive filtrate was used as the sample solution.

Quantitative UV/VIS spectrophotometric determination

Sample preparation

1 g traditional medicine was weighed to an accuracy of 0.001 g and extracted with 50 mL of 70% ethanol. After the extraction, the sample was filtered and the filtrate was transferred to a 100 mL volumetric flask and diluted with 70% ethanol to 100 mL.

Determination of total flavonoid content

3 mL of the extracts of the sample were accurately transferred to a volumetric flask (25 mL) and 3 mL distilled H_2O and 1 mL of 5% $NaNO_2$ were added. After standing for 6 min, 1 mL of the 10% $Al(NO_3)_3$ solution and 10 mL of 4% NaOH solution were added and then diluted with distilled water to the volume and the absorbance was measured using a spectrophotometer (UV-M51, UV/VIS, Italy) at 500 nm. Total flavonoid content was calculated as μg/g of rutin equivalent from the calibration curve using the equation: $Y = 0.0416x + 0.007$, $R^2 = 0.999$, where x is the absorbance and Y is the rutin equivalent.[16]

Determination of total phenolic compounds content

Folin-Ciocalteu reagent was used for analysis of total phenolic compounds content. 10 mL of the extract was transferred to 50 mL standart volumetric flask and added ethanol to the nominated volume to obtain 2 mg/mL solution. 0.5 mL aliquot from this solution was mixed with 10 mL distilled H_2O and 1 mL Folin-Ciocalteu reagent (diluted 1:10 in water), and 13.5 mL of 10.75% Na_2CO_3. After 30-40 min, the absorbance was measured at 620 nm. Results were expressed as μg/g of gallic acid equivalent using the calibration curve: $Y = ax–b$, R^2, where x is the absorbance and Y is the gallic acid equivalent. [17]

Validation of the developed UV-Spectrophotometric method for the quantitative determination of gallic acid

ICH guideline were followed for the validation of developed methods with precision, repeatability, accuracy, LOD, and LOQ.

Selectivity and specificity

The selectivity and specificity of the developed method to measure gallic acid in the in Khurtsiin deed-6 traditional medicine with 6 ingredients were defined.

Linearity

Preparation of stock solution (300 μg/mL)

An accurately weighed 15 mg of standard gallic acid was transferred to 50 mL volumetric flask and 30 mL 70% ethanol was added to it. The solution was shaken for 5 minutes to solubilize the compound and final volume was made up to the nominated volume with 70% ethanol.

Preparation of working standard solution

Appropriate aliquots were withdrawn from the stock solution and diluted up to 25 mL with 70% ethanol to obtain standard solutions of different concentrations (12, 36, 60, 84 and 108 μg/mL). 0.5 mL of each of those solutions were mixed with 10 mL distilled H_2O and 1 mL Folin-Ciocalteu reagent (diluted 1:10 in water), and 13.5 mL of 10.75% Na_2CO_3. After 30-40 min, the absorbance was measured at 620 nm. Calibration curve was generated by taking the absorbance verses concentration.

Precision

The precision of the method was determined by inter-day and intra-day precisions.

Inter-day precision

Aliquots of 0.25, 0.5 and 0.75 mL of working standard solution (2 mg/mL) were transferred to 25 mL volumetric flask and volume was adjusted to 10.25, 10 and 9.75 mL with distilled H_2O and 1 mL Folin-Ciocalteu reagent (diluted 1:10 in water), and 13.5 mL of 10.75% Na_2CO_3 to get concentration of 20, 40 and 60 μg/mL. The absorbance of solutions was measured spectrophotometry for three times and % RSD was calculated. For inter-day, the analysis was carried on different three days in a week.

Intra-day precision

Aliquots of 0.25, 0.5 and 0.75 mL of working standard solution (2 mg/mL) were transferred to 25 mL volumetric flask and volume was

adjusted to 10.25, 10 and 9.75 mL with distilled H_2O and 1 mL Folin-Ciocalteu reagent (diluted 1:10 in water), and 13.5 mL of 10.75% Na_2CO_3 to get concentration of 20, 40 and 60 µg/mL. The absorbance of solution was measured spectrophotometry three times and %RSD was calculated. For intra-day, the analysis was carried out at different intervals on the same day.

Accuracy

For the accuracy of proposed method, recovery studies were performed by standard addition method at three different levels (80%, 100% and 120% of final concentration). A known amount of standard gallic acid was added to preanalyzed tablet powder and the sample was then analyzed by proposed method.

Statistical analysis

The data were reported as the mean ± standard deviation. All experiments were repeated six times, and the concentration of flavonoid and total phenolic compounds in a sample were determined using linear regression analysis. Analyzes were performed using SPSS Statistics 20.0 software.[18]

Ethical statement

The study protocol was approved by the Research Ethics Committee of the Mongolian National University of Medical Sciences (№2020/3-03).

RESULTS

Microstructural study

Microstructures of four medicinal plants of Khurtsin Deed-6 traditional medicine and this traditional medicine were defined and compared. The plant ingredients can be identified in Khurtsin Deed-6 traditional medicine by the following anatomical characterizations: *Odontites vulgaris* can be identified by the hairs on the leaves surface, epidermis, stem sclerenchyma and xylem cells of vascular bundle of leaves; *Terminalia chebula* by the fruit epidermis, *Carthamus tinctorius* by the structure of the flower petals cells, epidermis cells of the columnar androecium, and *Saussurea lappa* L. by the epidermis cells of the rhizome (Figure1).

TLC fingerprinting

TLC fingerprinting of Khurtsiin deed-6 was developed in hexane-ethyl acetate-formic acid (10:5:1 v/v) solvent system. Seven spots (all dark bands) with Rf values in the range of 0.18-0.92 were observed under 254 nm and nine spots with Rf values in the range of 0.19-0.93 were visualized under 366 nm (Figure 2). Chromatographic bands corresponding to standards of gallic acid (Rf 0.27), costunolide (Rf 0.71), apigenin (Rf 0.46) and E-guggulsterone (Rf 0.5) were observed in the extract of Khurtsiin deed-6 traditional medicine (Figure 2).

HPLC analysis

Standard and Sample solutions were prepared according to the Section of preparation of sample solution and standard solution, and 20 µL of each of them were injected into the HPLC system according to the chromatographic conditions given in Section of chromatographic procedures, and the chromatograms were recorded. The retention times were 10.66, 31.52, 35.03 and 55.82 min for gallic acid, luteolin, apigenin and costunolide, respectively (Figure 3). The chromatogram of the traditional medicine indicated the presence of gallic acid with the retention time at 10.64 min, luteolin at 31.52 min, apigenin at 35.30 min, and costunolide at 55.82 min compared with their standard substances (Figure 4), and *Terminalia chebula*, *Carthamus tinctorius* L, *Saussurea lappa* L, *Odontites vulgaris,* and *Commiphora wightii* were identified in Khurtsiin deed-6 traditional medicine according to the retention times of above-mentioned compounds contained in those medicinal plants (Figure 5).

Standard and Sample solutions were prepared as mentioned in the Section of sample solution and muscone standard solution preparation, and 20 µL of each of them were injected into the HPLC system according to the chromatographic conditions given in Section of chromatographic conditions, and the chromatograms were recorded. The retention time was 19.57 min for muscone. The chromatogram of the traditional medicine indicated the presence of muscone with the retention time at 19.51 min, compared with their standard substances, and Musk was identified in Khurtsiin deed-6 traditional medicine according to the retention time of the muscone contained in this raw material (Figure 6).

The retention time was 6.35 min for hydroxysafflor yellow A. The chromatogram of the traditional medicine indicated the presence of hydroxysafflor yellow A with the retention time at 6.33 min, compared with standard substance, and *Carthamus tinctorius* was identified in Khurtsiin deed-6 traditional medicine according to the retention time of the hydroxysafflor yellow A contained in this raw material (Figure 7).

Total phenolic and flavonoid contents

The total phenolic content of Khurtsiin deed-6 traditional medicine, calculated from the calibration curve, was 65.95±1.05 mg/g expressed as gallic acid equivalents, and the total flavonoid content was 10.55±0.16 mg/g expressed as rutin equivalents (Table 1).

Method validation

The UV-Spectrophotometric method for the Estimation of gallic acid was developed and validated according to ICH Q2 (R1) guideline (Table 2).

Specificity

According to ICH Q2 (R1) guideline, specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Results of specificity are shown in Figure 8.

Linearity

According to ICH Q2 (R1) guideline, linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample. Results of linearity are shown in Figure 9.

Precision

Based on the ICH Q2 (R1) guideline, precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at two levels: repeatability and intermediate precision. Results of precision (repeatability and intermediate precision) are shown in Tables 3 and 4.

Accuracy

ICH Q2 (R1) guideline specified that the accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. Results of accuracy are shown in Table 5.

Quality and safety parameters

Quality and safety parameters of Khurtsin deed-6 traditional medicine were defined according to Mongolian National Standard

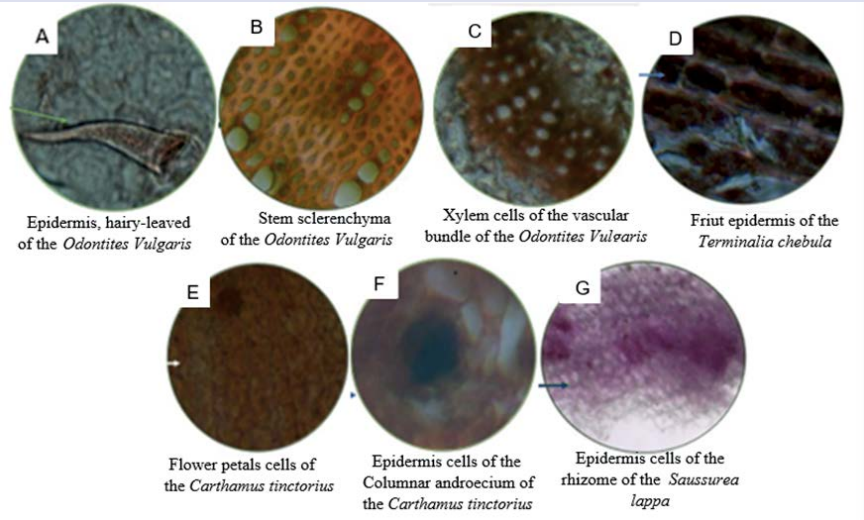

Figure 1: Microscopic analysis of *Odontites vulgaris* (A, B, C), *Terminalia chebula* (D) *Carthamus tinctorius* (E, F), *Saussurea lappa (G).*

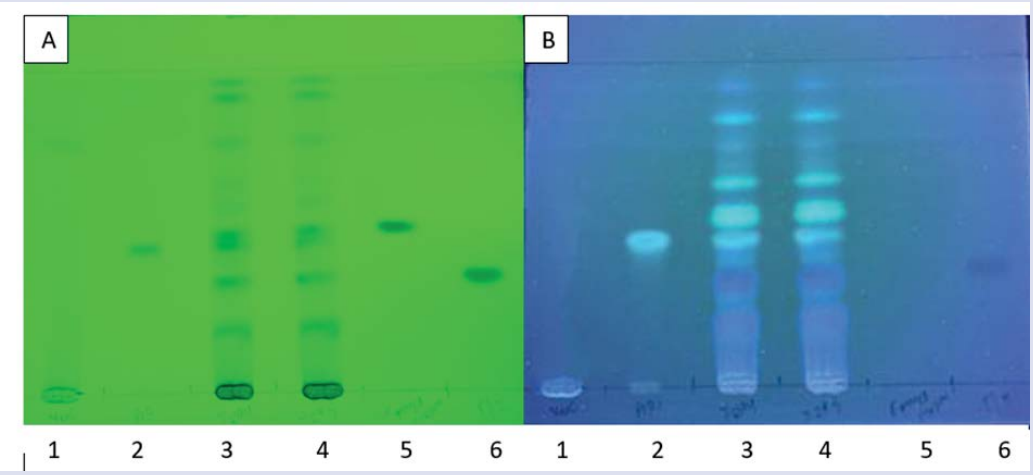

Figure 2: TLC fingerprint of extract of Khurtsiin deed-6 traditional medicine. 1. Costunolide standard, 2. Apigenin standard, 3-4. Extracts of Khurtsiin deed-6 traditional medicine, 5. E-guggulsterone standard, 6. Gallic acid standard; A. UV 254 nm, B. UV 365 nm.

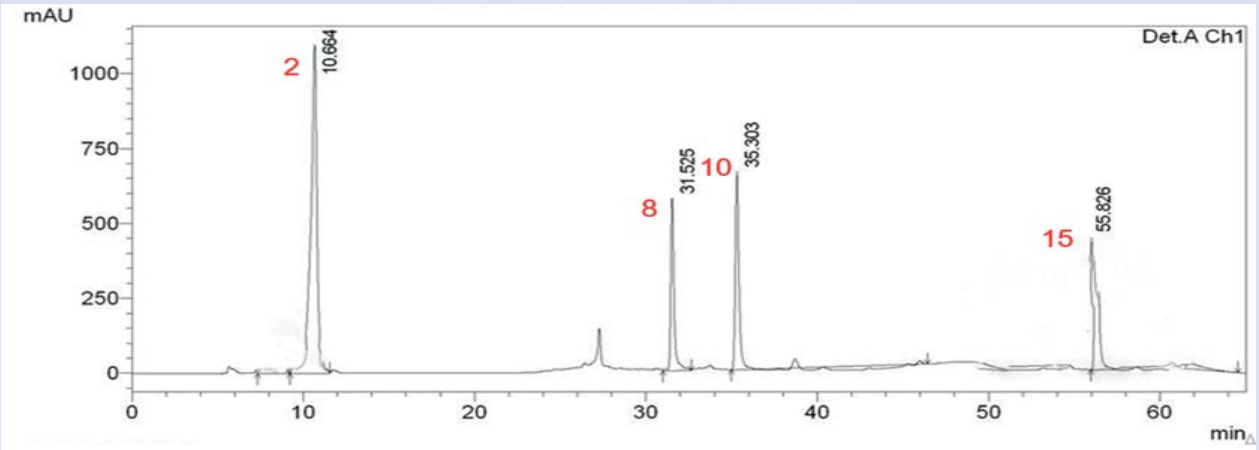

Figure 3: HPLC Chromatogram of standard solution.

Peaks: 2. Gallic acid, 8. Luteolin 10. Apigenin, 15. Costunolide

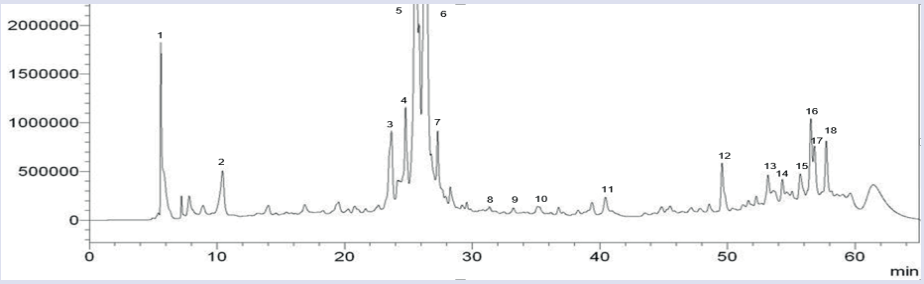

Figure 4: Chromatogram at 210 nm of the methanol extract of Khurtsiin deed-6.

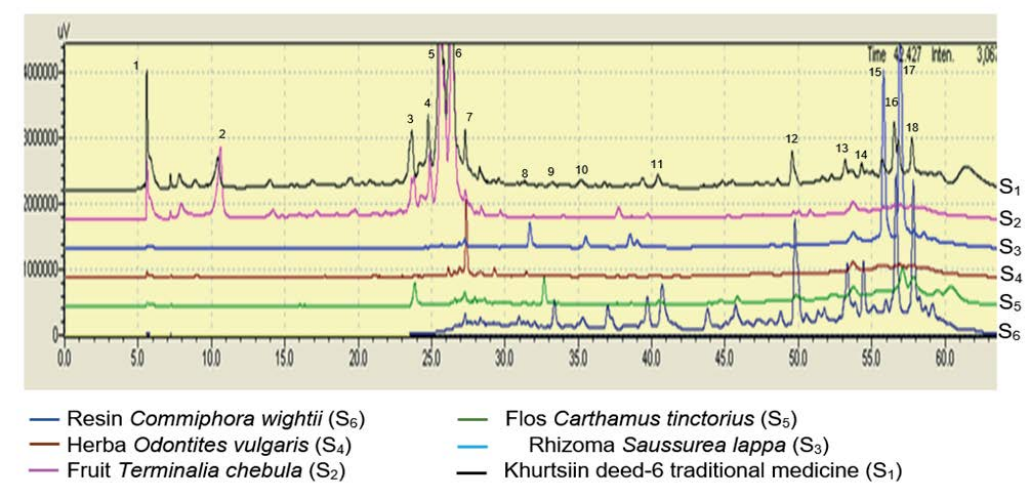

Figure 5: Comparison of Raw materials of each medicinal plant and Khurtsiin deed-6 traditional medicine's samples.

Peaks: 2. Gallic acid, 8. Luteolin 10. Apigenin, 15. Costunolide

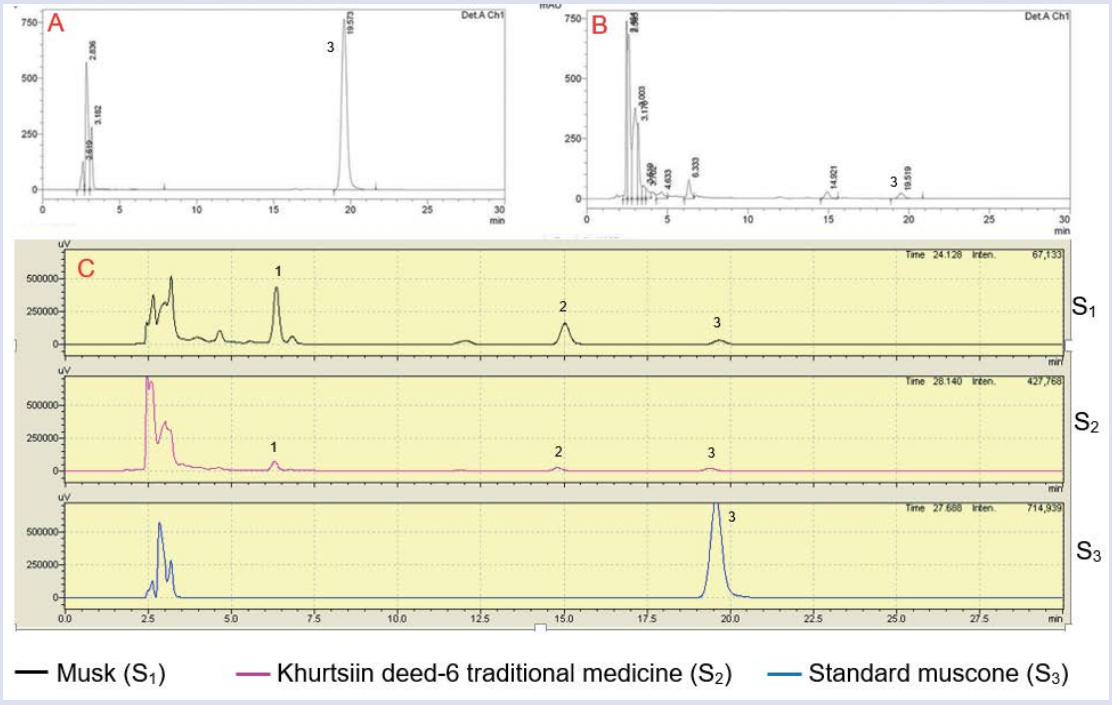

Figure 6: HPLC Chromatograms

A. Chromatogram of Standard solution. Peaks: 3. Muscone, B. Chromatogram at 365 nm of the ethanol extract of Khurtsiin deed-6, C. Comparison of Raw materials of Musk and Khurtsiin deed-6 traditional medicine's samples.

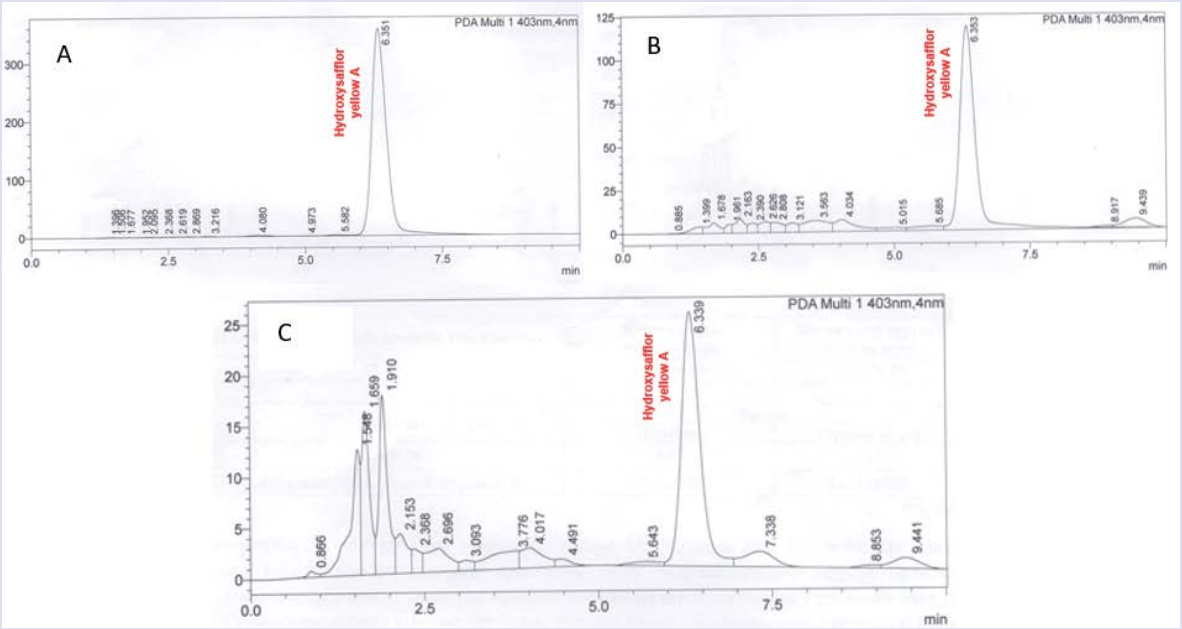

Figure 7: HPLC Chromatograms

A. Chromatogram of Standard solution. Peaks: Hydroxysafflor yellow A, B. Raw materials of *Carthamus tinctorius,* C. Chromatogram at 403 nm of the methanol extract of Khurtsiin deed-6 traditional medicine.

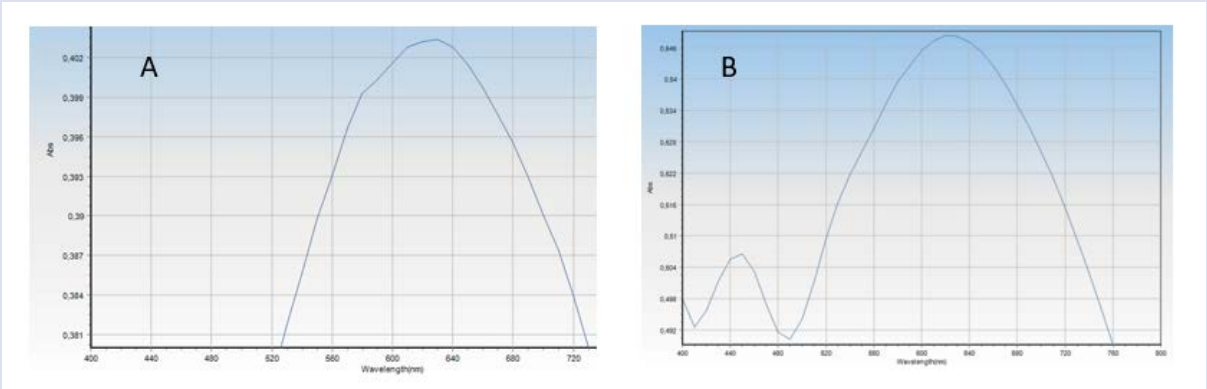

Figure 8: Absorption of standard solution (A) and sample solution of Khurtsiin deed-6 traditional medicine (B) in the UV range from 400 to 800 nm.

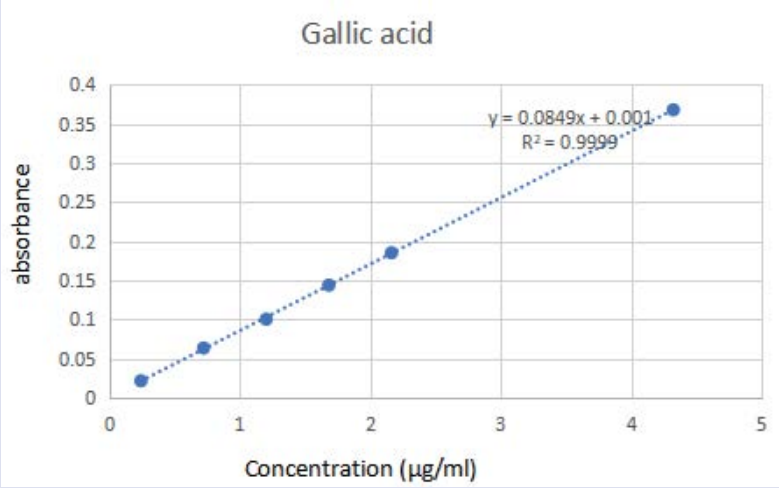

Figure 9: Calibration curve of gallic acid**.**

Table 1: Total phenolic and flavonoids contents in Khurtsiin deed-6 traditional medicine.

Biologically active compounds	Standard reagent	Standard curve equation	Content, mg/g	RSD, %
Total flavonoids	Rutin	Y = 0.0416x + 0.0071, r^2=0.999	10.55 ±0.16	1.51
Total phenolic compounds	Gallic acid	Y=0.0849x – 0.001, r^2=0.999	65.95±1.05	1.59

Table 2: Summary of validation parameters of the proposed UV method.

Parameters	Results
Absorption maximum (λ_{max})	620
Beer's law limit (µg/mL)	0.24-4.32
Regression equation (Y=ax+b)	$Y = 0.0849x - 0.001$
Slope	0.0849
Intercept	0.001
Coefficient of correlation	0.999
Accuracy (% RSD)	98.58-101.35, % RSD 0.54-1.18%
Precision (% RSD)	Inter-day = 1.04-1.75
	Intra-day = 0.46-1.42

Table 3: Repeatability (intra-day precision) of the proposed UV method.

Repeatability (intra-day precision) (n=3)		
Sample conc (µg/mL)	Absorbance at 620 nm (Mean±SD)	%RSD
20	0.1162±0.0009	0.77
40	0.2191±0.001	0.46
60	0.3274 ± 0.004	1.42

Table 4: Intermediate precision (Inter-day precision) of the proposed UV method.

Intermediate precision (Inter-day precision) (n=3)		
Sample conc (µg/mL)	Absorbance at 620 nm (Mean±SD)	%RSD
20	0.1187 ± 0.002	1.75
40	0.2175 ± 0.0022	1.04
60	0.3301 ± 0.0034	1.05

Table 5: Accuracy studies of the proposed UV method.

Level (%)	Amount of gallic acid in the sample (µg/mL)	The added amount of gallic acid standard (µg/mL)	Predicted concentration (µg/mL)	Observed concentration (µg/mL)	Recovery, %	Mean recovery, %	% RSD
	2.6	0.44	3.04	3.03	99.92		
80%	2.6	0.44	3.04	2.99	98.41	99.69	1.18
	2.6	0.44	3.04	3.06	100.73		
	2.6	1.2	3.8	3.86	101.66		
100%	2.6	1.2	3.8	3.87	101.97	101.35	0.82
	2.6	1.2	3.8	3.81	100.42		
	2.6	1.96	4.56	4.47	98.15		
120%	2.6	1.96	4.56	4.48	98.41	98.58	0.54
	2.6	1.96	4.56	4.52	99.18		

Table 6: Quality and safety parameters of Khurtsiin deed-6 traditional medicine.

№	Parameters		Results
1	Organoleptic		Color: Brownish-yellow Odor: distinctive odor Taste: Bitter
2	Moisture content		9.63±0.09%
3	Total ash		4.725±0.22%
4	Water-soluble extractive value		21.28±0.11%
5	Alcohol soluble extractive value		46.30±0.09%
6	Heavy metals:	Lead	0.2 mg/kg
		Cadmium	<0.1 mg/kg
7	Total aerobic microbial count		$5x10^3$
8	Total yeast and mold count		$3x10^2$
9	Bile-tolerant Gram-negative bacteria		Absent
10	*Staphylococous aureus*		Absent
11	E.*coli*		Absent

MNS5586:2006 "General requirements for traditional powder medicines" and Mongolian National Pharmacopeia, and the results are shown in Table 6.

DISCUSSION

Khurtsin Deed-6 traditional medicine is composed of six ingredients including rhizome of *Saussurea lappa* L, herba of *Odontites vulgaris*, and flower of *Carthamus tinctorius* L, the fruit of *Terminalia chebula* Retz, *Commiphora wightii,* and musk.[3] One of the quality indicators of medicinal plants and traditional powder medicines is the determination of microstructure based on anatomical characterization. Although the anatomical structure of each plant have been determined by the previous studies[19-22], there was a need to determine the specific anatomical characterization of those plants which will be used for the identification of them in Khurtsin Deed-6 traditional medicine. The microstructures of four medicinal plants, the rhizome of *Saussurea lappa* L, herba of *Odontites vulgaris*, and flower of *Carthamus tinctorius* L, the fruit of *Terminalia chebula* Retz, and Khurtsiin Deed-6 traditional medicine were studied on this purpose with the specific anatomical characterization. The microstructures of musk, because of animal raw material, and *Commiphora wightii* resin, because it easily loses its shape when grinding, were not possible to be determined. The hairs on the leaves surface, epidermis, stem sclerenchyma, and xylem cells of the vascular bundle of leaves of *Odontites vulgaris,* fruit epidermis of *Terminalia chebula*, flower petals cells, epidermis cells of the columnar androecium of *Carthamus tinctorius*, and the epidermis cells of the rhizome of *Saussurea lappa* L. are specific anatomical characterizations of those plants to be used for the identification of each plant ingredient in Khurtsin Deed-6 traditional medicine.

Quality consistency is one of the basic attributes of medicines, but it is also a difficult problem of natural medicines, especially with multicomponent content. A combination of several herbs might give rise to interactions with hundreds of natural constituents during the preparation of extracts, the fingerprints produced by the chromatographic instruments, which may present a relatively good integral representation of various chemical components of herbal medicines. TLC is a convenient method of determining the quality and possible adulteration of herbal products and TLC fingerprint is being widely used for quality control of herbal medicines because of its simplicity, versatility, high velocity, specific sensitivity and simple sample preparation.[23]

Biologically active substances in each plant were identified by other studies, but in this study, for the first time, biologically active substances in Khurtsiin Deed-6, a 6-ingredient traditional medicine, were identified and the study to develop Fingerprints of Khurtsiin Deed-6 traditional medicine was conducted because TLC and HPLC fingerprints have not been studied.

The main compositions of Khurtsiin Deed-6 traditional medicine are *Odontites Vulgaris* Moench and *Carthamus tinctorius* L, and they contain apigenin, flavonol-O-glycoside (hyperoside, isoquercetin), flavonoids (quercetin, kaempferol, rutin) and *Carthamus tintorius* L. also contains hydroxysafflor yellow A.[4,24,25]

The rhizome of *Saussurea lappa* L. contains biologically active compounds such as sesquiterpene, costunolide, and dihydrocostulactone.[26] Some study results of *Saussurea lappa* L. showed that costunolide can be an inhibitor of NO, iNOS, and TNF-a.[27]

The one ingredient of Khurtsiin Deed-6 traditional medicine is the musk of Musk deer, and it contains muscone, cholestanol, cholesterol, 5α-Androstane-3,17-dione, 5β-Androstane-3, 17-dione, 3α-Hydroxy-5α-androstan-17-one, 3β-Hydroxy-androst-5-en-17-one, 3α-Hydroxy-5β-androstan-17-one, 3β-Hydroxy-5α-androstan-17-one, Androst-4-ene-3,17-dione, Androsta-4, 6-diene-3,17-dione, 5α-Androstane-3β,17α-diol, 5β-Androstane-3α, 17β-diol, 5β-Androstane-3α,17α-diol, 17β-hydroxyandrost-4-ene-3-one, 2,6-nonamethylene pyridine, 2,6-decamethylene pyridine, hydroxymuscopyridine A, hydroxymuscopyridine B, wax, aliphatic long-chain alcohols, free fatty acids, and alkanes.[28] The musk of Musk deer raw materials are standardized with muscone in the Chinese pharmacopeia.[29]

The fruit of *T.chebula* is rich in hydrolyzable tannins (20-50%) including gallic acid, ellagic acid, chebulic acid and gallotannins such as 1,6 di-O-galloyl-β-D-glucose, 3,4,6 tri-O-galloyl-β-D-glucose, 2,3,4,6 tetra-O-galloyl-β-D-glucose, 1,2,3,4,6 penta-Ogalloyl-β-D-glucose.[30] The main components of *Commiphora wightii* are resins: Z-guggulsterone, E-guggul sterol, and guggul sterols and volatile oils: myrtle, myrcene, and polymyrcene. [31]

Our findings have established TLC and HPLC fingerprints to reveal apigenin, costunolide, gallic acid, and E-guggulsterone of 4 medicinal plants contained in Khurtsiin Deed-6 traditional medicine and this result will be applied for the quality control of this traditional medicine. *Terminalia chebula* raw materials are standardized with gallic acid in Chinese pharmacopeia.[29] Gallic acid, a biologically active substance with antioxidant properties, was revealed by TLC (Rf 0.27) and HPLC (10.64 min) methods in Khurtsiin Deed-6 traditional medicine, but this result differs from the result of the detection of gallic acid (Rf 0.58) in the Lider-7 traditional medicine[19], which contains the *Terminalia chebula* raw material, due to the use of solvent systems with different polarities in the two studies. E-guggulsterone of a resin of *Commiphora wightii* was detected in Khurtsiin Deed-6 traditional medicine with TLC result (Rf 0.5) and this result is approximate with the result of the detection of E guggulsterone (Rf 0.52) in the *Commiphora wightii* are resins.[32]

Apigenin of *Odontites vulgaris* Moench and *Carthamus tinctorius* L. was detected by TLC (Rf 0.46) and HPLC (RT 35.03 min), muscone of musk was detected by HPLC (RT 19.51 min) in Khurtsiin Deed-6 traditional medicine. TLC (Rf 0.71) and HPLC (RT 55.82 min) results indicated the presence of costunolide in *Saussurea lappa* L. and Khurtsiin Deed-6 traditional medicine and these results were the same as the study results of Gardi-5 traditional medicine.[33]

To develop HPLC fingerprint, it is critical to define a favorable mobile phase system, gradient elution system, and detection wavelength to obtain efficient separation of the numerous target components. The suitable condition of HPLC method was investigated by checking peak resolution and purity of Khurtsiin deed-6 traditional medicine. Different mobile phases of acetonitrile-water, methanol-water, methanol-water containing phosphoric acid at different concentrations, and acetonitrile-water with phosphoric acid at different concentrations were tested. Better resolution and reproducibility of fingerprint chromatograms were under the conditions of Section Chromatographic Procedures.

The peaks 1, 2 (gallic acid), 3, 5 and 6 belong to *Terminalia chebula*, 7, 8 (luteolin) and 10 (apigenin) belong to *Odontites vulgaris*, 3 and 9 belong to *Carthamus tinctorius*, 8, 15 (costunolide) and 17 belong to *Saussurea lappa* and the peak 9, 11, 12, 16 and 18 belong to *Commiphora wightii* (Figure 5).

The suitable condition of the HPLC method was investigated for the determination of muscone in samples by pre-column derivatization HPLC with 2, 4-Dinitrophenyl hydrazine (DNPH). The peaks 1, 2, and 3 (muscone) belong to Musk (Figure 6). According to The Chinese Pharmacopoeia the muscone content of natural musk must be not less than 2%.[29]

Carthamus tinctorius raw material is standardized with hydroxysafflor yellow A in Chinese pharmacopeia[29] and Hydroxysafflor yellow A was revealed by HPLC (6.33 min) method in Khurtsiin Deed-6 traditional medicine by this study.

The practical value of the standardization study of medicines is to improve to the quality and safety control of them, and for this purpose quality and safety parameters of Khurtsiin Deed-6 traditional medicine with the quantitative determination of biologically active compound content were determined and on the result of this study National Pharmacopeia Monograph for Khurtsiin Deed-6 traditional medicine was developed.

T.chebula raw materials have been found to contain large amounts of organic acids such as polyphenolic compounds, including gallic acid and chebulic acid, and *C.tinctorius* and *O.vulgaris* contain flavonoid, and the total content of phenolic compounds was defined as 65.95±1.05 mg/g and the total flavonoid content was defined as 10.55 ±0.16 mg/g in Khurtsiin deed-6 traditional medicines for the first time.

The UV-Spectrophotometric method for the quantitative determination of gallic acid was validated according to ICH guideline. The method showed good linearity ($r > 0.999$) in the concentration range (0.24-4.32 µg/ml). The limits of detection and quantification for the compounds were found to be 0.144 µg/ml and 0.43 µg/ml, respectively. Percentage recovery studies were performed for 80%, 100%, and 120% respectively, and percentage recovery of gallic acid was found to be between 98.58-101.35% and the relative standard deviation was found less than 2%. The developed method of determining the gallic acid content in the Khurtsiin deed-6 traditional medicine is valid.[34,35] The contents of biologically active compounds in traditional medicines are incomparable, because of ingredient difference and raw material proportions in the traditional medicine. The chemical compositions of herbal plants also may vary depending on the species, location of growth, age, harvesting season, drying processes, geographical regions, and some other factors.[36]

The study results show Khurtsiin deed-6 traditional medicine could alleviate the migraine-like headache induced by Nitroglycerine, which is related to the regulation of vasoactive substances.[37] Mansour Rezaei investigated the effect of Sodae herbal capsule, a product contains *Terminalia chebula*, Turpethum, Bdellium, Rhubarb, and Eyaraj fighara, for the treatment of migraine, and the study results confirmed the effectiveness of these plants in reducing the frequency of migraine attacks.[38] *Saussurea lappa* has been shown an anti-migraine effect and it decreases calcium, magnesium, serotonin in serum and increases phosphorus, and alkaline phosphatase concentrations in serum.[39] According to Arman Zargaran's study, apigenin showed the anti-migraine effect.[40] The research results show Mongolian Badanga musk has activity of protecting neuron cells, supporting neurogenesis against inflammation, improving neuro tissues regeneration, reducing ischemic areas through increasing brain tissues Arg-1 and BCL-2 protein's expressions, and reducing Iba-1 proteins expressions.[41]

These studies may prove the usage of Khurtsiin deed-6 traditional medicine for the treatment of bacterial fever headaches, bloody yellow headaches, and headaches caused by yam disease in traditional medicine, and this traditional medicine can be possible to be used to treat Migraines and Alzheimer's disease.

The moisture, ash, and water-soluble substances, microbiological purity, and heavy metal contamination of Khurtsin Deed-6 traditional medicine were determined and the National Pharmacopeia Monograph of this traditional medicine was developed and approved.

CONCLUSION

The standardization criteria for Khurtsiin deed-6 traditional medicine were defined and Mongolian National Pharmacopeia Monograph's draft for Khurtsiin deed-6 traditional medicine was developed and approved.

CONFLICTS OF INTEREST

The authors have declared no conflicts of interest.

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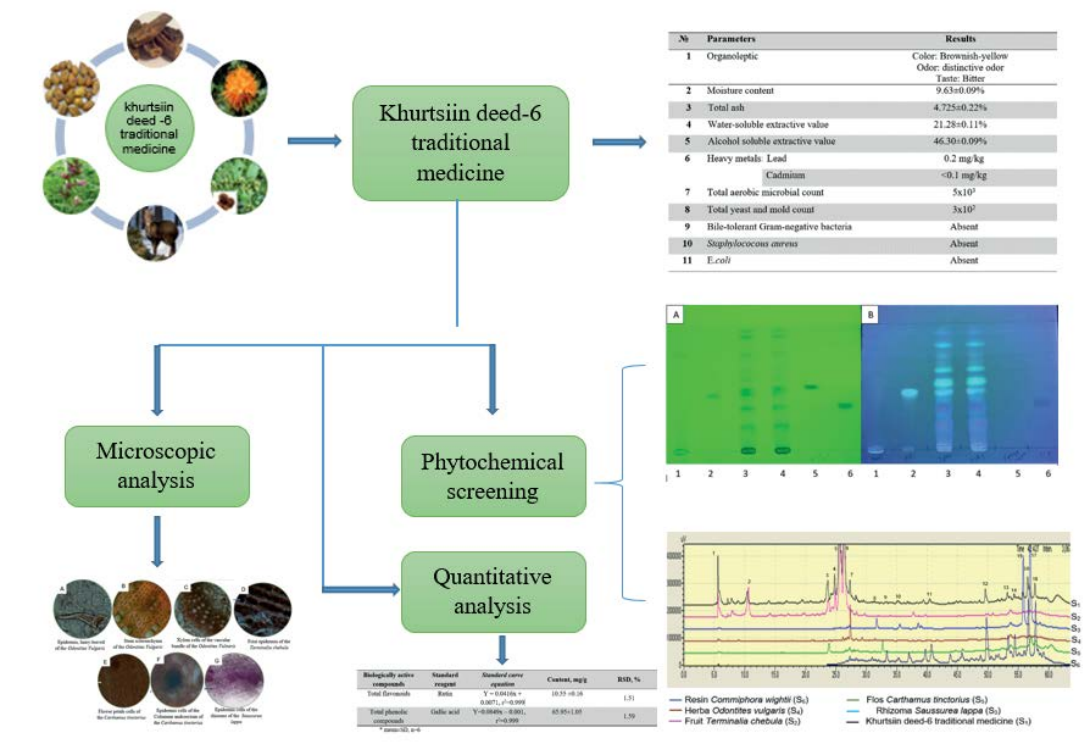
GRAPHICAL ABSTRACT



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11	*E.coli*	Absent

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