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Research Article

Analysis of active components of rhinoceros, water buffalo and yak horns using two-dimensional electrophoresis and ethnopharmacological evaluation

Cornu Rhinoceri Asiatici (rhinoceros horn, RH), *Cornu Bubali* (water buffalo horn, WBH), and *Cornu Bovis grunniens* (yak horn, YH) are traditional Chinese medicine (TCM), and have been used in China for thousands of years. In this study, ethnopharmacological experiments were used to evaluate and verify the traditional efficacies of horns. Area under curve (AUC) was used to quantify the pharmacological efficacy strength of three horns. Two-dimensional electrophoresis (2-DE) was used to analyze the protein components in horns, as a result, 14 common protein spots in rhinoceros horn, water buffalo horn, and yak horn electrophoresis gels were found by image analysis. Then linear regression analysis was used to establish the correlation between pharmacological efficacies and components in the horns, and five potential active components were selected from the 14 common protein spots. Finally, two protein spots from five were identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). In this study, a simple method to construct correlation between components and efficacy strength was explored by linear regression analysis, which could be applied to screen potential active components of animal horns.

Keywords: Animal horn-derived traditional Chinese medicine / Linear regression analysis / Pharmacological evaluation / Two-dimensional electrophoresis
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1 Introduction

Horns are pointed projections of the skin or hair on the head of animals, consisting of a covering of horn sheath surrounding a core of living bone. The horn-derived traditional Chinese medicine (TCM) such as *Cornu Rhinoceri Asiatici* (rhinoceros horn, RH), *Cornu Bubali* (water buffalo horn, WBH), and *Cornu Bovis grunniens* (yak horn, YH) were used in Chinese medicine for dispelling heat, counteracting toxins, and relieving convulsions [1, 2]. RH was first documented in *Shennong Bencao Jing* (about 200 B.C. to 200 A.D.) [1]. WBH was first documented to have medicinal use in *Ming Yi Bie Lu*, which was completed in the *Han* dynasty (about 220 A.D.). YH had been used in traditional Tibetan medicine for thousands of years, which was recorded in *Bencao Gangmu* (about 1590 A.D.).

From the 1970s, rhinoceros were listed by the Convention on International Trade in Endangered Species (CITES) of wild animals and plants and therefore RH was forbidden to be used in China [3]. In order to reduce the dependence on RH in Chinese medicine, researchers have started to look for other horns that could substitute for RH. Different animal horns have been investigated to determine their inorganic elements, amino acids constituents, and pharmacological properties in recent decades [4]. As a result, WBH and YH were shown to be similar to RH in all the aspects described above [5, 6]. Besides, WBH is currently widely used in China due to its abundant resources, low price, and eutherapeutic effects. Thus, WBH was used as a substitute for RH in clinical applications and produced satisfactory therapeutic efficacy, which was documented in the Chinese Pharmacopoeia in the 2010 Edition [7].

Based on the documented efficacy, ethnopharmacological experiments including antipyretic activity, spontaneous activity, and procoagulant activity test were used to evaluate and verify the biological efficacies of the three animal horns in this study. Despite their long use history, the components of horn-derived TCM are still unclear.

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Abbreviations: AUC, area under curve; **CT,** clotting time; **NCBInr,** non-redundant National Center for Biotechnology Information database; **RH,** rhinoceros horn; **TCM,** traditional Chinese medicine; **WBH,** water buffalo horn; **YH,** yak horn

Three bioactive peptides have been ever purified from aqueous extraction of WBH by different chromatographic methods in our previous study [8]. However, these peptides might be derived from the hydrolysis of original proteins, which could not represent the actual original components of horn.

It has been reported that animal horns consist of keratin and other soluble proteins; keratins are resistant to digestion by the pepsin or trypsin and are insoluble in dilute acids, alkalines, water, and organic solvents [9, 10]. As keratins in horns are particularly rich in cystine residues, which can provide disulfide cross-links between adjacent polypeptide chains, such cross-links are covalent and thus are very strong. The soluble proteins of horns could be dissolved only under harsh extraction procedure or enzymolysis conditions, which might destroy the original type of components in horns. Therefore, it is difficult to obtain the actual information of components in horns. Based on these facts, two-dimensional electrophoresis (2-DE) was chosen to analyze animal horns for the first time. 2-DE was a powerful tool for separating complex proteins [11–13], and could obtain the nearly full information in horns when combined with isoelectric focusing and SDS-PAGE in two directions without proteins degradation. The 2-DE lysis buffer could extract the soluble proteins from horns, which contain chaotropic compounds, detergents, surfactants, reducing agents, and carrier ampholytes, and could obtain sufficient proteins for the reasons as follows [14, 15]. The role of chaotropes (urea and thiourea) was to disrupt hydrogen bonding, leading to protein unfolding and denaturation; surfactants (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) and SDS) act synergistically with chaotropes. Reducing agents (DTT) were used to break disulfide bonds; carrier ampholytes could enhance protein solubility by charge–charge interactions [14, 16].

In this study, 2-DE was used to analyze the original components of the horns, and the main proteins of horns can be easily obtained by 2-DE analysis. Thereafter, based on the majority components information of the horns in electrophoresis gels, linear regression analysis was applied to establish correlation between the components and the therapeutic effects of horns. Thus, we can elucidate the possible components having therapeutic effects according to the coefficient of correlation. Furthermore, potential active component was identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) and non-redundant National Center for Biotechnology Information database (NCBInr) database.

2 Materials and methods

2.1 Reagents and materials

RH was made available from State Forestry Administration of China (2006, No. 1093), and identified as horn of *Rhinoceros sumatrensis* Fischer. WBH was collected from Wharf town of Huaian city, Jiangsu province, China, and YH was collected from Tibet, China. Horns were authenticated by Dr. Jin-ao Duan and were then pulverized into fine powder.

Paracetamol, estazolam, and aminomethylbenzoic were purchased from Baijingyu Pharmaceutical Co. Ltd., Nanjing, China. All 2-DE reagents were obtained from Amersham Biosciences, GE, USA. Other reagents were of analytical grade.

2.2 Animals

Adult New Zealand rabbits (2.0–2.5 kg) and mice (18–20 g) of both sexes were obtained from the Center of Experimental Animals, China Pharmaceutical University. Animals were housed under standard conditions of temperature ($22 \pm 2^{\circ}C$); relative humidity ($55 \pm 5\%$) and light (12 h light/dark cycles) were used. The animals were fed with standard diet and water ad libitum. Animal welfare and experimental procedures were strictly in accordance with the Guide for the Care and Use of Laboratory Animals (US National Research Council, 1996) and the related ethics regulations of our University.

2.3 Pharmacological experiment

2.3.1 Antipyretic activity

The antipyretic activity test was performed according to the method of Backhouse with slight modification [17]. Antipyretic activity was determined in rabbits using six animals of both sexes for each group. Initial rectal temperatures were recorded using a digital clinical thermometer (OMRON, Japan), and the thermometer was inserted about 3 cm into the rectum of each rabbit. Rabbits presenting an initial rectal temperature between 38.5 and 39°C were selected for the antipyretic tests. Hyperthermia was induced by i.v. injection of *Escherichia coli* endotoxin at a dose of 20 ng/kg. Rectal temperature was measured 6 h after the endotoxin injection, and rabbits developing only significant hyperthermia were used. Then the rabbits were orally administered with horn powder, and their negative control (saline), positive control (paracetamol at 20 mg/kg body weight), and rectal temperatures recorded at 15, 30, 45, 60, 90, 120, and 180 min after drug treatment.

2.3.2 Sedative activity

The test was performed according to the method of Connor with slight modification [18]. This test measuring exploration and voluntary locomotion within an enclosed area was used to evaluate the sedative activity of the horns. Objective values for spontaneous motor activity were obtained with a photoactometer. Mice were placed individually in a black chamber with a screen floor and a light-tight lid. Six beams of red light were focused 2 cm above the floor onto

photocells on the opposite side. Each beam interruption registered as an event on an external counter. The floor of the chamber was wiped clean with 5% v/v alcohol before each use. The mice were orally administered with horn powder, their negative control (saline), and positive control (estazolam at 0.5 mg/kg body weight). Mice were placed in the chamber 30 min after oral administration. They were allowed to acclimate for 2 min, and then light beam breaks were counted for the next 2 min.

2.3.3 Procoagulant activity

The blood clotting time (CT) experiment was performed according to the method of Singh to evaluate the procoagulant activity of horns, with slight modification [19]. Mice were divided into six groups of ten animals each. The mice were orally administered with horn powder, their negative control (saline), and positive control (4-(aminomethyl)benzoic acid at 36.7 mg/kg body weight) once a day for 3 days. One hour after last oral administration, blood samples were taken with the help of a glass capillary from orbital plexus of the eye of each mouse and the time was noted. Small pieces of capillary were broken from one end at every 15 s till fibrin threads of blood appeared between the broken ends of capillary.

2.4 2-DE analysis

2.4.1 Sample preparation

RH, WBH, and YH were solubilized in lysis buffer containing 7 M urea, 2 M thiourea, 2% m/v CHAPS, and 2% Pharmalyte pH 3–10. After 4 h of gentle stirring at room temperature, samples were centrifuged at 13 000 × *g* for 1 h and the supernatants were centrifuged at 13 000 × *g* for 20 min to remove precipitation. The protein concentration was determined by Bradford assay [20]. The protein samples were stored in 1.5 mL Eppendorf tubes in aliquots at −80°C until use.

2.4.2 2-DE

For 2-DE, proteins were solubilized in a rehydrated solution containing 8 M urea, 2% m/v CHAPS, 20 mM DTT, a trace of bromophenol blue, and 0.5% v/v immobilized pH gradient (IPG) buffer (pH 3–10) (Amersham Biosciences, Uppsala, Sweden). A total of 24 cm IPG strips (non-linear pH 3–10; Amersham Biosciences) were rehydrated overnight with 450 μL rehydrated solution and 80 μg proteins were loaded in IPG strips. Rehydration and isoelectric focusing were carried out using an IPGphor (Amersham Biosciences), with the following settings: 30 V for 6 h, 60 V for 6 h, 500 V for 1 h, 1000 V for 1 h, and 8000 V for 10 h (depending on the length and pH gradient of the IPG strips) at 20°C. After focusing, proteins were reduced by incubating the IPG strips with 1% m/v DTT and alkylated with 2.5% m/v iodoacetamide in 10 mL equilibration buffer (6 M urea, 30% m/v glycerol, 2% m/v SDS, and 50 mM Tris-HCl) with gentle shaking for 15 min. After equilibration, the IPG strips were blotted with filter paper for 1 min to remove excess equilibration solution and gently rinsed for 1 min using SDS electrophoresis buffer. Then, the IPG strips were transferred to 12.5% SDS-PAGE gels without a stacking gel for second-dimension electrophoresis with the Ettan DALT twelve gel system (Amersham Biosciences) using SDS electrophoresis buffer (250 mM Tris, pH 8.3, 1.92 M glycine, and 1% SDS). After the sealed agarose was cooled down, the second-dimensional SDS-PAGE was carried out (settings: step 1, 5 w/gel 30 min; step 2, 15 w/strip about 5 h) at 15°C until the dye front reached the bottom of the SDS-PAGE. After SDS-PAGE, the gels were fixed in 40% v/v ethanol and 10% v/v acetic acid for 2 h to remove any compounds (e.g. detergents) that might interfere with detection. For silver staining, gels were stained with a modified silver staining protocol as described previously without glutaraldehyde [21]. Two replicates were performed for each sample.

2.4.3 Image and data analysis

Gels were processed for image analysis of 2-DE protein patterns with scanners (Amersham Biosciences). Computerized 2-DE gel analysis was performed with IMAGE MASTER 2D ELITE 5.0 software package (Amersham Biosciences) for spot detection, spot editing, and pattern matching. The abundance of each protein spot was estimated by the percentage volume (the spot volumes were normalized as a percentage of the total volume in all the spots present in the gel to correct the variability because of loading and silver staining). The spots in common on three gels were selected.

2.5 Protein spots-efficacy analysis

To quantify the components and therapeutic effects of horns, value of abundance and area under curve (AUC) were introduced, respectively. Abundance represented the content of protein spots in gels. AUC was used to evaluate the effect and effective time in pharmacology experiments, which represented the efficacy strength of three horns. AUC was calculated by Graphpad Prism 5 software. Besides, linear regression analysis was used to establish the correlation between protein spots in the gels and efficacy of horns, and help to pick out potential active components.

2.6 In-gel digestion and MALDI-TOF MS analysis

Picked spots were washed with double-distilled water and then transferred to siliconized Eppendorf tubes. The digestion method was used according to the protocol described by Shevchenko et al. with minor modifications [21]. Briefly, the excised gel spots were destained and

dehydrated with acetonitrile (ACN). The proteins were reduced in 10 mM DTT/50 mM NH_4HCO_3 for 1 h at 56°C and alkylated in 55 mM iodoacetamide/50 mM NH_4HCO_3 for 1 h at room temperature. The gel pieces were washed several times in 50 mM NH_4HCO_3 followed by dehydration with ACN and dried in a Speed-Vac centrifugation. The proteins were digested with trypsin (10 ng/mL trypsin, 25 mM NH_4HCO_3, Promega, Madison, WI) at 37°C for 16–18 h. The digestion reaction was stopped by addition of 2 μL 10% v/v trifluoroacetic acid (TFA). The digestion products were desalted with a Millipore ZipTip C_{18} column. The resulting peptide mixtures were analyzed by MS. The samples were mixed on-target with an equal volume of the matrix solution, 10 mg/mL α-cyano-4-hydroxy-cinnamic acid (Bruker-Daltonics, Bremen, Germany) in 50% v/v ACN/0.1% v/v TFA, and spotting for MALDI-TOF MS was operated in the positive ion delayed extraction reflector mode for highest resolution and mass accuracy. Peptides were ionized with a 337-nm laser and spectra were acquired at 19 kV acceleration potential with optimized parameter. Calibration was carried out using a standard peptide mixture. The peptide spectra were automatically processed for baseline correction, noise removal, peak deisotoping, and threshold adjustment (2% base peak intensity) and collected from monoisotopic peaks in the mass-to-charge ratio (m/z) range of 500–3000 Da.

2.7 Database search

Peptide mass fingerprinting (PMF) was used for protein identification from tryptic fragment sizes by using the MASCOT search engine (http://www.matrixscience.com) base on the Swiss-Prot protein database, using the assumption that peptides are monoisotopic. Search criteria required the match of at least four peptides and the coverage of the matching peptides of a minimum of 15%. Monoisotopic masses were used and a mass tolerance of ±100 ppm was allowed. The maximum number of missed cleavages was set at one. Complete carbamidomethylation of cysteines and variable oxidation of methionines were assumed.

2.8 Statistical analysis

Effect values are expressed as mean ± SEM. They were further analyzed using one-way analysis of variance (ANOVA) test to calculate the significance of results. As $p < 0.05$ was considered as indication of significance.

3 Results

3.1 Pharmacological efficacy of three horns

According to Chinese Pharmacopoeia, dose of WBH was chosen as 1.5 g/kg for rabbit and 5.5 g/kg for mouse [7]. Besides, the dose of RH, WBH, and YH was selected as base on the protein concentration determined in Section 2.4.1.

As shown in Fig. 1, the antipyretic study results showed that the rectal temperatures in rabbits were significantly increased after injection of *E. coli* endotoxin, oral administration with RH, WBH, and YH caused significant lowering of body temperature, respectively. According to the calculation of AUC ($AUC_{0–180\,min}$ (RH) = 112.3, $AUC_{0–180\,min}$ (WBH) = 116.0, $AUC_{0–180\,min}$ (YH) = 128.0, $AUC_{0–180\,min}$ (Control) = 178.0, $AUC_{0–180\,min}$ (Paracetamol) = 149.4), the antipyretic efficacy strength order of three horns was RH > WBH > YH.

As shown in Fig. 2, oral administration with RH, WBH, and YH showed significant activity on decreasing mice voluntary movements. According to the calculation of AUC [$AUC_{0–120\,min}$ (RH) = 4168.5, $AUC_{0–120\,min}$ (WBH) = 4218.0, $AUC_{0–120\,min}$ (YH) = 4265.5, $AUC_{0–120\,min}$ (Control) = 5464.0, $AUC_{0–120\,min}$ (Estazolam) = 3412.5], the sedative efficacy strength order of three horns was RH > WBH > YH.

As summarized in Table 1, oral administration with RH, WBH, and YH could significantly decrease CT.

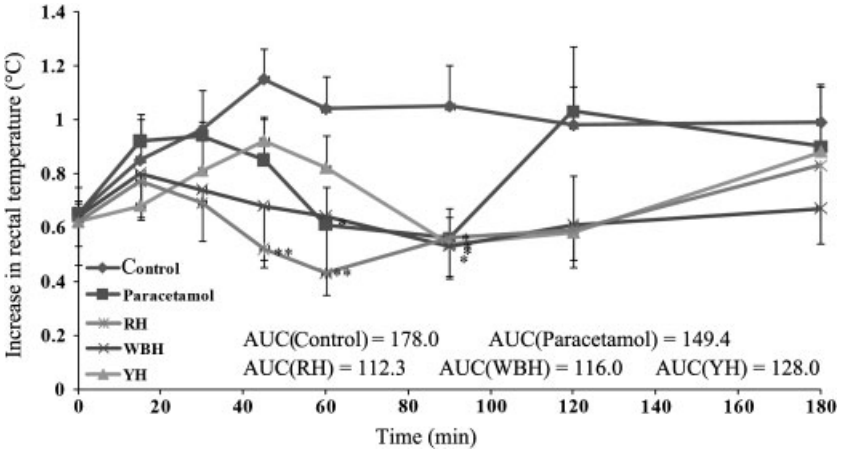


Figure 1. Effects of RH, WBH, YH, and paracetamol on endotoxin-induced hyperthermia in rabbits. Medicines were given (i.g.) 60 min after endotoxin injection. Values were expressed as mean ± SEM ($n = 6$). AUC was calculated by Graphpad Prism 5 software. *Significantly different from control ($p < 0.05$). **Significantly different from control ($p < 0.01$).

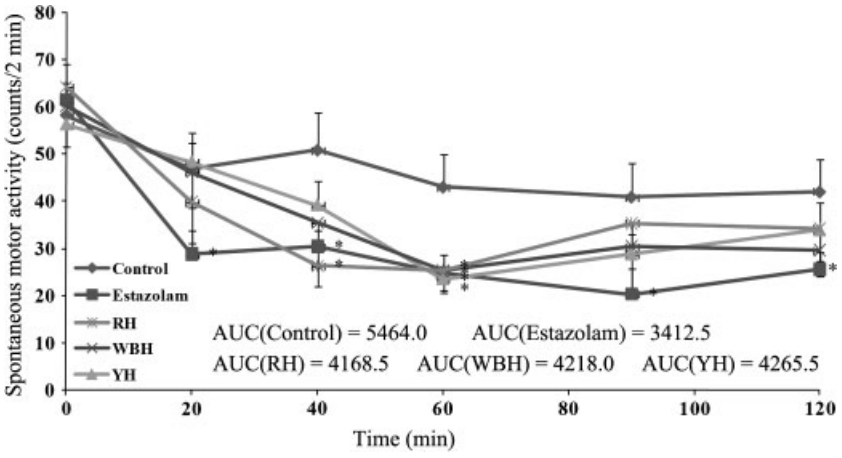


Figure 2. Effects of RH, WBH, YH, and estazolam given on spontaneous motor activity in mice. Values were expressed as mean ± SEM ($n = 10$). AUC was calculated by Graphpad Prism 5 software. *Significantly different from control ($p < 0.05$).

Table 1. Procoagulant effect of RH, WBH, YH, and 4-(aminomethyl)benzoic acid

Group	Blood CT (s)
Control	55.8 ± 4.9
4-(Aminomethyl)benzoic acid	42.4 ± 3.7*
RH	42.6 ± 3.7*
WBH	42.7 ± 2.4*
YH	43.4 ± 2.7*

Values were expressed as mean ± SEM ($n = 10$). *Significantly different from control ($p < 0.05$).

According to the results, although the procoagulant activity of three horns was similar, sequence of procoagulant activity absolute values was RH > WBH > YH.

Therefore, it can be elucidated that the pharmacological efficacy strength of three horns was diminished as the sequence of RH, WBH, and YH.

3.2 Image and data analysis

In our 2-DE image analysis, as shown in Figs. 3 and 4, 14 common protein spots existing in RH, WBH, and YH electrophoresis gels were found by IMAGE MASTER 2D ELITE 5.0 software.

3.3 Protein spots-efficacy analysis

Linear regression analysis with evaluation of the linearity of abundance–efficacy curves was drawn. For 14 common protein spots, linear regression analyzes the relationship between two variables, spot abundance, and efficacy. The goal of linear regression is to find the line that best-predicts spot abundance from efficacy, which means the spot abundance correlates with efficacy. For each ethnopharmacology experimental unit, the best straight line through the spots abundance and absolute values of efficacy should be found, respectively. The value *r* is a measure of goodness-of-fit of linear regression. According to the linear regression analysis, spot correlating with all three main efficacies of horn was picked. As shown in Fig. 5, the absolute *r*-value of spots 6, 7, 10, 11, and 14 was all larger than 0.9. It was showed that these five spots were more correlated with main efficacies compared with the other nine spots. The five spots might be the potential active components with therapeutic effects.

3.4 Efficacy-related protein spots identified by MALDI-TOF MS

Spots 6, 7, 10, 11, and 14 were picked from 14 spots, which were correlated with efficacies. Then five spots were excised repeatedly and digested with trypsin, and then analyzed by MALDI-TOF MS. The identities of five protein spots were determined by PMFs using PROFOUND and MASCOT search engine according to the similarity of sequences with the previously characterized proteins in the NCBInr database. As summarized in Table 2, spots 6 and 11 were identified as zinc finger CCCH-type containing 12D and peptide methionine sulfoxide reductase, respectively. The MALDI-TOF peptide mass map of spots 6 and 11 is shown in Figs. 6 and 7. Spots 7, 10, and 14 did not yield any match in the present protein NCBInr database, which might be identified by MS/MS in further experiments.

4 Discussion

Horn-derived TCMs are an indispensable part of TCM in China. Currently, more and more modern technologies are applied to investigate the mechanism of action and active components of horn-derived TCM. However, how to find the most effective components from animal horns is a difficult work. First, the features of horns make it difficult to find out a proper way to obtain the fingerprint of horns which include the information of components in horns as

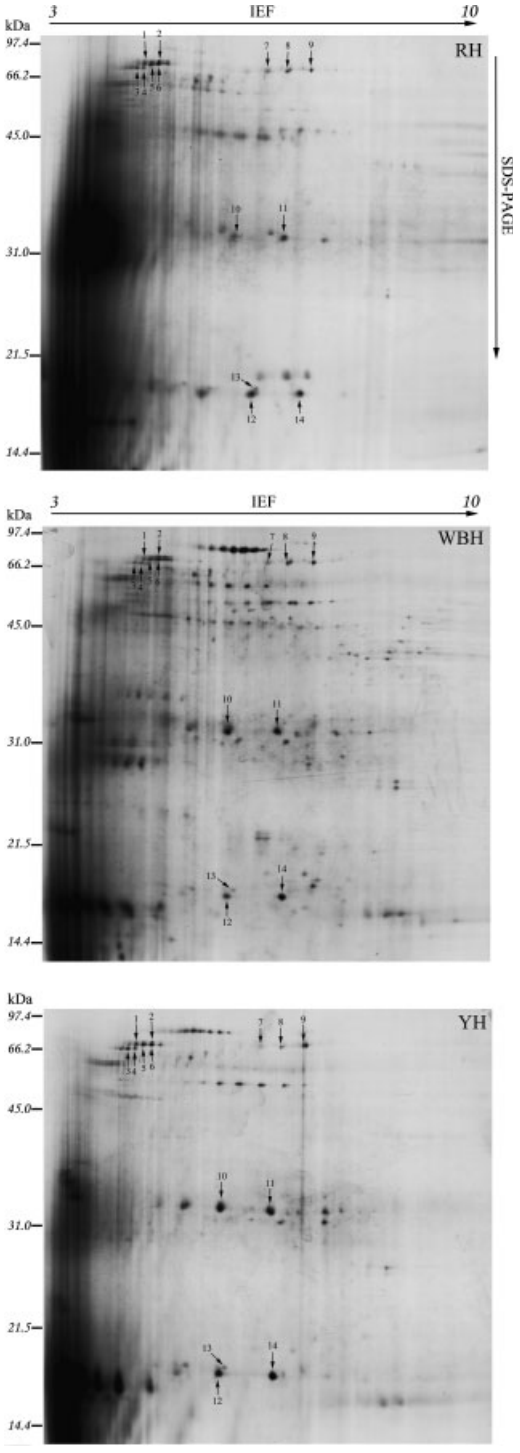


Figure 3. 2-DE was performed using 80 μg of protein, non-linear 24 cm IPG strips (pH 3–10), and 12.5% SDS-PAGE gels for second-dimension electrophoresis. Gels were stained with silver nitrate. (A) RH, (B) WBH, and (C) YH.

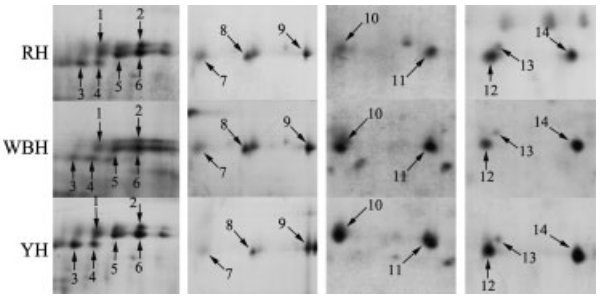


Figure 4. Enlarged 2-DE patterns showing the common protein in three horns. Protein spot numbers were marked.

much as possible. Second, because of the complex construction of horns, purification and identification of original components from horns are difficult. Third, efficacy evaluation of components is based on the pharmacological experiments; however, the content of components in horns is often too low to purify and for the pharmacological research.

In this study, it was shown that the oral treatment with RH, WBH, and YH powder could reduce the febrile response induced by injection of *E. coli* endotoxin, reduce the spontaneous motor activity movements of mice, and reduce the CT, respectively. These studies confirmed the antipyretic, sedative, and procoagulant efficacy of three kinds of horn, which verified their traditional effects.

Bradford assay was used to determine the proteins' concentration. First, exact protein concentration of each sample can help to homogenize total protein level in each gel. Second, the contents of soluble proteins can also help to define the dose relationship of three horns in pharmacology experiments. For instance, the proteins concentration of RH (5.0 μg/μL) was determined 25 times higher than that of WBH (0.2 μg/μL) and YH (0.2 μg/μL). As a result, the doses of WBH and YH (1.50 g/kg for rabbit, 5.50 g/kg for mice) should be selected 25 times higher than that of RH (0.06 g/kg for rabbit, 0.22 g/kg for mice) in pharmacology experiments according to the protein concentration determination. Therefore, the total soluble proteins of each horn taken by animals in pharmacology experiments were equal.

It has been reported that some researchers had established correlation between fingerprints and efficacy to control quality or screen effective components from complex system of TCM. For example, a strategy was proposed to evaluate efficacy strength of TCM and help to screen candidate herbal drugs according to their UV spectra fingerprints [22]. Principal component analysis (PCA) and logistic stepwise regression were applied to build relationship between the chemical compounds of the volatile oils from a TCM Formulae Shao-Fu-Zhu-Yu decoction, five constituent herbs and their bioactivity, finally selected nine compounds which might contribute to the bioactivity of the TCM Formulae [23]. These investigations on components-efficacy analysis were valuable for evaluating the efficacy strength of TCM samples' base on their fingerprints, even screening and finding active components from the samples.

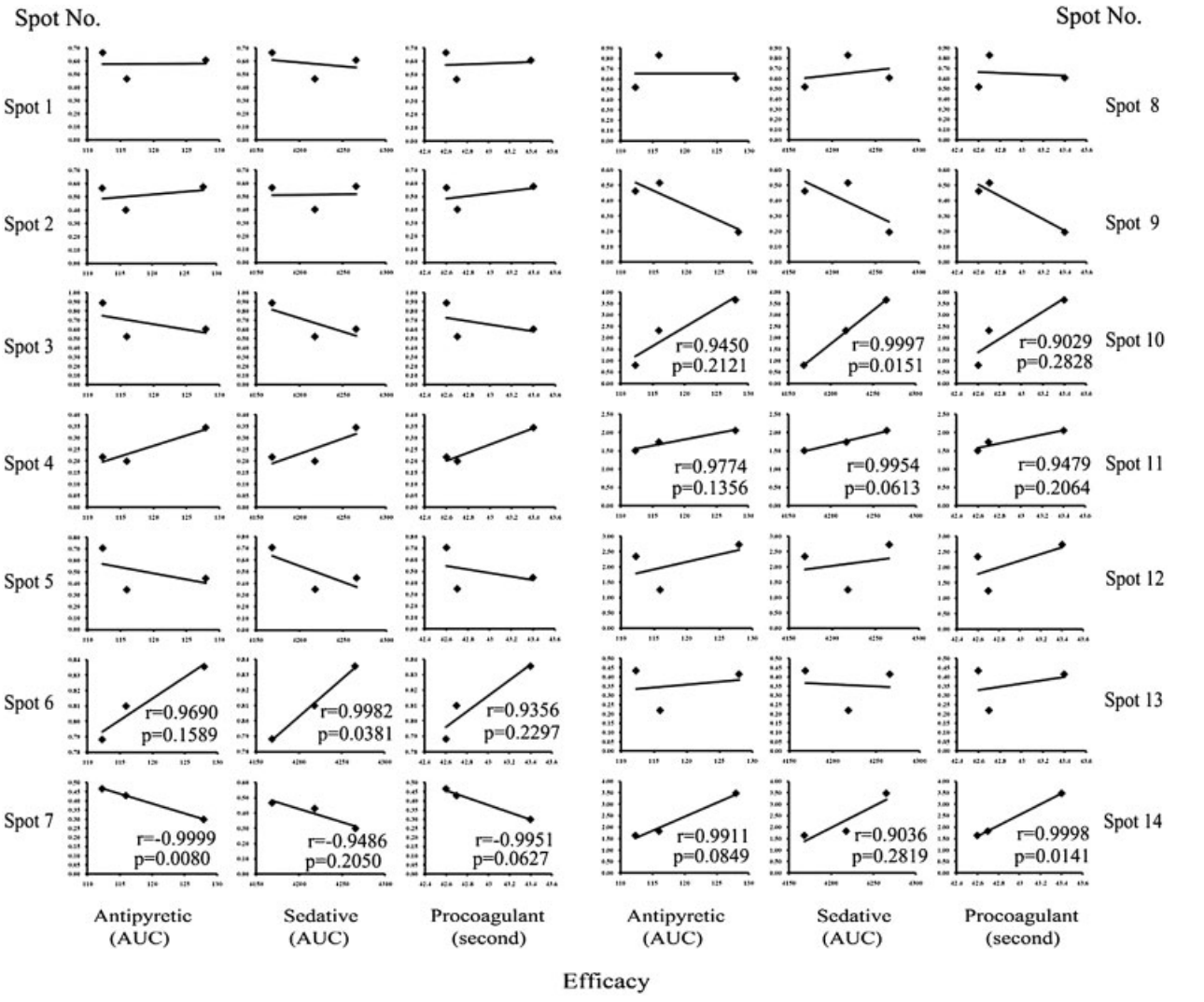
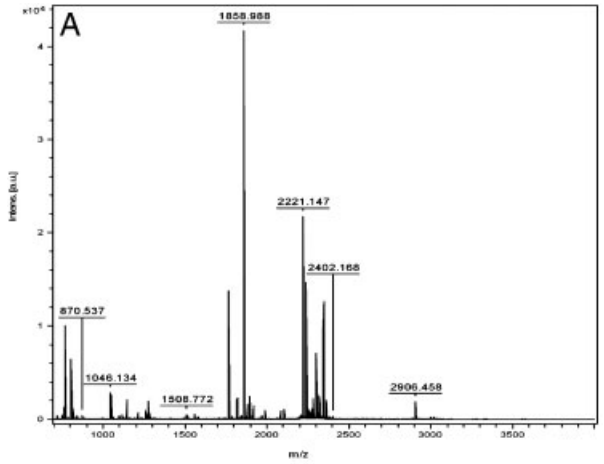


Figure 5. Results of linear regression analysis. Each panel was shown as efficacy for *x*-axis and spot abundance for *y*-axis. The panels whose coefficient correlation was larger than 0.9 were chosen and the corresponding spots were considered correlated with efficacy.

Table 2. Identification of proteins isolated from horns by PMF analysis

Spot no.	Matched proteins	Accession no.	Theoretical kDa/p*I*	Matched peptides	Sequence coverage (%)
6	Zinc finger CCCH-type containing 12D	CAX11887	12 433/8.01	8	86
7	No match	–	–	–	–
10	No match	–	–	–	–
11	Peptide methionine sulfoxide reductase	YP_001256455	35 800/6.01	15	60
14	No match	–	–	–	–

In order to test the correlation between therapeutic effects and common components of horns, the method of linear regression analysis was used. AUCs represent the antipyretic efficacy strength and sedative efficacy strength. The efficacy strength was quantified by calculating the AUC of rectal temperature changing curve and spontaneous motor activity changing curve according to the pharmacology data. Then common components of three horns were correlated to their therapeutic effects by using linear regression analysis. Finally, five proteins which might contribute to the efficacy were picked. A total of five potential active proteins were then identified by MALDI-TOF MS, and only two PMFs of protein could be matched in the NCBInr database: spot 6 was identified to be zinc finger

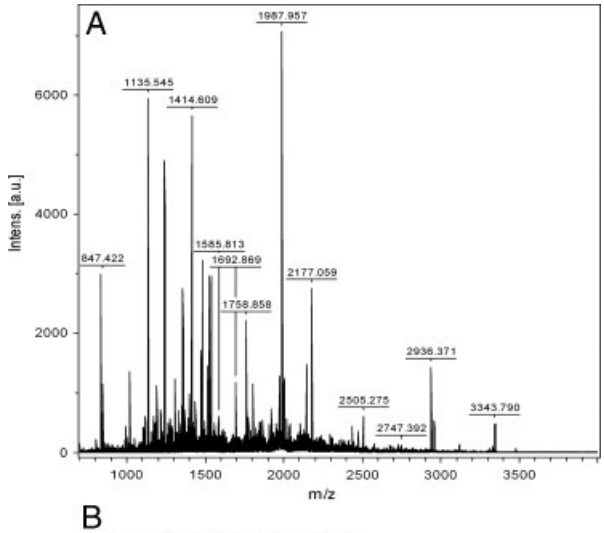


B

Match to: gi|220675444; Score: 93; Expect: 0.0045
Nominal mass (Mr): 12433; Calculated pI value: 8.01;
zinc finger CCCH-type containing 12D;
Number of mass values matched: 8; Sequence Coverage: 86%;
Matched peptides shown in Bold:

1 AVLVYTPSRK VHGK**RLVCYD DRYIVKVAYE QDGVIVSNDN YRDLQSENPE**
51 **WKWFIEQRLL MFSFVNDRFM PPDDPLGRHG PSLSNFLSRK PKPPEPSWQH**
101 **CPYGG**

Figure 6. Identification of protein by MALDI-TOF peptide mass map. (A) Mass spectra of spot 6 obtained from gels. (B) Search results with the corresponding mass data.



B

Match to: gi|148377579; Score: 116; Expect: 2.2e-05;
Nominal mass (Mr): 35800; Calculated pI value: 6.01;
peptide methionine sulfoxide reductase;
Number of mass values matched: 15; Sequence Coverage: 60%;
Matched peptides are shown in bold:

1 MQK**TIYLAGG CFWGVEAYFK RVKGVLDTQV GYANGKDENA TYTNLK**NSLH
51 AETVKVVYDS EITSVEELVL HLFKIIDPTS LNK**QGNDVGV QYRTGVYYQD**
101 **VNDVLTIEKL** FSYLKK**QYNE FHVELKVLNH FIDAEAYHQD YLTK**NPTGYC
151 HVNLNTDYSL SK**DDYQHIKQ VR**NELKLSQL SYDILK**NSAT ERPHTSVLNN**
201 **EYRR**GIYVEK **ITGEPLFSSS TK**FNSGCGWP SFSEPIFKDT VK**YLDDTSHN**
251 **MFRIEVRSGQ GDHHLGHVFN DGPKDMGGKR YCINGAAIDF IPYEEMDEKG**
301 YSEFKKFVK

Figure 7. Identification of protein by MALDI-TOF peptide mass map. (A) Mass spectra of spot 11 obtained from gels. (B) Search results with the corresponding mass data.

CCCH-type containing 12D, and spot 11 was identified to belong to *Mycoplasma agalactiae* PG2.

In this study, a simple method to construct correlation between components and efficacy strength was explored by linear regression analysis first. The results of analysis between 14 common components of 2-DE gels and pharmacological data of three horn samples illustrate that five spots are mostly correlated to the efficacy of samples, and may be the potential active components. Of course, the study in this article is only a new, primary investigation on developing novel method to search possible active components of horn-derived TCM. Further study with advanced technique would be providing more beneficial way to screen and evaluate horn-derived TCM.

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