Screening for performance enhancing substances and quantification of ethanol in different arishta manufactured in Sri Lanka

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Abstract

Background Arishta have been used in ayurveda medicine for over thousands of years in Sri Lanka to treat various diseases. Ashwagandharishta, Balarishta and Dashamoolarishta are usually prescribed to obtain an anabolic effect, and Ashwagandharishta and Dashamoolarishta for androgenic effect in males. Thus, these arishta have shown similar effects as anabolic androgenic steroids and stimulants in western medicine. Therefore, arishta could potentially be used by athletes to improve their performance in sports leading to unintentional doping. Additionally, ethanol develops in-source during arista fermentation, which can affect athletes health.

Objective The aim of this study was to investigate whether the anabolic androgenic steroids or stimulants banned by World Anti-Doping Agency (WADA) were present in these arishta, and to determined their ethanol content.

Methods Methanol extractions of Ashwagandarishta, Balarishta and Dashamoolarishta from four different manufacturers were screened for 21 stimulants and 22 anabolic androgenic steroids banned by WADA, using Gas Chromatography Mass Spectrometer (GC/MS). Ethanol content of the twelve arishta samples were also measured.

Results Anabolic androgenic steroids or stimulants were not present in the tested arishta samples, and percentage volume by volume (v/v) ethanol content of all arishta samples were between (5.80-8.35) ±0.5.

Conclusion The tested brands of Ashwagandharishta, Balarishta and Dashamoolarishta did not contain stimulants or anabolic androgenic steroids banned by WADA.

Ceylon Medical Journal 2020; **65:** 112-117

DOI: http://doi.org/10.4038/cmj.v65i4.9282

Introduction

Athletes around the world use different techniques to win medals. Among them, commercially available supplements, ayurvedic medications, traditional remedies, and designer drugs are very popular. WADA operates with the aim of bringing consistency to anti-doping policies and regulations within sport organizations and governments across the world [1]. They publish annually a list of substances prohibited for use by athletes, considering that they may artificially increase an athlete's performance [1]. In Sri Lanka, the ayurveda medicinal system has been well established for thousands of years and has been successfully treating various diseases. According to the ayurveda, the human body consists of three dosa (parts) that is watha, pitha, sema. Watha refers to the energy generated by vibration and movement [2]. In western medicine this is referred to as boosting body strength, which is done by stimulants and anabolic androgenic steroids [2]. In ayurvedic practice, watha diseases are treated by Ashwagandarishta, Balarishta and Dashamoolarishta [3]. In addition to that Dashamoolarishta is prescribed for improving sperm count in males [3], similar to the use of anabolic androgenic steroids in western medicinal practice [2]. These three arishta are available in ayurvedic drug stores as over-the-counter medications. Therefore, sportsmen and sports women are able to consume these arishta in Sri Lanka in order to enhance their performance, or as a form of treatment. Arishta is produced by fermentation. Thus, the exact chemical composition of these fermented products is not known by the user. The ingredients of all these three arishta are given by the name of the plant material [3]. Athletes who use these arishta would be yielded a positive result in a urine test due to the consumption of these arishta. This unintentional or intentional doping might lead to the

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athlete being found guilty of an adverse analytical finding, which would have adverse effects on the athlete's reputation. Therefore, it is important to screen these arishta for anabolic androgenic steroids and stimulants prohibited by WADA. During the arishta manufacturing process, plant material undergoes self-fermentation leaving self-generated ethanol as the end product. Ashwaganda arishata manufactured in Sri Lanka contains a percentage mean ethanol content of 6.55 ±0.87 (v/v) [4]. The long-term usage of arishta with high ethanol content, may affect the liver and other organs of humans. The sportsmen might be consumed arishta for long time to enhance performance, being unaware of the ethanol content. Another objective of our study was to determine the ethanol content in these three arishta preparations manufactured in Sri Lanka.

There are several ayurvedic drug manufacturers in Sri Lanka who export the ayurvedic products to highly competitive foreign market. With the commercial pressure some manufacturers tend to adulterate these products, in order to increase the efficacy of the products, as compared with western medicine. Four brands from three arishta, manufactured by ayurvedic drug manufacturers in private sector and the state sector were screened in this study.

Materials and Methods

Chemicals and reagents

Four brands from each arishta (coded as A, B, C, D) manufactured according to the ayurvedic recipes, were used for this study (Table 1).

Certified reference standards of the stimulants and anabolic androgenic steroids used in this research were purchased from National Measurement Institute (Australia). Analytical-grade sodium chloride, potassium hydroxide, hydrogen chloride (36% w/w), methanol (99.8% w/v) was from Sigma Aldrich, ethanol (99.8% w/v), tert-butyl methyl ether (100% w/v) and n-pentane (99% w/v) were from VWR BDH Chemicals. Ammonium iodide and N-methyl-N-trimethylsilyl-trifluoroacetamide were purchased from Fluka Analytical. Deionized water was produced for this study from ultra high-water purification system (Thermo Fisher Scientific).

Qualitative analysis of anabolic androgenic steroids and stimulants in arishta

For the determination of anabolic androgenic steroids and stimulants in arishta, were performed by gas GC/MS [5,6].

Table 1. Arishta taken for the study: Ashwagandarishta, Balarishta and Dashamoolarishta

Arishta	*Code of the Manufacturer*	*Nature of the sample*	*Batch No*
Ashwagandaarishta	A	Dark brown colour liquid	022938
Ashwagandaarishta	B	Dark brown colour liquid	483
Ashwagandaarishta	C	Dark brown colour liquid	6175
Ashwagandaarishta	D	Dark brown colour liquid	11444
Balarishta	A	Dark brown colour liquid	021836
Balarishta	B	Dark brown colour liquid	577
Balarishta	C	Dark brown colour liquid	6093
Balarishta	D	Dark brown colour liquid	11487
Dashamoolarishta	A	Dark brown colour liquid	022712
Dashamoolarishta	B	Dark brown colour liquid	714
Dashamoolarishta	C	Dark brown colour liquid	1254
Dashamoolarishta	D	Dark brown color liquid	11390

Extraction of anabolic androgenic steroids

For the extraction of anabolic androgenic steroids in arishta, 5.0 mL sample from each arishta was vortexed with 5.0 mL of methanol. Then, 500.0 µL of methanolic layer was separated and evaporated to dryness using nitrogen gas. Then, 0.1 M potassium hydroxide and 5.0 mL of n-pentane were added into the residue and resultant solution was vortexed for 10 minutes. The n-pentane layer was separated and again vortexed with 2.0 mL of methanol for another 10 minutes. The resultant methanol layer was separated and evaporated to dryness on a steam bath and the residue was derivatized using 50.0 µL N-methyl-N-trimethylsilyl-trifluoroacetamide/ammonium iodide/ ethanol (1000:2:3) solution for 20 minutes at 60°C. The derivatized mixture was analysed using GC/MS.

Extraction of stimulants

For the extraction of stimulants in arishta, 5.0 mL sample from each arishta was vortexed with 5.0 mL of methanol. Then 500.0 µL of methanolic layer was separated and mixed with a drop of hydro chloric acid in ethanol. It was evaporated to dryness using nitrogen gas. Next 5.0 mL of 5 M potassium hydroxide and one spatula of sodium chloride and tert-butyl methyl ether were added in to the residue. The resultant mixture was vortexed for 10 minutes. The organic layer was separated and analysed using GC/ MS after concentration.

Separation of anabolic androgenic steroids and stimulants using gas chromatography – mass spectrometry

Separation of stimulants and anabolic androgenic steroids were performed using an Agilent 7890A gas chromatography coupled to an Agilent 5975 mass spectrometry detector from Agilent Technologies, equipped with a 30 m HP-5 mass spectrometry capillary column (internal diameter 0.25 mm, film thickness 0.25 µm). Helium was the carrier gas at a linear velocity of 1 mL per minute. The injection volume was 1.0 µL and injection temperature was 250°C, spitless. The GC/MS oven temperature was programmed as follows: initially at 60°C for 30 seconds and increased up to 280°C at a rate of 12°C per minute. It was held isothermally for 30 minutes. The total running time for this separation was 48.33 minutes. The temperature of the mass spectrometry detector's transfer line, mass spectrometry source and mass spectrometry quadrupole were maintained at 280°C, 230°C, 150°C respectively.

Quantification of ethanol in arishta

Quantification of ethanol in arishta [5], was carried out using Agilent 7890A gas chromatography coupled with Agilent 7697A head space analyser and flame ionization detector from Agilent Technologies, equipped with a 50 m HP-FFAP capillary column (internal diameter 0.2 mm, film thickness 0.33 µm). Concentration of 1% solution were prepared from each arishta mixing with deionized water. Extraction of ethanol from arishta was carried out injecting 5.0 mL of each solution in to the head space analyzer. The filling pressure of the sample was maintained at 103 K Pa with single extraction of 1 mL loop size. Nitrogen was used as the carrier gas at a linear velocity of 30 mL per minutes. Vial equilibration, injection duration and gas chromatography cycle were set as 15 minutes, 0.5 minutes, 35 minutes respectively during the extraction. The temperature of the oven, loop, transfer line (DB-ProSteel, diameter 0.53 mm) was maintained at 85°C, 85°C, 100°C respectively during the extraction.

The extract was injected into the gas chromatography for the separation of ethanol at injector temperature of 225°C, using split injection mode (split ratio 50:1). The carrier gas used in the gas chromatography was nitrogen, at a linear velocity of 1.20 mL per minute. The oven temperature of gas chromatography was held isothermally at 60°C for 4 minutes and increased at a rate of 6°C per minute up to 200°C and it was again held at 200°C for another 2 minutes. Total running time for this separation was 34.33 minutes and the detector temperature were kept at 285°C during the separation.

Preparation of calibration curve

A series of calibration standards with percentage concentration of 0.004%, 0.01%, 0.02%, 0.05%, 0.1%, 0.2%, 0.5% and 1% v/v were prepared mixing ethanol and deionized water. Then 5.0 mL of each solution was injected in to gas chromatography – head space analyser and chromatogram was recorded.

Results

Qualitative analysis of arishta

The GC/MS analysis revealed that all three arishta from four different brands contain glycerol, while they were free from anabolic androgenic steroids and stimulants listed in Table 2.

Quantification of ethanol in arishta

The data of the calibration curve is given in the (Table 3 and the calibration curve of ethanol standards is given in the Figure 1.

Table 2. Anabolic androgenic steroids and stimulants screened in arishta

Name of the Anabolic Androgenic steroids or Stimulant	*Presence*	*Name of the Anabolic Androgenic AAS or Stimulant*	*Presence*
17a-Hydroxyethyl-5a-estrane-3a,17b-diol	No	6b-Hydroxymethandienone	No
Boldenone (1,4-Androstadiene-17b-ol-3-one)	No	5b-Androst-1-en-17b-ol-3-one	No
Androstedione	No	4-Chloro-4-androsten-3a-ol-17-one	No
Testosterone	No	17-Epimethandienone	No
2a-Hydroxymethylethisterone	No	17a-Ethyl-5a-estrane-3a,17b-diol	No
Methyldienolone	No	1a-Methyl-5a-androstan-3a-ol-17-one	No
17b-Hydroxy-17a-methyl-5a-androst-1-ene-3-one	No	1a-Methyl-5a-androstan-3a-17b- diol	No
1,4-Androstadiene -3, 17-dione (Boldendione)	No	19-Norandrosterone	No
Gestrinone	No	Etiocholanolone	No
4-Hydroxynandrolone (Oxabolone)	No	Androsterone	No
Norbolethone (13b,17a-diethly-gonan-4-ene-17b-ol-3-one)	No	Epitestosterone	No
1-Testosterone (5a-Androst-1-en-3-one-17ß-ol)	No	Mestanolone	No
19-Norandrostendione (Estrendione)	No	Oxymetholone	No
17a-Trenbolone (Epitrenbolone)	No	Fencamine	No
Stanozolol	No	Fenproporex hydrochloride	No
5a-Androstane-3b,17b-diol	No	Fenethylline hydrochloride	No
5a-Androstane-3b,17b-diol	No	Famprofazone	No
7b,17a-Dimethyl-5b-androstane-3a,17b-diol	No	Heptaminol hydrochloride	No
2-Hydroxymethyl-17a-methylandrostadiene-11a, 17b-diol-3-one	No	Isometheptene mucate	No
16b-Hydroxystanzolol	No	Mefenorex hydrochloride	No
17-Epioxandrolone	No	(+)-4-Methylamphetamine hydrochloride	No
1-Methylene-5a-androstan-3a-ol-17-one	No	(+)- Norpseudoephedrine hydrochloride(Cathine)	No
Calusterone	No	Selegiline hydrochloride	No
9a-Fluoro-17a-methyl-4-androsten-3a,6b,11b,17b-tetra-ol	No	Benzylpiperazine	No
6b-Hydroxy-oral turinabol	No	Methylephedrine	No
7a,17a-Dimethyl-5b-androstane-3a,17b-diol	No	Etilamphetamine	No
Oral turinabol	No	Methylenedioxyamphetamine	No
16b-Hydroxyfurazabol	No	Parahydroxyamphetamine	No
3'- Hydroxystanozolol	No	Oxilofrine hydrochloride	No
9a-Fluoro-17,17-dimethyl-18-nor-androstan-4,13 -diene-11b-ol-3-one	No	Modafinil	No
2a-Methyl-5a-androstan-3a-ol-17-one	No	Fenfluramine	No
		Phentermine	No
		Methylphenidate	No
		Sibutramine	No

Table 3. Quantification results of ethanol standards

Calibration Levels	*Percentage of ethanol % (v/v)*	*Area*
Level-1	0.004	20.89680
Level-2	0.01	51.85415
Level-3	0.02	104.37607
Level-4	0.05	263.82648
Level-5	0.1	538.66351
Level-6	0.2	1094.35632
Level-7	0.5	2788.11865
Level-8	1	5571.76074

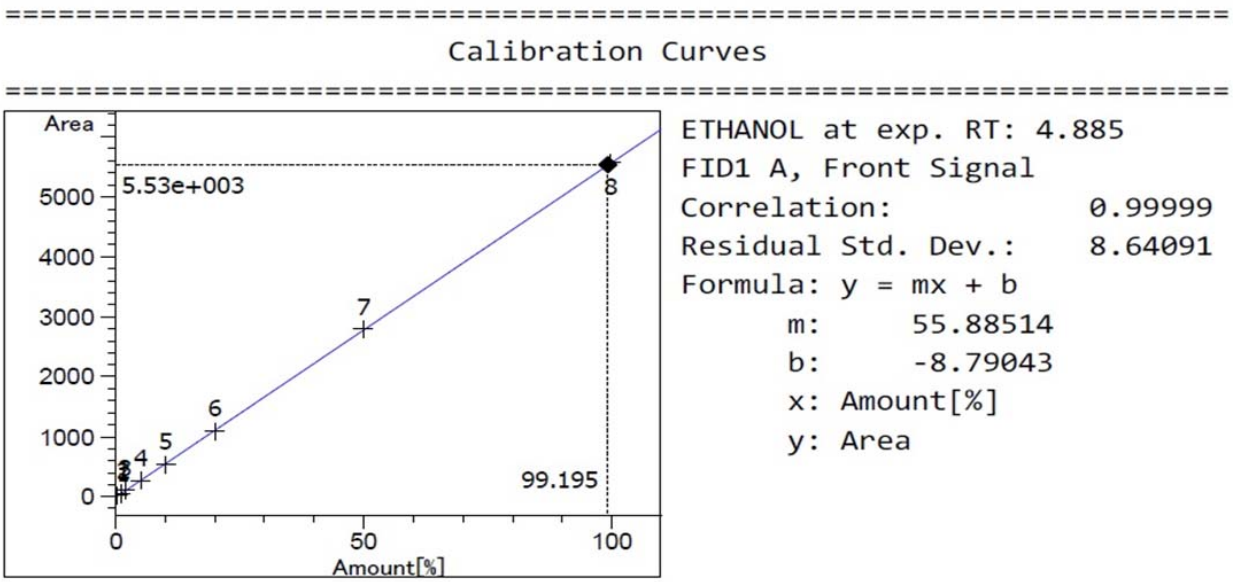

Figure 1. **Calibration curve of ethanol standards.**

Ethanol was identified in all arishta samples and ethanol content in each arishta is given in the Table 4. Percentage of ethanol content of all arishta samples were in between (5.80-8.35) ±0.5 v/v and the limit of detection of ethanol in the gas chromatography method was 2 µg/ml. The gas chromatogram of an arishta sample is given in the Figure 2.

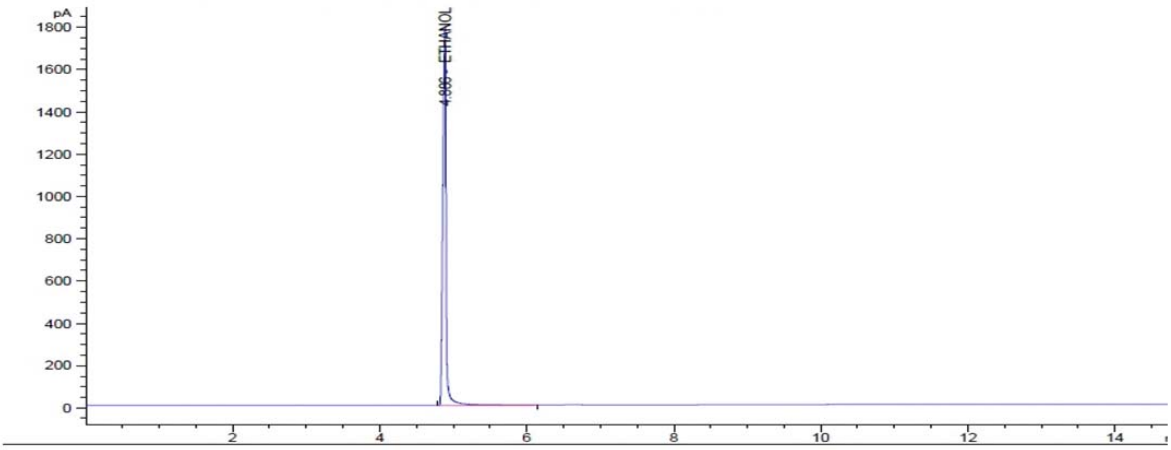

Figure 2. **GC-HS chromatogram of an arishta samples.**

Table 4. Quantity of ethanol in Arishta samples

Sample name	*Percentage of ethanol (% ± 0.50, v/v)*
Ashwagandarishta A	6.96
Ashwagandarishta B	7.84
Ashwagandarishta C	9.52
Ashwagandarishta D	9.59
Balarishta A	7.05
Balarishta B	7.84
Balarishta C	8.80
Balarishta D	9.65
Dashamoolarishta A	5.84
Dashamoolarishta B	5.58
Dashamoolarishta C	5.98
Dashamoolarishta D	5.80

Discussion

Ashwagandharishta, Balarishta and Dashamoolarishta had been prescribed to get anabolic and androgenic effects in humans from ancient times [3]. Even though the effects were claimed by the ayurvedic doctors in their clinical practices there were no clinical studies to prove these biological effects in arishta. This study reveals that none of the WADA prohibited anabolic androgenic steroids and stimulants were present in the tested arishta formulas. The aforesaid biological effects might be generated either by the prohormone or precursor molecules of anabolic androgenic steroids or due to the effect of some other chemical. Further studies should be carried out to identify the chemical species which are responsible for those effects.

All these three arishta contained glycerol, which was a prohibited substance for sportsman according to the WADA list published in year 2017 [7]. However, WADA revises its prohibited list every year, and the list published in year 2018 [8] did not declare glycerol as a prohibited substance.

The alcohol content in the same type of arishta was observed to be varied with the manufacturer. This might be due to difference in plant matter used to manufacture arishta. There are slight variations in the composition of the plant matter based on geological factors or season of the year of cultivation.

Conclusion

This study showed the absence of anabolic androgenic steroids or stimulants in Ashwagandarishta, Balarishta and Dashamoolarishta samples as per the WADA prohibited list. There were no evidence of adulteration of the tested batches of different arishta brands with anabolic androgenic steroids or stimulants prohibited by the WADA. However, the percentage ethanol content was between (5.80-8.35) ±0.5 v/v in all three arishtas.

Conflicts of interest

There are no conflicts of interest.

Funding

No source of funding.

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