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Results of Standardization and Antipyretic Effect studies of the Musk Raw Material

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Abstract— Musk is a biological secretion with a unique odor produced by the *Moschus moschiferus* Linnaeus. Although musk was used as an anti-inflammatory, antibacterial, analgesic, antipyretic, anti-ischemic, and anti-infectivein Traditional Mongolian Medicine, it has not been standardized until now. This study aimed to conduct a standardization study with focus on the determination of the quality and safety parameters of musk and evaluate the antipyretic effect of the musk by yeast-induced model of fever. The musk was extracted from the domesticated musk deer from the Musk Deer Breeding Center of the ITMT was used in this study. The content of the muscone in the natural musk was determined by HPLC method. Mice were fevered by injecting 20 mg/kg of Brewer's yeast subcutaneously. Then, rectal temperature was recorded by thermometer for six (6) hours. In this study, the musk reduced the body temperature significantly after yeast injection compared to the control group (*$p<0.01$). Suitable conditions of HPLC revealed the presence of muscone in natural musk and the content of muscone in natural musk was determined at 0.31±0.005%. The quality and safety parameters of natural musk were determined: moisture content is at 24.88±0.85%, total ash at 2.62 ± 0.32% while aerobic microbes, yeast, and mold were not detected in the Musk. The standardization indicators of natural musk were defined and the Mongolian National Pharmacopeia Monograph's draft for natural Musk was developed.

Keywords: Moschus moschiferus L, Muscone, HPLC, Quality parameter, Antipyretic

1. Introduction

The Siberian musk deer (*Moschus moschiferus* Linnaeus) from the genus Moschus has a glandular sac between the genitals and umbilicus with a size of 4-6 cm, which produces biological greasy secretion with a unique odor. This secretion obtained from the sac is called the musk[1]. It is not only one of the most famous and precious ingredients in Traditional Mongolian Medicines (TMM), but also an important raw material in the perfumery industry over the world. The musk has been mainly used to treat white channel disorder for thousands of years in TMM and 573 of about 4,000 TMM prescriptions contain musk according to the studies of Boldsaikhan B and et al (1993)[2]. Among them, Gardi-5, Gardi-13, Baragshun-9, Agar-35, Bontag-25, Managchenmo, Sugmel-10, Senden-25, Zachun-13, Saffron-13, Sariichun, Sampilnorov, and

Notsog-6 prescriptions are widely used in TMM[3]. Musk is one the intriguing subjects for researchers and several studies on the chemical composition and pharmacological effects of musk were conducted. Studies found that the musk of Musk deer mainly contains macrocyclic ketones, pyridine, steroids, fatty acids, amino acids, and proteins, whilst the main active ingredient is muscone[4]. Musk demonstrates wide pharmacological effects such as but not limited to the reduction of inflammation, acceleration of respiratory rate, enhancement of heartbeat, elevation of blood pressure and correction of physiological disorders in patients who need resuscitation[1, 5].Modern pharmacological studies have proven that musk and muscone, the main active ingredient of musk, possess potent anti-inflammatory, neuroprotective, anticancer, antioxidant and other pharmacological effects[6]. Since 1989, several research on musk, musk milking methods and its pharmacological effects were conducted in Mongolia. In traditional medicine, musk has been used as an anti-inflammatory, antibacterial, analgesic, antipyretic and an anti-ischemic agent for centuries[2]. Studies have proven its traditional use of anti-inflammatory, antibacterial, analgesic and anti-ischemic effects, but the antipyretic use of musk in TMM is still needed to be proven.

Siberian musk deer (*Moschus moschiferius* L), the sole species of musk deer in Mongolia,is distributed on the upper, northern slopes of the Khentii and Khuvsgul mountains and along the tops of the Khangai and Khankhukhii mountains ranges[1]. For over a thousand years, musk has been obtained by hunting wild musk deer, but this practice threats to the species and to a valuable natural resource. Therefore, new methods were adopted to the keep the musk deer from becoming extinct. In addition to strengthening protection measures against possible damage to wildlife, Mongolia haset up musk-deer farms in the area Khentii Mountain since 1989. The collection of musk from domesticated musk deer could at the same time encourage the protection of musk deer population and their habitat[7].

Standardization of herbal and animal formulation is essential in order to assess the quality, purity, safety and efficacy of drug which is based on amount of their active constituents[8, 9].

Musk deer is domesticated in Mongolia[7] and it is necessary to standardize this raw material since it is included in many complex traditional medicine prescriptions. For these reasons, we aimed to study the antipyretic effect and determine the quality and safety parameters of the musk of *Moschus moschiferus***.**

2. Material and methods

2.1 Animal material

The musk extracted from the domesticated 4-year old musk deer (*Moschus moschiferus* L) from the Musk Deer Breeding Center of the Institute of Traditional Medicine and Technology (ITMT) was used for this study**.**

2.2 Standards and Chemicals

Reference muscone from Sigma Aldrich (USA) and Brewer’s yeast from Now Foods., (USA) were used for the study.All other reagents and solvents were of analytical grade.

2.3 Physical methods

Foreign matter percentage, total ash, and moisture were determined by physical method.

2.4 Chemical method

2.4.1 HPLC identification of muscone

Muscone standard solution: 10 mg of a muscone reference substance was accurately weighed and put 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 minutes 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

10 mg musk of *Moschus moshiferius* 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. The mixture was put in a constant temperature water bath at 65°C for 30 minutes 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 the solution and centrifuged at 13000 r/min for 10 minutes. 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[10].

2.4.2 Method validation

The HPLC method was validated as per ICH guidelines. System suitability tests were carried out on freshly prepared standard muscone by injecting six replicate and % relative standard deviation (%RSD) of peak area were determined. The linearity of the developed method was evaluated according to the correlation coefficient (r^2) of the calibration curve of each standard compound using eight serial concentrations of 0.4-30μg/mL. Inter-day and intra-day precisions were

determined by analyzing the sample solution with one concentration on five (5) consecutive days, respectively. Accuracy of the analytical method was studied by recovery experiments. LOD & LOQ were calculated from the calibration curve. The LOD was calculated as 3.3σ/s and LOQ was 10σ/s respectively, where s=slope of the calibration curve and σ= standard deviation of the response[11-14].

2.4 Pharmacology method

2.5 Suspension preparation

The sample used for the study of an antipyretic effect of the musk was prepared from the dried musk, ethanol 96% and desiccated goat liver. The dried musk was mixed with 96% ethanol and desiccated goat liver in a proportion of 1:5:49 by weight, and the mixture was milled into a fine powder. Then, a 10% suspension of musk was prepared in 1% of carboxymethyl cellulose (CMC).

Experimental animals

Male adult laboratory non-breed mice (healthy, 25-30 g) were randomly selected from The Animal House of the Research Center Institute of Traditional Medicine and Technology, Ulaanbaatar. The mice were kept in the laboratory under the constant condition of light/dark (12:12) and temperature (20±2°C) in an animal cage with libitum feedingof a standard animal diet and tap water for 7 days, before and during the experiment. The experiment protocols were approved by the Ethical Committee of the Ministry of Health to minimize an animal's suffering.

Yeast induced pyrexia mice model

In this experiment, the animals were randomly divided into four groups. Briefly, pyrexia was induced by injecting 20 mg/kg of Brewer's yeast suspension subcutaneously in the back behind the neck except for the normal group (pyrogen-free 0.9% NaCl). The rectal temperature was recorded by a thermometer in the rectum. The rise in rectal temperature was recorded for 17 hours since injecting Brewer's yeast suspension. The experiment groups were treated by oral administration with Musk of *Moschus moschiferus* (MM) at 118 mg/kg.The comparative group received Aspirin at a dose of 90 mg/kg. Mice groups administrated with saline were set as normal and control groups[15-20].

2.6 Statistical analysis

The data were shown as the mean ±SD. An analysis of variance (TWO-way ANOVA followed by Tukey's post hoc test) was performed to determine significance using software Graph Pad Prism 7.0 and values of *$p<0.05$ and **$p<0.01$ were considered significant.

3. Results

3.1 Chemical study

Standard and sample solutions were prepared as mentioned in the Section on sample solution and muscone standard solution preparation. 20 µL of each of them were injected into the HPLC system according to the chromatographic conditions given in the Section on chromatographic conditions and the chromatograms were recorded. The retention time was 19.57 minutes for muscone. The chromatogram of the musk indicated the presence of muscone with a retention time of 19.66 minutes compared with their standard substances.

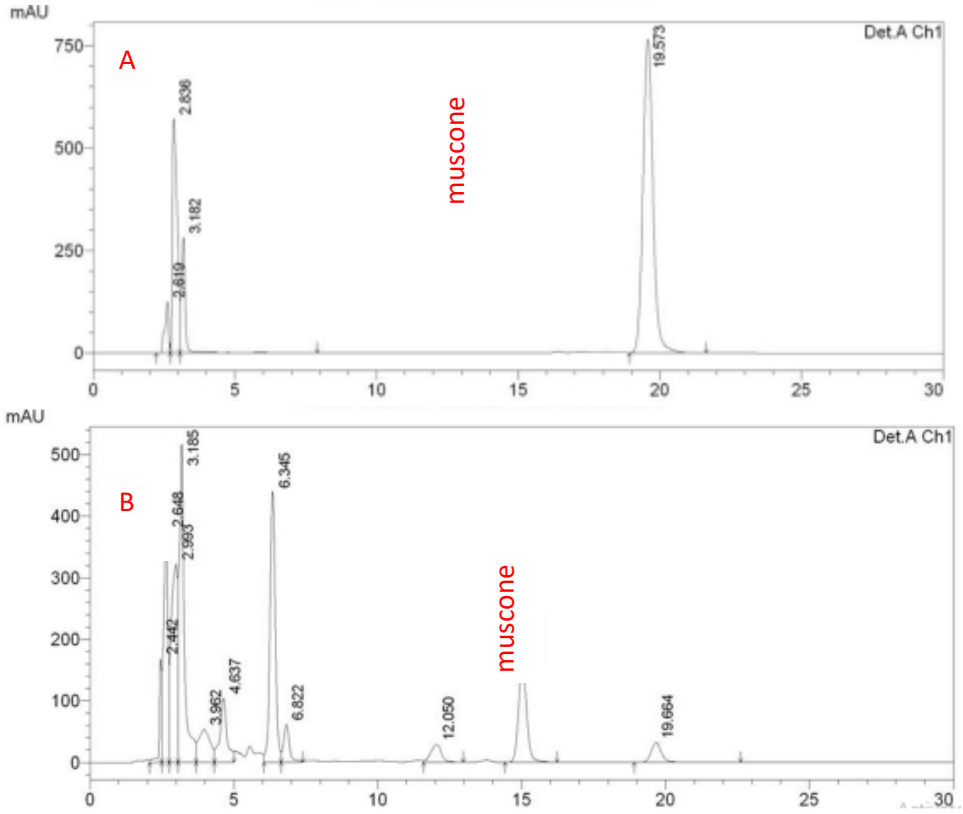

Figure 1: HPLC Chromatograms
Chromatogram of standard solution of muscone. B. Chromatogram at 365 nm of the ethanol extract of Musk.

The regression equation obtained from the calibration curve ($Y = 127913x - 3390.4$) can be used to determine the muscone content in the musk (Figure 2). From the results of these calculations, it can be seen that the muscone content in the musk of *Moschus Moschiferus*is 0.31±0.005%.

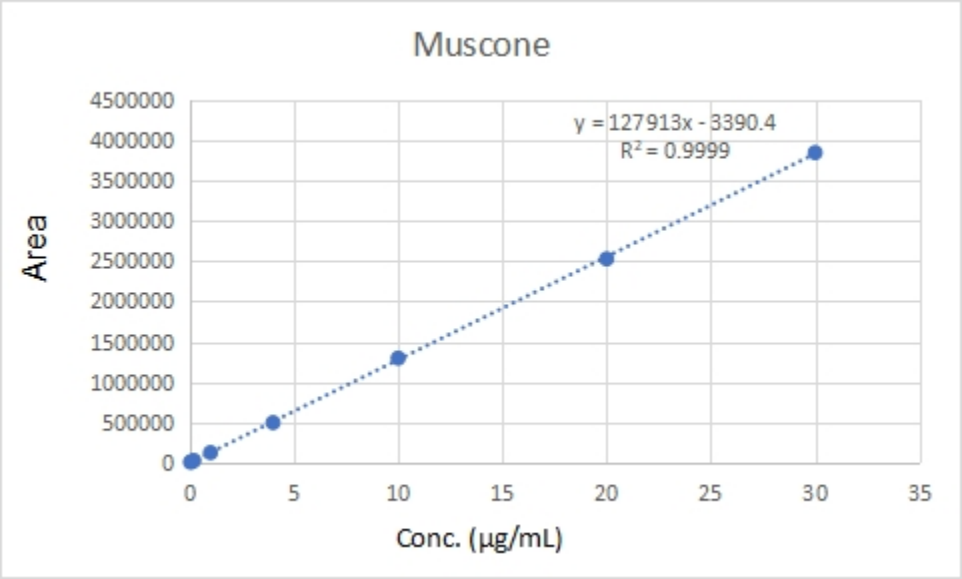

Figure 2: Calibration curve of muscone

3.2 Method validation

The HPLC method for the Estimation of muscone was developed and validated according to ICH Q2 (R1) guideline (Table 1).

Table 1: Validation parameters of the proposed HPLC method

Parameters	**Results**
Retention time	19.36 min
Beer's law limit (µg/mL)	0.4-30
Wavelength	365 nm
Regression equation (Y=ax+b)	$Y = 127913x - 3390.4$
Slope	127913
Intercept	-3390.4
Coefficient of correlation(r^2)	0.999
Limit of detection (LOD)	0.44 µg/mL
Limit of quantification (LOQ)	1.33 µg/mL
Accuracy (% RSD)	98.37-100.32%, % RSD 0.57-1.32%
Precision (% RSD)	Inter-day = 0.75
	Intra-day = 1.6

3.3 Quality and safety parameters

The quality and safety parameters of the musk of *Moschus moschiferius*were defined according to Mongolian National Pharmacopeia and the results are shown in Table 2.

Table 2: Quality and safety parameters musk of *Moschus Moschiferius*

№	Parameters	Analytical methods	Results
1	Appearance(taste, odor, color)	Sensory method	The powder is usually brown or yellowish-brown, purplish-black, odor, characteristic and strongly aromatic, taste, slightly pungent, bitter and salty
2	Quality analysis	HPLC	19.6 min
3	Foreign matter		Absent
4	Loss on drying	Weight method	24.88±0.85%
5	Total ash	Weight method	2.62 ± 0.32%
6	Muscone	HPLC	0.31±0.005%
7	Total aerobic microbial count	Microbiological quality	absent
8	Total yeast and mold count		absent
9	Escherichia coli		absent
10	Salmonella spp		Absent

3.4 Pharmacology study

The effect of the musk of *Moschus moschiferius* (MM) on body temperature using yeast-induced pyrexia mice model was investigated. The line graph illustrates that in the control group, body temperature was significantly increased by subcutaneous injection of yeast during the time period, and it reached a peak within 19 h (37.92 ± 0.66^0C) compared to the normal group (35.42 ± 0.35^0C). In the treatment group, rectal temperature was significantly reduced by Musk of MM at the dose of 118 mg/kg from the onset of the pyrexia for up to 6 hours after treatment. It decreased at 35.86±0.11 at 17 hours and 35.7±0.31 at 18 hours respectively (**$p<0.01$). Also, Aspirin (90 mg/kg) administrated by the oral group, significantly reduced (**$p<0.01$) by subcutaneous injection of yeast all over the period.

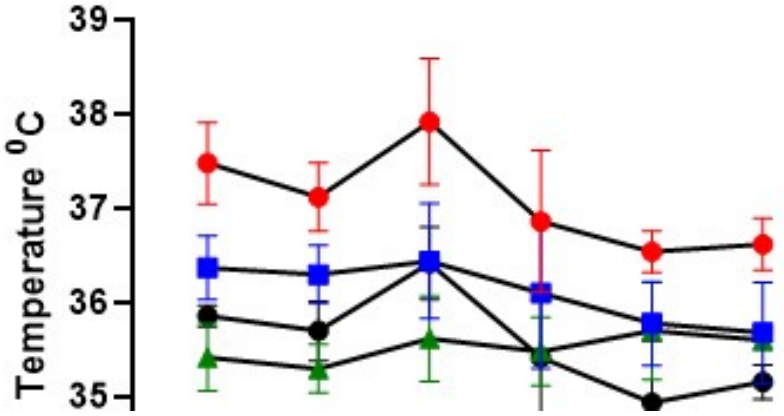

Figure 3: The musk of MM attenuates yeast induced increase in rectal temperature

4. Discussion

Traditional medicine contains many natural ingredients and their safety, therapeutic activity and quality need to be studied and validated. Musk of musk deer is still widely used in traditional medicine as a raw material.

The musk of musk deer 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. Musk's main active ingredient is muscone[6] and musk of musk deer is standardized using the Chinese pharmacopeia[21]. Muscone is highly volatile and has poor UV absorption, which limits the application of the commonly used HPLC method with a UV detector. The methods for the determination of muscone could be classified into two categories: the indirect methods with derivatization of muscone (pre or post column derivatization) and the direct methods without derivatization. The methods used for the direct determination of muscone are GC, GC/MS with refractive index detector (RI) and the GC method is well established. LC/MS provides a higher selectivity and sensitivity and gives unambiguous identification of muscone. While the LC/MSand GC methods may work well for the identification and quantification of the wide range of muscone levels, the equipment is relatively expensive and may not be available in every laboratory[22].

2.4-Dinitrophenylhydrazine (DNPH) is a commonly used derivatization reagent for aldehydes or ketones. Its derivatized product, phenylhydrazine, has a good UV response at around 360 nm. Muscone is a natural 3-methylcyclopentadecanone that can be combined with DNPH. The quantitative conversion relationship between the product muscone benzoquinone and the reaction substrate muscone can be used for HPLC quantitative determination of muscone. In the previous study, we optimized the appropriate conditions for the DNPH derivatization reaction of muscone[10]and for this study, we validated a method to determine the muscone content in the

musk by HPLC. In the validation procedure, typical validation characteristics such as precision, selectivity, linearity, and accuracy were evaluated. The precision was evaluated by carrying out six independent assays. The RSD of the intra-day precision was 1.6% and within the acceptable limit of 2.0%. The intermediate (inter-day) precision was 0.75%, within the acceptable limit of 2.0%[12]. Linearity regression data show a good linear relationship between concentration and peak area over a concentration range of 0.4-30µg/mL for muscone (Fig 1). The correlation coefficient was found to be 0.999. Percentage recovery studies were performed for 50%, 100%, and 150% respectively, and percentage recovery of muscone was found to be between 98.37-100.32% and the relative standard deviation was found less than 2%.[11, 12]. The developed method satisfies the acceptance criteria and ensures the accuracy of the method. LOD and LOQ were found to be 0.44µg/mL & 1.33µg/mL, respectively.

The content of muscone in natural musk obtained from the domesticated musk deer in Mongolia was determined as 0.31±0.005%. According to The Chinese Pharmacopoeia, the muscone content of natural musk must be not less than 2%[21]. Muscone content in musk may vary depending on the species, age, drying processes, geographical regions,different genetic sources, processing methods, storage time, and some other factors[22]. The moisture, ash, and microbiological purity of Musk of *Moschus moschiferius* L were determined and the National Pharmacopeia Monograph of this traditional medicine was developed and approved[23].

The musk has many beneficial effects such as immunity-enhancing, antibacterial, and anti-inflammatory effects. In addition, musk has significant effects on the central nervous system (CNS) that protects neurons, supports neurogenesis against inflammation, reduces ischemic areas and improves nervous tissues regeneration[24]. Fever is complex physiologic response triggered by infection due to the illness or some reasons. Elevation of the body temperature occur when level of prostaglandin (PGE) increases within certain area of the brain. These elevations alter the rate of neurons that control thermoregulation in the hypothalamus[25-27].Result of the present study demonstrate that musk of *Moschus moschiferus* low dose of 118 mg/kg alleviated fever of yeast-induced pyrexia in mice. Yeast-induced fever is called pathogenic fever by increasing the synthesis of prostaglandin and several cytokines[15, 20]. In the present study, we guess that Musk of *Moschus moschiferus* significantly reduced the rectal temperature of yeast-induced mice because of attenuated prostaglandin(PGE).

5. Conclusion

The standardization criteria for Musk of *Moschus moschiferius* were defined and the Mongolian National Pharmacopeia Monograph's draft for Musk of *Moschus moschiferius* was developed and approved. In addition, the musk of *Moschus moschiferus* has shown antipyretic effects yeast induced fever at a low dose of 118 mg/kg.

6. Conflict of Interest

The authors have declared no conflict of interest.

7. Acknowledgements

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