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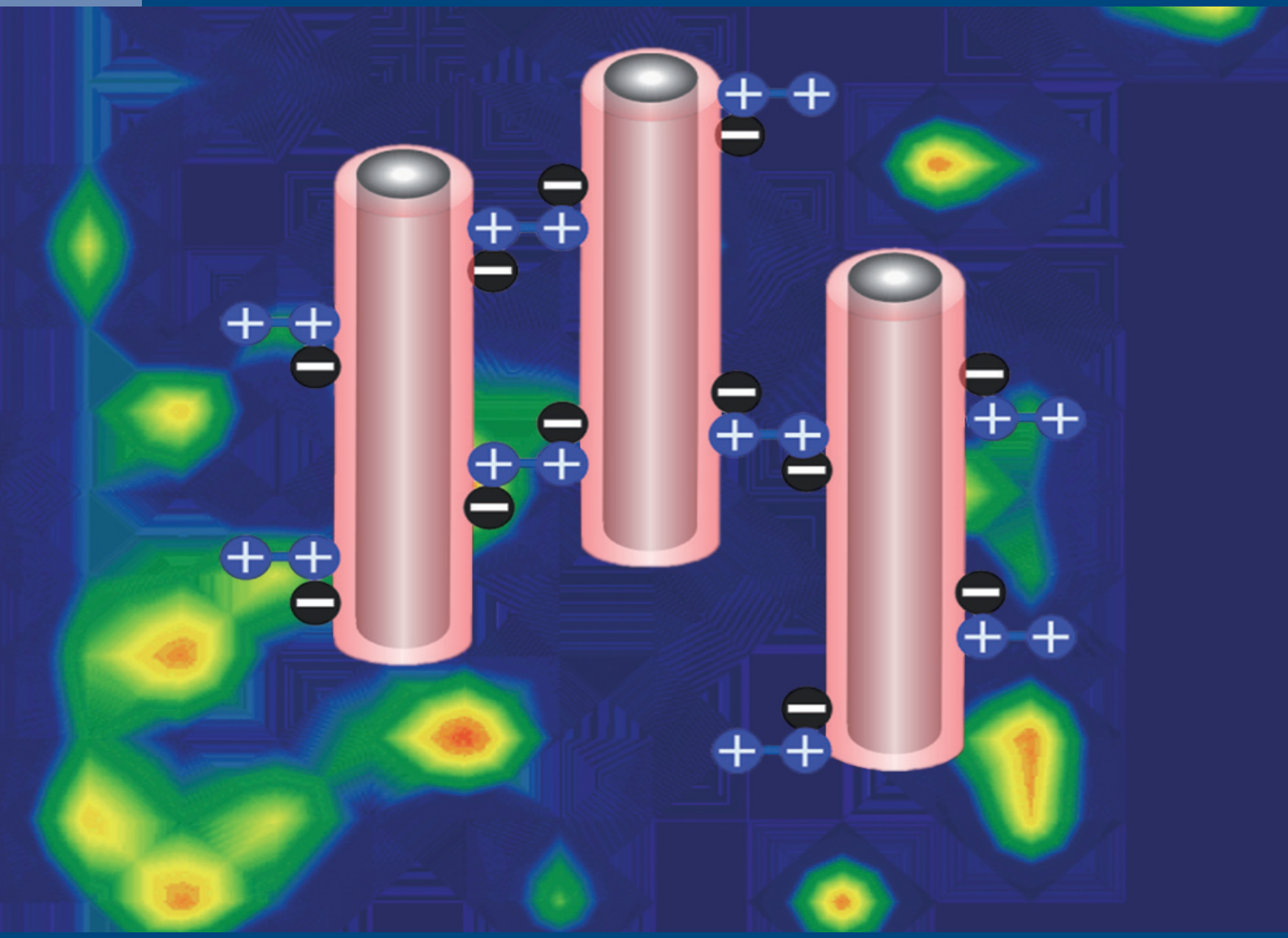

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RESEARCH ARTICLE

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Identification of short-chain poly-3-hydroxybutyrates in Saiga horn extracts using LC–MS/MS

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Saiga horn extracts were analyzed with the goal of obtaining new information about compounds present in it. The purpose of this study is to find synthetic alternatives to Saiga horn extract, which is used in traditional Chinese medicine, by identifying potentially biologically active compounds in the extracts. Using high-performance liquid chromatography coupled with high-resolution mass spectrometry, we have been able to identify a series of short-chain polyhydroxybutyrates in alcoholic extracts of Saiga horn. Optimized high-performance liquid chromatography coupled with tandem mass spectrometry methods for analysis of short-chain poly-3-hydroxybutyrates were developed and subsequently applied to investigate Saiga horn extract for the presence of these compounds, which might explain its biological actions, particularly for its antipyretic and procoagulant properties.

KEYWORDS

liquid chromatography, polyhydroxybutyrates, Saiga horn, tandem mass spectrometry, traditional Chinese medicine

1 | INTRODUCTION

Saiga horn, also known by its popular name as Lin Yan Jiao, is one of the most renowned materials used in traditional Chinese medicine (TCM). In the early 1990s, conservation organizations advocated the use of Saiga horn in TCM in an effort to reduce the losses of rhinoceros to poaching. In 2001, it was reported that the Saiga population had declined dramatically [1]. At the same time, Saiga antelope had joined rhinoceros on the endangered species list. In China, it is now classified as a class I protected species.

Saiga horn is prescribed by Chinese physicians for fevers, dizziness, blurred vision, headaches and convulsions [2]. The Wildlife Conservation Society states on its website that: "Saiga horn is mentioned in more than 30 TCM prescriptions or patent medicines. In "New-Edited Chinese Patent Medicine" published in 2002, Saiga horn is the main component of 18 kinds of medicines for detoxification, cold treatment and lung disease." [3].

The damping, antipyretic, fever-lowering effects of Saiga, and other animal horns have been known for a long time and the mechanisms of their action have been investigated.

For example, a metabolomic study of the antipyretic effect of water buffalo horn (WBH) in rats showed that WBH affects arachidonic acid metabolism and oxidative stress in yeast-induced pyrexia [4]. Another study showed that the levels of several cytokines, namely IL-1β, TNF-α, PGE2 and cAMP, in plasma were significantly decreased 2 h after the treatment of yeast-induced fever rats with WBH extract [5]. A study published in 2016 evaluated available sustainable alternatives by comparing bio-effects and the proteomes of seven types of animal horns and concluded that both Saiga and rhinoceros horns can be substituted by other animals' horns in relieving

Artice Related Abbreviations: AHB, alpha-hydroxybutyric acid; AHIB, alpha-hydroxyisobutyric acid; BHB, beta-hydroxybutyric acid; BHIB, beta-hydroxyisobutyric acid; cP2HB, poly-2-hydroxybutyrate; cP3HB, poly-3-hydroxybutyrate; Cphb, polyhydroxybutyrate; GHB, gamma-hydroxybutyric acid; MRM, multiple reaction monitoring; TCM, traditional Chinese medicine; WBH, water buffalo horn

bleeding and treating fever and convulsion, respectively [6]. Biologically active substances contained in Saiga horn have a pronounced membranotropic effect. The effect depends on the dose of the drug and on the part of the horn from which the substances are extracted. In acute ethanol intoxication, twofold injections of the horn extract from the upper part of the horn in a dose of 0.4 mg per animal had a protective effect on erythrocytes' membranes. The higher concentration of the "upper" part of horn extract, as well as both studied concentrations of the extract from the lower part of the horn, did not possess such protective actions [7]. In addition, a complex of biologically active substances contained in Saiga horn helps protect the gastrointestinal mucosa from damage [8] and reduces alcohol consumption in rats [9]. Because Saiga is now classified as a critically endangered species, the search for possible substitutes continues [6,10–13]. Uncovering the identity of biologically active compounds in the horns of these species may lead to cheaper, more effective, synthetic alternatives to animal horns and save these animals from extinction.

The primary components of any horn are keratin and collagen [14,15]. The differences in amino acid sequences in keratins of various animals can be used to determine the origin of horn in TCM preparations [16]. Attempts have been made in the past to identify biologically active compounds in horns of various species, but to date, there are only very limited, if any, results that would explain the efficacy thereof.

For example, 2-DE and MALDI–TOF mass spectrometry were used to analyze the protein components in horns of rhinoceros, water buffalo, and yak. Out of 14 protein spots, five were found to be potentially connected with potential pharmacological efficacy. Two of them were identified as Zinc finger CCCH-type containing 12D and Peptide methionine sulfoxide reductase [17].

Three peptides exhibiting antioxidant properties were purified from an aqueous extract of WBH using chromatographic methods, which according to the authors may be derived from the hydrolysis of original proteins and thus may not represent the actual original components of the matrix [17,18].

A chemical analysis of Saiga horn has revealed that its main constituents are proteins (keratins and collagens) and calcium phosphate. Saiga horn has high zinc content, so it is believed that its consumption can help to delay aging. The amino acid composition of Saiga horn has already been described [13]. However, to date, no specific compound has been isolated or scientifically described that would explain the efficacy of the Saiga horn extracts [10].

LC–MS/MS analysis of Saiga horn extracts confirmed that the main components are keratin type I microfibrillar, keratin type II microfibrillar and collagen type I (α1 and α2 chains) [15].

The aim of this study is to identify potentially biologically active compounds in the extracts of Saiga horn.

2 | MATERIALS AND METHODS

2.1 | Samples and chemicals

Seven Saiga horn extracts (1.5 DGV, 2.7 PGV, 3.7 PGV, 3.7 DGV, III extr. 2.6 DGN, 3.7 DGV solub H_2O, 3.7 DGV solub Hlf:MeOH; numerals indicate the age of the animal: 1.5–young, 2.7–3.7 – adult; DG – before rutting season; PG – after rutting season; V – upper part (2/3) of the horn, N – lower part (1/3) of the horn) were kindly supplied by Kalmykian State University, Elista, Russia. Extracts were prepared as follows: Saiga horn shavings were ground and extracted in 50% ethanol in a ratio of 1:1 v/v as this provides a greater concentration of the active substances in an extract. After 24 h, the extract was separated from the chips. Typically, the extraction was performed three times, and the combined extracts were distilled off under vacuum. If the number of extractions is not specified, all three extracts were combined. Solub H_2O indicates the part of the extract that is soluble in water. Solub Hlf/MeOH means that the dry extract was treated with a mixture of chloroform/methanol (1:1 v/v) and the methanol layer was taken and evaporated.

Water used was Milli-Q water (Millipore, Bedford, MA, USA). Formic acid for MS, acetonitrile LC–MS Ultra Chromasolv, 2-hydroxybutyric acid (AHB) and 3-hydroxybutyric acid (BHB) were purchased from Fluka (Sigma-Aldrich, St. Louis, MO, USA). Ammonium bicarbonate, trypsin TPCK Bovine and ethanol were obtained from Sigma (Sigma-Aldrich) and sulfuric acid was purchased from Penta (Prague, Czech Republic). Anhydrous sodium sulfate was supplied by Lachema. Ethanol used for the extraction was purchased from Sigma (Sigma-Aldrich).

2.2 | Preparation of Saiga horn extracts for LC–MS/MS analyses

2.2.1 | Pre-prepared extracts

Saturated solutions of Saiga horn extracts in 2% formic acid at room temperature were prepared and filtered through Ultrafree-MC 0.45 μm (Millipore, Bedford, MA, USA) in a centrifuge (Heathrow Scientific, IL, USA) using centrifugal force of 6000 *g*.

2.2.2 | Trypsin digests of Saiga horn extracts

Approximately 1 mg of Saiga horn extracts were dissolved in 20 mmol/L ammonium bicarbonate buffer (pH 7.8) to achieve the concentration of 5 mg/mL and treated with trypsin (1:50 enzyme/substrate ratio). The incubation was done at 37°C for 24 h. The samples were centrifuged and 40 μL of the solution were lyophilized and dissolved in 200 μL of 2% formic acid. Blank samples were prepared by incubating and treating the enzyme solution alone in the same way.

2.2.3 | Preparation of short-chain polyhydroxybutyrate standards

The preparation of short-chain polyhydroxybutyrate (cPHB) standards was done according to Fischer esterification in which the carboxyl group of one molecule of hydroxybutyric acid reacts with hydroxyl group of the second molecule of hydroxybutyric acid in the presence of concentrated sulfuric acid as a catalyst [19].

Anhydrous sodium sulfate (100 mg) was added to 400 µL of BHB and 100 µL of sulfuric acid (96% w/w) was added to the mixture. The mixture was then vortexed for 1 h at room temperature. Five microliters of supernatant were diluted with 95 µL of acetonitrile and 40 µL aliquots of this solution were lyophilized and reconstituted in 40 µL of 2% formic acid.

For AHB, the procedure described above was repeated with a slight modification: AHB, which is a solid compound at room temperature, was first heated to its melting point and 100 µL of AHB was added to 25 mg of anhydrous sodium sulfate. Subsequently, 25 µL of sulfuric acid were added and the resulting mixture was vortexed. After 1 h, 5 µL of supernatant were diluted with 95 µL of acetonitrile and 40 µL of this solution were lyophilized and reconstituted with 40 µL of 2% formic acid.

The two resulting solutions contain mixtures of short-chain poly-2-hydroxybutyrates (cP2HB) and short-chain poly-3-hydroxybutyrates (cP3HB). For LC-MS/MS analyses, the c2PHB and c3PHB standard solutions were diluted with 2% formic acid 1:100 and 1:5000 v/v, respectively.

2.3 | Nano-HPLC/maXis QTOF analyses

The nano-HPLC apparatus used for the analysis was a Proxeon Easy-nLC (Proxeon, Odense, Denmark) coupled to a maXis QTOF mass spectrometer with ultrahigh resolution (Bruker Daltonics, Bremen, Germany) by nanoelectrosprayer. The nLC-MS/MS instruments were controlled with the software packages HyStar 3.2 and oTOFControl 3.4. The data were collected and processed with the software packages DataAnalysis 4.2 and ProteinScape 3.0 (Bruker Daltonics).

The samples were injected into an NS-AC-11-C18 Biosphere C18 column (particle size: 5 µm, pore size: 12 nm, length: 150 mm, inner diameter: 75 µm) with an NS-MP-10 Biosphere C18 precolumn (particle size: 5 µm, pore size: 12 nm, length: 20 mm, inner diameter: 100 µm), both manufactured by NanoSeparations (Nieuwkoop, Holland). The injection volume was 1 µL. All analyses were done in duplicate.

2.3.1 | Identification of short-chain polyhydroxybutyrates in Saiga horn extracts using LC–MS/MS in positive mode

Separation was achieved via a linear gradient between mobile phase A (water) and B (acetonitrile), both containing 0.1% v/v formic acid. Separation was started by running the system with 5% mobile phase B, followed by a gradient elution to 50% B at 70 min. The next step was a gradient elution to 70% B in 10 min, and then a gradient to 100% B in 10 min. Finally, the column was eluted with 100% B for 10 min. Equilibration before the next run was achieved by washing the column with 5% mobile phase B for 10 min. The flow rate was 0.25 µL/min and the column was held at ambient temperature (25°C).

On-line nano-ESI (easy nano-ESI) in positive mode was used with the following settings:

Source – End Plate Offset: 500 V, Capillary: 4500 V, Nebulizer Pressure: 100 kPa, Drying Gas (N_2): 4 L/min, Dry Temperature: 150°C; Tune – Transfer: Funnel 1 RF: 300 Vpp, isCID Energy: 0 eV, Multipole: 250 Vpp, Quadrupole: Ion Energy: 4 eV, Low Mass *m/z*: 100, Collision Cell: Collision Energy: 10 eV, Pre Pulse Storage: 10 µs, Stepping: On, Collision RF: 500–1200 Vpp, Transfer Time: 65–90 µs. Timing: 50/50%, Mass Range *m/z*: 50–1500, Spectra Rate: 1 Hz. CID settings for monocharged ions were as follows: *m/z*: 100, Width: 6, Collision energy: 14 eV; *m/z*: 500, Width: 8, Collision energy: 20 eV; *m/z*: 1000, Width: 10, Collision energy: 30 eV.

The reference ion used (internal mass lock) was a monocharged ion of $C_{24}H_{19}F_{36}N_3O_6P_3$ (*m/z* 1221.9906).

The ChemCalc software (http://www.chemcalc.org/mf_finder/mfFinder_em_new) was used to assign exact masses and exact mass differences in MS/MS spectra to molecular formulas for cPHBs analyses.

2.3.2 | Identification of short-chain polyhydroxybutyrates in Saiga horn extracts using LC–MS/MS in negative mode

Separation was achieved via a linear gradient between mobile phase A (5 mM ammonium bicarbonate in water) and B (90% acetonitrile/water containing 5 mM ammonium bicarbonate). The gradient used was the same as described in the previous section with a slight change in the flushing phase, which was prolonged from 10 to 20 min, so the total run was 110 min.

MS and MS/MS settings were the same as described in the previous section except that the mode was set to negative and CID settings for monocharged ions were modified as follows: *m/z*: 100, Width: 6, Collision energy: 3 eV; *m/z*: 500, Width: 8, Collision energy: 11 eV; *m/z*: 1000, Width: 10, Collision energy: 20 eV.

2.3.3 | Identification of 3-hydroxybutyrate moiety using multiple reaction monitoring in a negative mode

The LC settings were the same as described in the previous section.

Multiple reaction monitoring (MRM) in a negative mode was used for identification of the monomer units. The method

was optimized using the two prepared standard solutions of cPHB mixtures to achieve desirable intensity of signal in low masses. For the identification of cP3HB, the following settings were used:

Source – End Plate Offset: 500 V, Capillary: 4500 V, Nebulizer Pressure: 100 kPa, Drying Gas (N_2): 4 L/min, Dry Temperature: 150°C; Tune – Transfer: Funnel 1 RF: 300 Vpp, isCID Energy: 0 eV, Multipole: 250 Vpp, Quadrupole: Ion Energy: 4 eV, Low Mass *m/z*: 100, Collision Cell: Collision Energy: 10 eV, Pre Pulse Storage: 5 µs, Mass Range *m/z*: 50–1500, 1 Hz, Stepping: On, Collision RF 100–500, Transfer Time: 65–90 µs, Timing: 80–20%. MRM settings: *m/z* 103.040, collision energy: 10 eV, timing: 0–25 min; *m/z* 189.07, collision energy: 30 eV, timing: 0–25 min; *m/z* 275.11, collision energy: 50 eV, timing: 0–40 min; *m/z* 361.15, collision energy: 70 eV, timing: 0–40 min; *m/z* 447.19, collision energy: 90 eV, timing: 25–50 min; *m/z* 533.22, collision energy: 110 eV, timing: 40–63 min; *m/z* 619.26, collision energy: 130 eV, timing: 40–63 min; *m/z* 705.30, collision energy: 150 eV, timing: 50–70 min; *m/z* 791.33, collision energy: 170 eV, timing: 63–110 min; *m/z* 877.37, collision energy: 190 eV, timing: 63–110 min; *m/z* 963.41, collision energy: 200 eV, timing: 70–110 min.

For cP2HB standard, the MRM settings were modified to take into account the different retention times of cP2HB oligomers: 0–25 min: *m/z* 103.04, 189.07, 275.11; 25–40 min: *m/z* 275.11, 361.15, 447.19; 40–50 min: *m/z* 361.15, 447.19, 533.22; 50–63 min: *m/z* 447.19, 533.22, 619.26; 63–78 min: *m/z* 533.22, 619.26, 705.30; 78–85 min: *m/z* 705.30, 791.33, 877.37 and 85–110 min: *m/z* 791.33, 877.37, 963.41.

2.3.4 | Proteomic analyses of Saiga horn extracts

Free peptide analyses of saturated solutions of Saiga horn extracts in 2% formic acid and proteomic analyses of tryptic digests were carried out.

Separation was achieved via a linear gradient between mobile phase A (water) and B (acetonitrile), both containing 0.1% v/v formic acid. Separation was started by running the system with 5% mobile phase B, followed by a gradient elution to 30% B at 70 min. The next step was a gradient elution to 50% B in 10 min, and then a gradient to 100% B in 10 min. Finally, the column was eluted with 100% B for 10 min. Injection volume was 5 µL.

On-line nano-ESI (easy nano-ESI) in positive mode was used with the following settings:

Source – End Plate Offset: 500 V, Capillary: 4500 V, Nebulizer Pressure: 100 kPa, Drying Gas (N_2): 4 L/min, Dry Temperature: 180°C; Tune – Transfer: Funnel 1 RF: 400 Vpp, isCID Energy: 0 eV, Multipole: 400 Vpp, Quadrupole: Ion Energy: 4 eV, Low Mass *m/z*: 320, Collision Cell: Collision Energy: 10 eV, Collision RF: 1200 Vpp, Transfer Time: 120 µs, Pre Pulse Storage: 10 µs, Mass Range *m/z*: 20–1500, Spectra Rate: 1 Hz. The reference ion used (internal mass lock) was a monocharged ion of $C_{24}H_{19}F_{36}N_3O_6P_3$ (*m/z* 1221.9906).

2.4 | Database searching

Data were processed using ProteinScape software. Proteins were identified by correlating the tandem mass spectra of Saiga samples to the SwissProt (January 2016) database using the MASCOT search engine (v. 2.3.0) (http://www.matrixscience.com). The taxonomy was restricted to mammals to remove protein identification redundancy. Enzyme parameter was set to none to reflect that free peptides (nontryptic digests) are also present in the samples. Three missed cleavages were allowed and an initial peptide mass tolerance was set to ±15.0 ppm and ±0.05 Da for MS and MS/MS, respectively. The monoisotopic peptide charge was set to 1+, 2+ and 3+. The Peptide Decoy was selected to remove false-positive results. Lysines and prolines were assumed to be hydroxylated and methionine was allowed to be oxidized. Only significant hits were accepted (MASCOT score ≥80 for proteins and MASCOT score ≥20 for peptides).

3 | RESULTS

The goal of this work was to identify potentially biologically active compounds in Saiga horn extracts that are not yet described in the literature.

Initial LC–MS/MS experiments performed on Saiga horn extracts revealed the presence of several unknown polymer compounds with the same fragmentation pattern exhibiting similar characteristics, i.e. multiple neutral loss of *m/z* 86. The interpretation of high-resolution tandem mass spectra led us to the idea that these spectra might be assigned to cPHBs. To confirm this hypothesis, two standard mixtures of cP2HB and cP3HB were synthesized and analysed in addition to Saiga samples. The standards were also used to optimize the LC method to achieve desirable separation efficacy of oligomers. The samples of Saiga horn extracts were again measured using the optimized method.

LC–MS and LC–MS/MS experiments performed in both positive and negative modes revealed the presence of cPHBs in the samples (Figure 1). The cPHBs were identified as cP3HB by comparing their negative MRM spectra with standards of cP2HB and cP3HB. MRM spectra are shown in Figure 2. The results are summarized in Table 1.

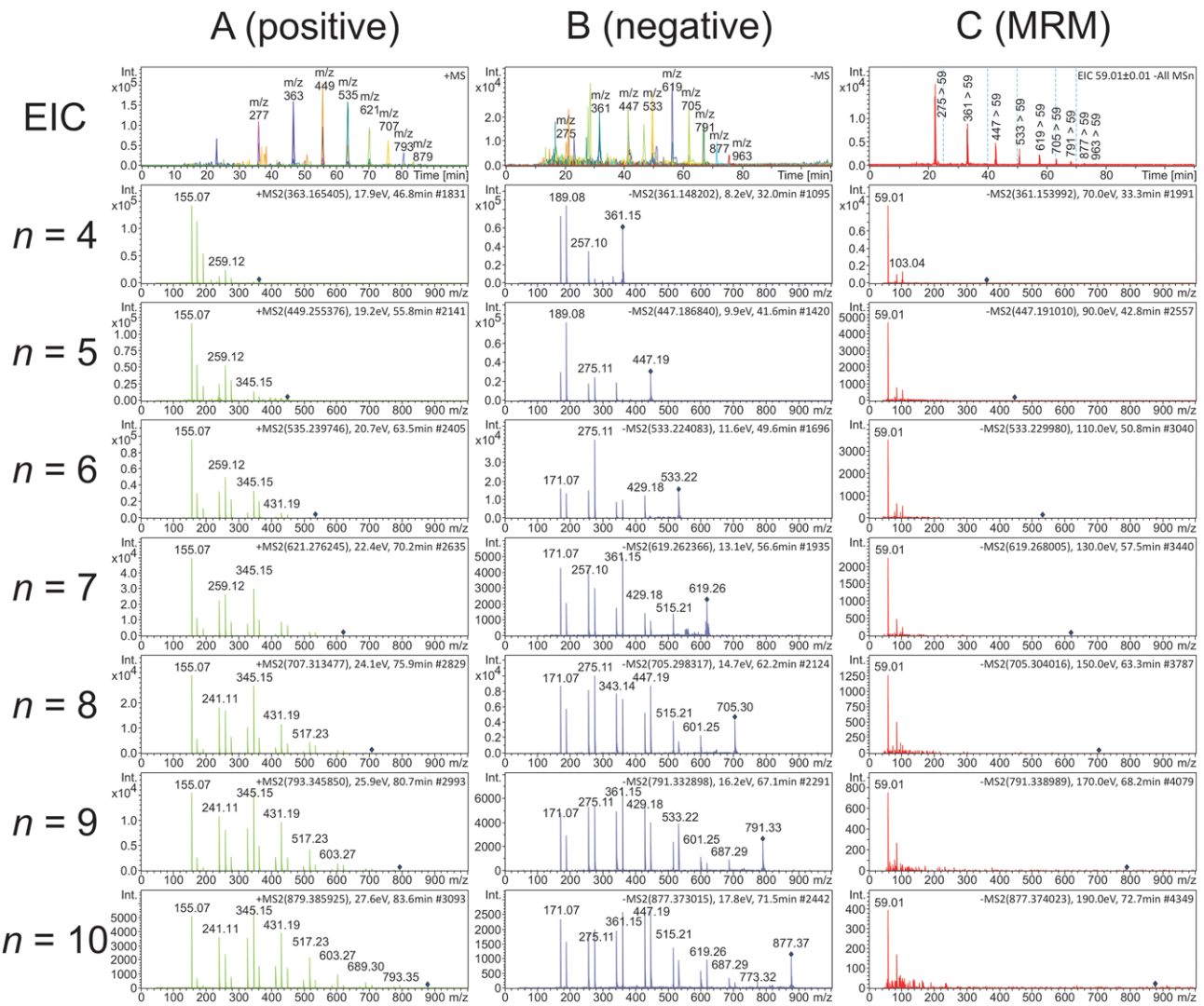


FIGURE 1 LC–MS EIC chromatogram of cPHBs found in Saiga horn extract (1.5 DGV) and their (A) corresponding MS/MS spectra in positive mode, (B) MS/MS spectra in negative mode and (C) MRM spectra in negative mode; spectra presented for cPHBs with $n = 4$ to 10

3.1 | Identification of cPHBs in Saiga horn extracts using LC–MS/MS analysis in a positive mode

LC–MS and LC–MS/MS analysis of saturated solutions of Saiga horn extracts in a positive mode revealed the presence of cPHBs. Representative MS/MS spectra of cPHBs ($n = 4$ to 10) identified in a positive mode in a 1.5 DGV sample are shown in Figure 1A.

MS/MS spectra showed a multiple neutral loss of crotonic acid ($\Delta m/z$ 86.0382, $C_4H_6O_2$) – the loss typical for cPHBs [20]. A representative positive MS/MS spectrum of a cPHB ($n = 9$) for the 1.5 DGV sample is described in Table 2.

3.2 | Identification of short-chain polyhydroxybutyrates in Saiga horn extract using LC–MS/MS analysis in a negative mode

LC–MS/MS analyses of Saiga horn extracts in negative mode also confirmed the presence of cPHBs in the samples. The MS/MS spectra of cPHBs identified in a negative mode are shown in Figure 1B. Because of the lower sensitivity, only samples that exhibited the most intense signals in the positive mode (1.5 DGV) were analyzed in negative mode.

The retention times are shifted due to the different composition of the mobile phase used to enhance the signal intensities in negative mode but the peaks are still well resolved.

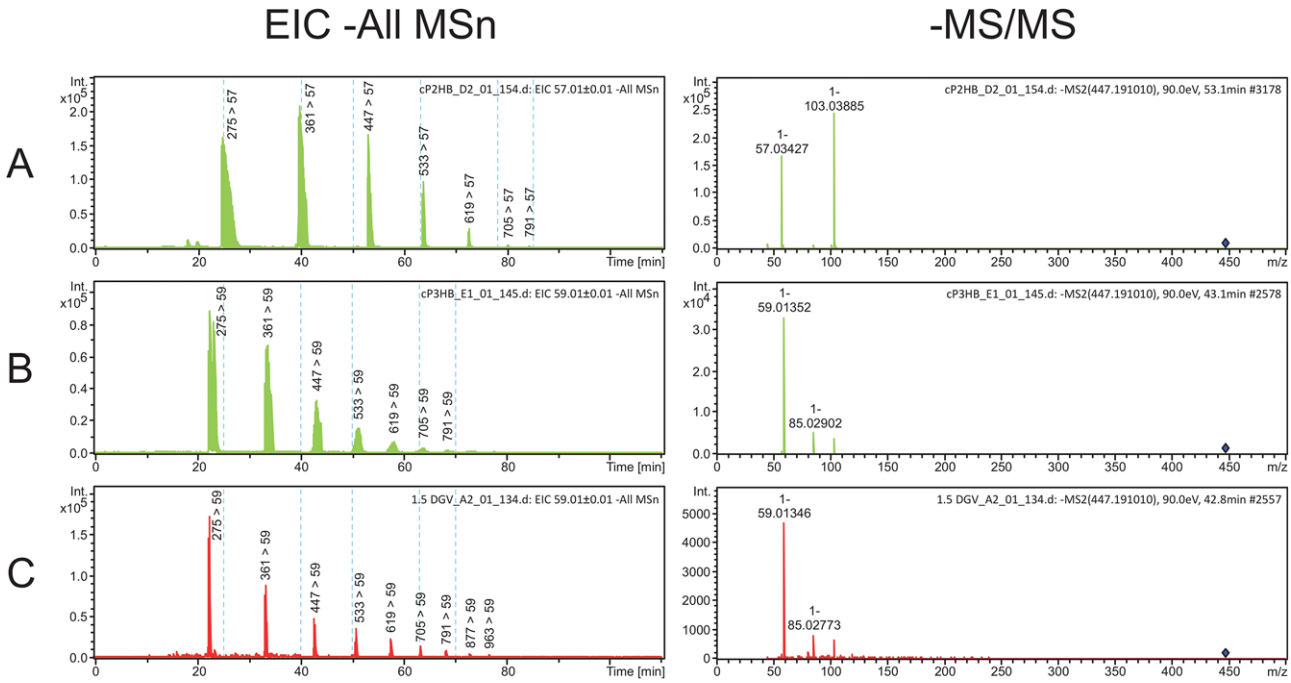


FIGURE 2 EIC chromatograms and representative fragmentation spectra of cPHB ($n = 5$) m/z 447 (collision energy: 90 eV) in negative mode for cPHBs contained in (A) cP2HB standard mixture, (B) cP3HB standard mixture and (C) Saiga horn extract

TABLE 1 Summary of cPHBs identified in Saiga horn extracts based on the number of hydroxybutyrate moieties and MS method

	Identified cPHBs based on the number of HB moieties (n)[b] (EIC–MS positive/MRM negative)										
Sample[a]	**1 (BHB)**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
1.5 DGV	-/-	-/-	Y/Y	**Y**/Y	**Y**/Y	**Y**/Y	**Y**/Y	**Y**/Y	**Y**/Y	**Y**/Y	Y/Y
2.7 PGV	-/-	-/-	-/-	Y/-	Y/-	Y/-	Y/-	Y/-	Y/-	-/-	-/-
3.7 PGV	-/-	-/-	-/-	Y/-	Y/-	Y/-	Y/-	Y/-	Y/-	Y/-	Y/-
3.7 DGV	-/-	-/-	-/-	-/-	Y/-	Y/-	Y/Y	Y/Y	Y/Y	-/-	-/-
III extr. 2.6 DGN	-/-	-/-	-/-	-/Y	-/Y	Y/Y	Y/Y	Y/Y	Y/Y	Y/Y	Y/-
3.7 DGV solub H_2O	-/Y	-/-	-/-	-/-	-/-	Y/Y	Y/Y	Y/Y	Y/Y	Y/Y	Y/Y
3.7 DGV solub Hlf:MeOH	-/-	-/-	-/-	-/-	-/-	-/Y	Y/Y	Y/Y	Y/Y	Y/Y	Y/Y

[a]Numerals indicate the age of the animal: 1.5 – young, 2.7–3.7 adult, DG – before rutting season; PG – after rutting season. V – upper part of the horn (2/3), N – lower part of the horn (1/3); Solub H_2O indicates part of extract soluble in water, Solub Hlf:MeOH means that the dry extract was treated with a mixture of chloroform:methanol (1:1 v/v) and the methanol layer was taken and evaporated.

[b]For the purpose of identification, the EIC signal-to-noise ratio is equal to or greater than 3; bold indicates that MS/MS spectra were obtained as well.

"Y" indicates that the oligomer was identified based on its exact mass and retention time in the case of MS method and by its MRM transition (parent ion → m/z 59) in case of MRM experiments; the "Y" letter presented in bold indicates that the oligomer was also identified by its MS/MS spectrum.

A representative negative MS/MS spectrum of a cPHB ($n = 9$) for the 1.5 DGV sample is described in Table 3.

3.3 | Identification of 3-hydroxybutyrate moiety within short-chain polyhydroxybutyrate oligomers in an alcoholic extract of Saiga horn using LC–MS/MS analysis in negative MRM mode

Because MS/MS in positive mode is not able to distinguish among the five possible hydroxybutyrate isomers (AHB, AHIB, BHB, BHIB and GHB), we used ionization in the negative mode, where BHB can be distinguished from its isomers (AHB, AHIB, BHIB and GHB) based on its characteristic product ion. While all isomers have the same mass to charge ratio of m/z 103 in negative mode, the fragmentation mechanism is different for BHB, thus leading to a characteristic transition 103 → 59 (AHB, AHIB and GHB form a product ion of m/z 57 whereas BHIB forms a product ion of m/z 73) [21–32]. MRM transitions for hydroxybutyric acid isomers are listed in Table 4.

To verify the product ions for cPHBs, two standard mixtures of cPHBs were prepared and used for the optimization of the LC–MS/MS method. Figure 2 shows extracted ion chromatograms (EIC) of standard cPHB mixtures and representative fragmentation spectra of cPHB containing

TABLE 2 ESI–MS/MS spectrum of poly-3-hydroxybutyrate ($n = 9$) in Saiga horn extract (positive mode)

Exact mass (theoretical)	Mass (experimental)	Charge	Relative Intensity	Ion Formula
155.07027	155.06989	1+	100.0%	$[C_8H_{15}O_5 - 2H_2O]^+$
173.08084	173.08026	1+	15.9%	$[C_8H_{15}O_5 - H_2O]^+$
191.09140	191.09105	1+	4.2%	$[C_8H_{15}O_5]^+$
241.10705	241.10644	1+	69.5%	$[C_{12}H_{21}O_7 - 2H_2O]^+$
259.11761	259.11717	1+	52.0%	$[C_{12}H_{21}O_7 - H_2O]^+$
277.12818	277.12736	1+	17.4%	$[C_{12}H_{21}O_7]^+$
327.14383	327.14344	1+	54.1%	$[C_{16}H_{27}O_9 - 2H_2O]^+$
345.15439	345.15440	1+	99.5%	$[C_{16}H_{27}O_9 - H_2O]^+$
363.16496	363.16451	1+	31.0%	$[C_{16}H_{27}O_9]^+$
413.18061	413.18087	1+	16.9%	$[C_{20}H_{33}O_{11} - 2H_2O]^+$
431.19117	431.19177	1+	62.2%	$[C_{20}H_{33}O_{11} - H_2O]^+$
449.20174	449.20185	1+	16.2%	$[C_{20}H_{33}O_{11}]^+$
499.21739	499.21810	1+	6.0%	$[C_{24}H_{39}O_{13} - 2H_2O]^+$
517.22795	517.22847	1+	26.9%	$[C_{24}H_{39}O_{13} - H_2O]^+$
535.23852	535.24010	1+	8.3%	$[C_{24}H_{39}O_{13}]^+$
585.25417	585.25391	1+	1.9%	$[C_{28}H_{45}O_{15} - 2H_2O]^+$
603.26473	603.26513	1+	9.0%	$[C_{28}H_{45}O_{15} - H_2O]^+$
621.27530	621.27592	1+	7.1%	$[C_{28}H_{45}O_{15}]^+$
671.29095	N/A	1+	N/A	$[C_{32}H_{51}O_{17} - 2H_2O]^+$
689.30151	689.30405	1+	2.8%	$[C_{32}H_{51}O_{17} - H_2O]^+$
707.31208	707.31276	1+	2.6%	$[C_{32}H_{51}O_{17}]^+$
793.34886	793.34585	1+	1.0%	$[C_{36}H_{57}O_{19}]^+$

TABLE 3 ESI–MS/MS spectrum of poly-3-hydroxybutyrate ($n = 9$) in Saiga horn extract (negative mode)

Exact mass (theoretical)	Mass (experimental)	Charge	Relative Intensity	Ion Formula
171.06519	171.06579	1-	74.2%	$[C_8H_{13}O_5 - H_2O]^-$
189.07575	189.07599	1-	44.7%	$[C_8H_{13}O_5]^-$
257.10306	257.10207	1-	81.0%	$[C_{12}H_{19}O_7 - H_2O]^-$
275.11363	275.11251	1-	82.1%	$[C_{12}H_{19}O_7]^-$
343.13984	343.13996	1-	75.5%	$[C_{16}H_{25}O_9 - H_2O]^-$
361.15041	361.15043	1-	100.0%	$[C_{16}H_{25}O_9]^-$
429.17662	429.17651	1-	82.5%	$[C_{20}H_{31}O_{11} - H_2O]^-$
447.18719	447.18713	1-	61.4%	$[C_{20}H_{31}O_{11}]^-$
515.21340	515.21385	1-	36.9%	$[C_{24}H_{37}O_{13} - H_2O]^-$
533.22396	533.22421	1-	60.5%	$[C_{24}H_{37}O_{13}]^-$
601.25018	601.24812	1-	17.4%	$[C_{28}H_{43}O_{15} - H_2O]^-$
619.26074	619.25913	1-	9.6%	$[C_{28}H_{43}O_{15}]^-$
687.28696	687.28972	1-	13.5%	$[C_{32}H_{49}O_{17} - H_2O]^-$
705.29752	N/A	1-	N/A	$[C_{32}H_{49}O_{17}]^-$
791.33430	791.33290	1-	37.0%	$[C_{36}H_{55}O_{19}]^-$

five hydroxybutyrate moieties demonstrating the differences in product ion formation and retention times for cPHBs prepared from AHB (Figure 2A) and BHB (Figure 2B) and for Saiga horn extract (Figure 2C).

The MRM analysis of Saiga horn extract showed the formation of a product ion of m/z 59, confirming that cPHBs in Saiga horn consist of BHB moieties. The MRM spectra of cPHBs identified in a negative mode are shown in Figure 1C.

TABLE 4 MRM transitions for hydroxybutyric acid isomers AHB, BHB, GHB, AHIB and BHIB

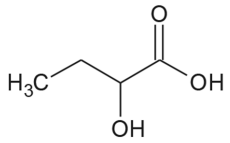
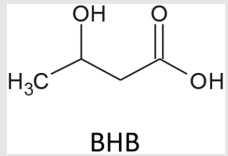
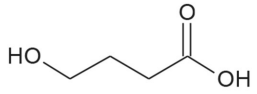
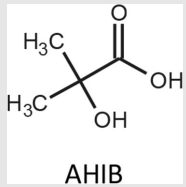
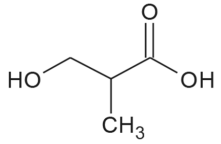
Isomer	MRM transition	Product ion	Ionization	Instrument	Reference
 AHB	103→57	$[C_3H_5O]^-$	ESI	Quattromicro (Waters)	[24]
			ESI	LCQ Advantage quadrupole-ion trap (Thermo Finnigan)	[25]
			ESI	Quattro Ultima triple quadrupole (Waters)	[25]
			not specified	LTQ XL (Thermo Scientific)	[27]
			ESI, APCI	API3200 (Applied Biosystems)	[31]
 BHB	103→59	$[C_3H_7O]^-$	ESI	4000 QTRAP (Sciex)	[26]
			not specified	LTQ XL (Thermo Scientific)	[27]
			ESI, APCI	API3200 (Applied Biosystems)	[31]
 GHB	103→57	$[C_3H_5O]^-$	ESI	Xevo TQ MS UPLC-MS/MS (Waters)	[28]
			ESI, APCI	API3200 (Applied Biosystems)	[31]
			ESI	Agilent 6460 Triple Quadrupole (Agilent Technologies)	[32]
	103→85	$[C_4H_5O_2]^-$	not specified	LTQ XL (Thermo Scientific)	[27]
			ESI	Xevo TQ MS UPLC-MS/MS (Waters)	[28]
			ESI	Esquire 3000 plus (Bruker Daltonics)	[29]
			ESI	LCQ ion trap (Finnigan)	[30]
			not specified	API3200 (Applied Biosystems)	[31]
			ESI	Agilent 6460 Triple Quadrupole (Agilent Technologies)	[32]
 AHIB	103→57	$[C_3H_5O]^-$	ESI	Xevo TQ MS UPLC-MS/MS (Waters)	[28]
	103→85	$[C_4H_5O_2]^-$	ESI	Xevo TQ MS UPLC-MS/MS (Waters)	[28]
 BHIB	103→73	$[C_3H_5O_2]^-$	ESI	4000 QTRAP (Sciex)	[26]
			not specified	LTQ XL (Thermo Scientific)	[27]
			ESI	Xevo TQ MS UPLC-MS/MS (Waters)	[28]

TABLE 5 Proteins assigned to free peptides found in Saiga horn extracts (data compiled from seven samples)

Accession	Protein	MW [kDa]	Mascot Score	#Peptides	SC [%]
P02453	Collagen alpha-1(I) chain OS = Bos taurus GN = COL1A1 PE = 1 SV = 3	138.9	6922	169	46.7
P02465	Collagen alpha-2(I) chain OS = Bos taurus GN = COL1A2 PE = 1 SV = 2	129.0	4016	96	42.0
P02534	Keratin, type I microfibrillar 48 kDa, component 8C-1 OS = Ovis aries PE = 1 SV = 2	46.6	1212	30	28.4
P25691	Keratin, type II microfibrillar, component 5 OS = Ovis aries PE = 1 SV = 1	54.6	874	27	25.7

3.4 | Proteomic analysis of free peptides and tryptic digests by LC–MS/MS

LC–MS/MS analyses of free peptides were performed, and the peptides were identified and assigned to proteins (Table 5). We found that the free peptides mainly consist of those belonging to collagen alpha-1 (I), collagen alpha-2 (I), keratin type I (microfibrillar) and keratin type II (cuticular).

Tryptic digests were also analyzed. Data obtained for seven samples containing cPHBs are presented in Table 6. In addition to collagen and keratin, we have identified a macrophage migration inhibitory factor.

4 | DISCUSSION

We have successfully identified cP3HB in Saiga horn extracts—compounds that may explain the biological activity of Saiga horn extracts. We developed and optimized LC–MS/MS methods for identification and characterization thereof.

cPHBs, generally referred to as those consisting of <20 residues, have been found in intracellular fluids and in prokaryotic and eukaryotic cells where they are conjugated to proteins [33–39]. cPHBs were found to be covalently bound to specific proteins in the membranes and the cytoplasm of *E. coli* cells [40]. Another study showed that prokaryotic histone-like protein, E. coli H-NS, and eukaryotic calf thymus histone proteins, H1, H2A, H2B, H3 and H4, are post-translationally modified by conjugation with cPHB [34].

As for the identification of cPHBs, a Western blot immunoassay using anti-PHB IgG is widely employed for the detection of cPHBs covalently bound to specific proteins [34,37,38]. According to the authors, this method is not able to recognize oligomers of less than four residues [34]. In one study, the authors employed MALDI/MS method for identification of cPHB modified peptide bearing six to ten cPHB units [37]. On the contrary, using our method we have been able to identify free oligomers of three to eleven residues and even the monomer unit.

BHB is one of three compounds known as ketone bodies, which is synthesized in the liver from acetyl-CoA during the fasting period or carbohydrate restriction and is used by the brain as a source of energy when blood glucose level is low. It also plays an important role in a ketogenic diet—a high-fat, low-carbohydrate and low-protein diet widely used to treat epilepsy in children [41]. A recent study has shown that endogenous BHB has an antiepileptic effect in suckling infant rats [42].

BHB treatment extends lifespan and protects against metabolic, proteotoxic and thermal stress in *C. elegans* [43]. Aside from that, BHB was also found to have anesthetic properties [44].

TABLE 6 Proteins found in tryptic digests of Saiga horn extracts (data compiled from seven samples)

Accession	Protein	MW [kDa]	Mascot Score	#Peptides	SC [%]
P02453	Collagen alpha-1(I) chain OS = Bos taurus GN = COL1A1 PE = 1 SV = 3	138.9	10033	220	59.0
C0HJN9	Collagen alpha-1(I) chain (Fragments) OS = Equus sp. GN = COL1A1 PE = 1 SV = 1	79.2	6732	140	72.7
P02465	Collagen alpha-2(I) chain OS = Bos taurus GN = COL1A2 PE = 1 SV = 2	129.0	6244	125	50.2
Q148H4	Keratin, type II cuticular Hb1 OS = Bos taurus GN = KRT81 PE = 2 SV = 1	54.6	3606	75	45.0
P25691	Keratin, type II microfibrillar, component 5 OS = Ovis aries PE = 1 SV = 1	55.2	1312	22	30.5
A4FUZ0	Keratin, type II cuticular Hb3 OS = Bos taurus GN = KRT83 PE = 2 SV = 1	54.0	1863	42	37.3
P02534	Keratin, type I microfibrillar 48 kDa, component 8C-1 OS = Ovis aries PE = 1 SV = 2	46.6	2737	55	46.1
P04258	Collagen alpha-1(III) chain OS = Bos taurus GN = COL3A1 PE = 1 SV = 1	93.6	372	8	7.7
B0LKP1	Keratin, type I cuticular Ha5 OS = Ovis aries GN = KRT35 PE = 2 SV = 1.	50.4	665	13	16.7
Q5XQN5	Keratin, type II cytoskeletal 5 OS = Bos taurus GN = KRT5 PE = 1 SV = 1	62.9	726	17	24.0
A1L595	Keratin, type I cytoskeletal 17 OS = Bos taurus GN = KRT17 PE = 2 SV = 1	48.7	480	11	19.5
Q1ZZU7	Macrophage migration inhibitory factor OS = Ovis aries GN = MIF PE = 3 SV = 1	12.3	169	3	27.8

The presence of BHB and cP3HB in Saiga horn extract might explain its antipyretic and procoagulant activities.

BHB has been shown to reduce significantly LPS-induced protein and mRNA expression levels of iNOS, COX-2, TNF-α, IL-1β and IL-6 both in BV-2 cells and in rats [45,46].

In vivo study of endotoxin-induced rabbit fever found that Saiga horn extract inhibited the production of TNF-α and PGE2, thereby relieving fever [12]. Another study that evaluated the effect of animal horns on yeast-induced fever in rats found that one hour after the oral administration, Saiga horn extract significantly reduced the levels of TNF-α and PGE2. Two hours after the oral administration, the levels of TNF-α and IL-6 were significantly reduced while the level of PGE2 returned to a level similar to the fever group [6]. Again, this indicates that the fever-lowering effect may be attributed to BHB.

Finally, a metabolomic study showed that WBH extract affects arachidonic acid metabolism and oxidative stress in yeast-induced pyrexia rats. Arachidonic acid is stereospecifically oxygenated by COX-2 to prostaglandins or by lipoxygenase to leukotrienes. Therefore, the inhibition of COX-2 will lead to decreased production of prostaglandins, an effect observed for both Saiga and WBHs. This may indicate that not only Saiga horn but also other animal horns produce their antipyretic activity due to BHB and cP3HB. In principle, it is in agreement that biologically active compounds are the same in horns of many animals [10].

On the other hand, it should be noted that macrophage migration inhibitory factor found in some extracts stimulates the expression and secretion of the proinflammatory cytokines such as TNF-α, IL-6 or COX-2 [47].

Saiga horn extract was also shown to exhibit significant procoagulant activity [6]. Interestingly, poly-3-hydroxybutyrate was reported to activate the coagulation system [48]. We can speculate that the procoagulant effect of Saiga horn extract may be attributed to COX-2 inhibition by BHB, which suppresses endothelial prostacyclin (PGI2) synthesis [49].

Surprisingly, one study dealing with possible substitutes for endangered medicinal animal horns and shells suggested goat horn is explored as a substitute for Saiga horn based on their alleged antithrombotic and anticoagulation effects. However, in our opinion, data and results published in the study do not support this conclusion as they show that Saiga horn does not significantly increase thrombolysis percentage and SPBC values, neither does it prolong plasma recalcification time and thrombin time compared with the saline group. Goat horn, on the other hand, caused highly significant increases of all of these values compared with the saline group [11].

In vivo study on mice revealed a decreased concentration of cAMP in brain tissue and in serum (compared with the control group) after seven days of administration to Saiga horn extract [12]. Similarly, BHB decreased intracellular cAMP levels in dairy cow anterior pituitary cells [50].

The results of this study also confirmed that collagens (α1, type I and α2, type I) and keratins (type I cuticular, type I microfibrillar, type I cytoskeletal, type II cuticular, type II microfibrillar and type II cytoskeletal) are the main constituents of Saiga horn as we previously reported in another study, which was performed on an ion-trap mass spectrometer [15]. In addition to that, we have now been able to identify two other proteins – collagen α1, type III and macrophage migration inhibitory factor.

5 | CONCLUDING REMARKS

It is, perhaps, not a coincidence that the biological effects of Saiga horn extract and BHB are similar to each other. In fact, we strongly believe that BHB and cP3HB, which were detected in Saiga horn extracts, can be responsible for many biological actions thereof.

In this case, 3-hydroxybutyrate appears to be a cheap substitute for Saiga horn. Using synthetic 3-hydroxybutyrate or cP3HB instead of Saiga horn would also have numerous other advantages. One of them is higher concentration and effectiveness, which is achievable with synthetic hydroxybutyrates. The other one is, of course, the possibility easily to achieve higher purity of the final product, which can be a major problem regarding some TCM preparations. This would make 3-hydroxybutyrate an ideal substitution for Saiga horn.

Although the results of this work are encouraging, more research should be done, particularly on comparing biological actions of animal horns' extracts with respect to the content of BHB and cP3HB and establishing a possible correlation between the content of cP3HB and the degree of efficacy.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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