Journal of Pharmaceutical Research International

21(4): 1-15, 2018; Article no.JPRI.39497
ISSN: 2456-9119
(Past name: British Journal of Pharmaceutical Research, Past ISSN: 2231-2919, NLM ID: 101631759)

Studies of Heavy Metal Contents and Microbial Profile in Selected Pediatric Oral Liquid Preparations Available in Bangladesh

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Authors' contributions

This work was carried out in collaboration between all authors. Author PS conceived of the presented research project. Author MMH performed the experimental work (microbiological work was supervised by SN and analytical work by TRC). Authors MMH, NU, AFMMH and AS managed the literature searches and performed the statistical analysis. Author MMH wrote the first draft of the manuscript which was corrected by SN. Overall research project was supervised by PS and MS. Authors PS, MS, SN and TRC finalized the final drafting of the manuscript. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JPRI/2018/39497
Editor(s):
(1) Nawal Kishore Dubey, Professor, Centre for Advanced Studies in Botany, Banaras Hindu University, India.
Reviewers:
(1) Falusi Bamidele Ayodeji, Federal College of Education, Nigeria.
(2) Kritika Subramanian, St. George's University School of Medicine, Grenada.
(3) Izharul Hasan, India.
Complete Peer review History: http://www.sciencedomain.org/review-history/23602

Original Research Article

Received 5th December 2017
Accepted 17th February 2018
Published 12th March 2018

ABSTRACT

Thirty different Pharmaceutical, Complementary and Alternative Medicine (CAM) products were tested for heavy metal contents and microbiological profile using standard methods. Among the investigated eleven heavy metals, seven (Cr, Co, Cu, Mn, Fe, Ni and Zn) were in detectable level.

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However, according to the manufacturers' recommended dose, 27.78% of pharmaceutical products (A-02, A-05, A-07, A-08, A-09) have crossed the oral Permissible Daily Exposure limit of ICH (International Conference on Harmonization) guideline for cobalt. However, in all CAM products, level of all tested heavy metals was within the permissible limit of United States Pharmacopoeia (USP), ICH and European Medical Agency (EMEA) guidelines. Most pharmaceutical and CAM products crossed the USP, British Pharmacopoeia (BP) and World Health Organization (WHO) acceptable limit for the total aerobic microbial count (TAMC). Pathogenic *Escherichia coli* was found in one Pharmaceutical (A-07) and two CAM products (D-06, D-08). *Salmonella* and *Shigella* spp. were absent in all tested products. In total combined yeast and mould count (TYMC) few pharmaceutical (A-03, A-07, A-14), as well as CAM products (D-02, D-06, D-08), were beyond USP, BP and WHO acceptable limits. Both pharmaceutical and CAM manufacturers should strictly follow the Current Good Manufacturing Practice to ensure the quality and safety of pediatric preparations.

Keywords: Heavy metals; microbial profile; pediatric preparations; complementary and alternative medicine.

1. INTRODUCTION

Toxic exposures from contaminated everyday items like medicines are of increasing concern for the safety of public health [1,2]. Studies were done to find various harmful heavy metals like lead, cadmium, arsenic and mercury as well as other synthetic agents in our everyday used products [3,4]. More reports were also published and found different types of toxic metals in our life-saving drugs at an alarming level [5]. In other studies, toxic heavy metals were found in pharmaceuticals [6] and another traditional system of medicines [7,8] in Bangladesh, India and Pakistan. Heavy metals can cause a toxic effect like chronic degenerative changes in different organs [9] by accumulating in our body. Moreover, they are also responsible for carcinogenic and teratogenic effects [10]. However, some of the heavy metals like iron, cobalt, copper etc. have very important biochemical and physiological role in the human body but still, they exert toxicity when they cross a certain limit [11].

Besides microbial contamination of pharmaceuticals and Natural Health Products (NHPs) are also become very common. The use of contaminated medicinal preparations has proved hazardous to the health of the users. There have been reports of drug-borne human infections worldwide [12]. Contamination of medicinal products with microorganisms can also bring about changes in their physical characteristics, including breaking of emulsions, thinning of creams, the appearance of turbidity or deposit, and changes in odour and colour [13].

Nowadays it has become a dangerous issue for the people especially for the children because they are the most sensitive to toxic heavy metals and more prone to microbial infections. Moreover, microbial contamination in pediatric preparations has become a burning issue especially in developing and underdeveloped countries. It is imperative to investigate the presence of heavy metal and to determine microbial profile in the medicines that are commonly used in children. Unfortunately, to our best knowledge, there is no specific study done till now on heavy metal contents, and only a few studies have been carried out to determine microbial profile in pediatric preparations available in Bangladesh. Our current endeavour is to investigate the presence of heavy metals and to determine microbial profile in pediatric preparations and finally compare the findings with the current guideline of regulatory authorities for ensuring the safety of children.

2. MATERIALS AND METHODS

2.1 Study Area and Sampling

Among thirty different types of pediatric oral liquid preparations, 18 of them were pharmaceutical, and 12 were Complementary and Alternative Medicine (CAM) products. Total 120 samples (30x4; four samples from each product having the same batch number) were collected from different pharmacy shops of Savar area, Dhaka. The pharmaceutical preparations include nine paracetamol and nine anti-histamine products prepared by different pharmaceutical companies. Pharmaceutical products are coded with A (A-01 to A-18). The CAM includes two herbal, two ayurvedic and eight Unani systems of medicines. CAM products are coded with B (B-01, B-02) for Herbal products; C (C-01, C-02) for

Ayurvedic products and D (D-01 to D-08) for Unani products.

2.2 Evaluation of Metallic Contents

The study was designed to investigate heavy metal contents of both pharmaceutical and CAM products. The level of eleven heavy metals lead (Pb), cadmium (Cd), chromium (Cr), cobalt (Co), copper (Cu), manganese (Mn), nickel (Ni), zinc (Zn), iron (Fe), arsenic (As) and mercury (Hg)) in the products were studied. The study was conducted in Analytical Chemistry Laboratory, Chemistry Division, Atomic Energy Centre, Dhaka.

2.2.1 Chemicals

An individual standard solution of target element was supplied by Varian Inc, the USA with highest purity level (99.98%). The HNO_3, $HClO_4$ and other chemicals were extra pure or supra pure and purchased from Merck, Germany.

2.2.2 Sample preparation

At first 1 g samples was taken in a clean glass beaker. About eight mL acid mixture (HNO_3**:** $HClO_4$**=** 4**:**1) were added to the sample. This sample and acid mixtures were heated on a hot plate at 110^{o}C. The heating was continued for about 6 hours to make it near to dryness. Sufficient deionized water was added to bring it ten mL and transfer into 25 mL vial. Finally, the sample was prepared for heavy metal analysis by filtering through Whatman filter paper. For heavy metal analysis, the samples were aspirated through a nebulizer, and the absorbance was measured against a blank as a reference. Specific hollow cathode lamps were used to analyze Pb (wavelength 217.0 nm), Cd (wave-length 228.8 nm), Cr (wavelength 357.9 nm), Co (wavelength 240.7 nm), Cu (wavelength 324.8 nm), Mn (wavelength 279.5 nm), Fe (wavelength 248.3 nm), Ni (wavelength 232.0 nm), Zn (wavelength 213.9 nm), As (wavelength 193.7 nm) and Hg (wavelength 253.7 nm). Before analysis, the samples were diluted to the appropriate factor according to the detection limit of the Atomic Absorption Spectrophotometer. A calibration curve was obtained using reference standard, and all the measurements were run in triplicate for the samples and standards solutions. Flame Atomic Absorption Spectroscopy (FAAS) (Model No.- AA240FS, manufacturer- Varian, USA) was used for the detection of Pb, Cd, Cr, Co, Cu, Mn, Fe, Ni and Zn. The As and Hg levels in the samples were measured by using Hydride Generation Atomic Absorption Spectroscopy (HGAAS) (Model No.- AA240, manufacturer- Varian, USA) and Cold Vapor Atomic Absorption Spectroscopy (CVAAS) (Model No.- novAA350, manufacturer- Analytik Jena, Germany), respectively.

2.3 Evaluation of Microbial Profile

2.3.1 Media used in microbiological tests

Different growth media like Nutrient Agar (NA) (Himedia, India), MacConkey Agar (MAC) (Himedia, India), Eosine-methylene blue agar (EMB) (Himedia, India), Xylose lysine deoxycholate (XLD) (Oxoid, England) and Mannitol Salt Agar (MSA) (Oxoid, England) were used for the evaluation of total viable aerobic bacteria, gram-negative bacteria, *Escherichia coli* (*E. coli*)*/Enterobacter*, *Salmonella* and *Shigella, Staphylococcus aureus* (*S. aureus*) count, respectably. Besides, Tetracycline-Potato Dextrose Agar (PDA) was used for total fungi count. The microbial profiles of the products were investigated in the lab of the Department of Microbiology, Jahangirnagar University, Savar, Dhaka-1342.

2.3.2 Sample preparation

Each sample was shaken properly then 1 mL of the liquid sample was aseptically transferred into a sterile tube containing 9 mL of Trypsin Soya Broth (TSB). This tube was incubated in an incubator at 37^{o}C for 20 min to resuscitate but not to promote the growth of microbial species. Then vortexed it and 1 mL was transferred from this TSB tube into another tube containing 9 mL sterile distilled water [14] and subsequently ten-fold serial dilution was carried out up to 10^{-10}.

2.3.3 Enumeration of total viable aerobic bacteria, gram-negative bacteria, E. coli / Enterobacter, Salmonella & Shigella, S. aureus and fungi

From every dilution of each sample, 100 µL samples were spread into different media. All the plates were incubated at 37 °C for 24 hours except PDA which was incubated at 27°C for 72 hours. Suitable dilutions yielding <300 colonies were counted. The procedure was repeated for another sample of the same batch of each type of product. The arithmetic mean of the counts

was taken, and a number of colonies forming units per mL (CFU/mL) were calculated [15].

2.3.4 Identification of bacteria

Identification was performed morphologically, microscopically and biochemically [15]. Morphological identification was based on size, diameter, colour and elevation of the colonies [15]. Bright pink colonies on MacConkey agar media and yellow colonies with yellow zones on the Mannitol media were suspected as the growth of *E. coli* and *S. aureus*, respectively. Microscopical identification was performed by gram staining. Red or pink stained microorganisms indicated gram-negative bacteria; on the other hand, dark-purple stained microorganisms indicated gram-positive bacteria. The bacterial isolates were confirmed as *E. coli* and *S. aureus* by various biochemical tests such as IMViC tests (indole, methyl red, Voges–Proskauer and citrate utilization tests), catalase test and oxidase test. To perform indole test organism was inoculated into broth media and incubated at 37^0C for 36 h. After incubation, Kovac's reagent was added to the broth, development of cherry red colour indicated a positive result. Indole test was carried out to detect *E. coli*. The methyl red (MR) and Voges–Proskauer (VP) test were read from a single inoculated tube of MR–VP broth. After 24–48 h of incubation the MR–VP broth was split into two tubes. One tube was used for the MR test; the other was used for the VP test. Upon addition of pH indicator methyl red, development of red indicated a positive MR test. The reagents used for the VP test were Barritt's A (alpha-naphthol) and Barritt's B (potassium hydroxide), development of pink-burgundy color indicated a positive VP test. For citrate utilization test, bacteria were inoculated on a media containing sodium citrate and a pH indicator bromothymol blue. In presence of enzyme citrate, the medium pH turned into alkaline. This alkaline pH changed bromothymol blue indicator incorporated into the medium from green to deep Prussian blue indicated a positive citrate utilization test. To perform catalase test, a few drops of hydrogen peroxide were added onto the clean microscopic slide. Then bacterial colonies were touched and smeared using inoculation loop into the drop of hydrogen peroxide. Production of any bubbles, the organism was assumed as 'catalase positive'. If not, the organism was 'catalase negative'. To perform oxidase test, a drop of oxidase test reagent was added onto a filter paper. Using an inoculation loop, a large mass of pure bacteria was aseptically transferred to the filter paper. The site of inoculation was observed for up to 10–30 s. The area of inoculation turned into pink to maroon to almost black, indicated the organism was 'oxidase positive'. The unchanged color of the area of inoculation indicated the organism was 'oxidase negative'[16].

2.4 Statement of Human and Animal Rights

This article does not contain any studies with human and animal subjects performed by any of the authors.

2.5 Statistical Analysis

Data were expressed as Mean ± SD (Standard deviation). Statistical program used was Microsoft Office Excel 2007.

3. RESULTS

3.1 Results of Heavy Metal Contents

All the pharmaceutical and CAM products were tested to determine the heavy metal contents of the samples (Fig. 1). When comparing the number of heavy metals in the 18 pharmaceutical products, Iron was found to have the greatest mean and largest standard deviation. In case of CAM products, Iron was also found to have the greatest mean and largest standard deviation. The detected metals were Cr, Co, Cu, Mn, Fe, Ni and Zn. In case of pharmaceutical products, the highest Cr level was found in A-03 (1.480 ppm), and the lowest was in A-02 (0.030 ppm). The Co level ranged from 0.030 to 0.400 ppm. Only one pharmaceutical product (A-17) had a detectable level of Cu (0.140 ppm). About 80% of the pharmaceutical products had no detectable level of Mn. The presence of Fe, Ni and Zn were detected in all the tested samples. The maximum levels of Fe, Ni and Zn, were 7.870, 1.100 and 0.790 ppm respectively. All the pharmaceutical products have no detectable level of Pb, Cd, As and Hg.

Among the 11 heavy metals, the presence of 07 (Cr, Co, Cu, Mn, Fe, Ni and Zn) heavy metals were detected. All the CAM products have the no detectable level of Pb, Cd, As and Hg.In case of CAM products, D-02 (0.700 ppm) and B-02 (0.580 ppm) showed the highest level of Cr and

Co, consecutively. About 16.66% CAM products have no detectable level of Cr, but all the products have a detectable level of Co. Seven products (58.33%) have detectable Cu level and the highest level was 0.200 ppm. Product D-03 (3.370 ppm) showed the highest level of Mn. All the CAM preparations have a detectable level of Fe, Ni and Zn. The highest detected level of Fe, Ni and Zn were 13.850, 0.760 and 1.863 ppm respectively.

3.2 Results of Microbial Profile

Fig. 2a, Fig. 3a and Table 3 represents the result of the microbial profile of pharmaceutical products. Highest TAMC 2.3×10^6 CFU/mL was contained in product A-07. About 3 out of 18 products did not show any aerobic count. Only one product (5.56%) showed the growth of *E. coli/ Enterobacter. Salmonella* and *Shigella* spp. were absent in all the tested products. About 16.67% products showed the presence of *S. aureus.* The fungus was present in 3 out of 18 pharmaceutical products (Table 3).

Microbial profile of CAM products is presented in Figure 2b, Figure 3b and Table 4. Product D-06 shows the highest TAMC (4.8×10^8 CFU/mL). Eleven products out of 12 showed total microbial growth in Nutrient Agar media. The *E. coli/ Enterobacter* were found in 16.67% products. All of the CAM products were free from *Salmonella* and *Shigella* sp. The *S. aureus* and fungus were present in 33.33% and 25% of the CAM products respectively.

4. DISCUSSION

4.1 Evaluation of Heavy Metallic Contents

Metallic elements having a specific density of more than 5 g/cm^3 are normally known as heavy metals [17]. It is assumed that heaviness and toxicity are inter-related [18]. Some heavy metals like mercury, cadmium and lead exert a toxic effect, but unfortunately, they have no useful biochemical and physiological functions [19]. Toxic metals pose particular risks to the very young, as exposures early in life compromise development, with lifelong physical, intellectual, and behavioural impairments [19]. The International Agency for Research on Cancer (IARC) classifies cadmium as a known carcinogen, inorganic lead a probable carcinogen, and methylmercury a possible carcinogen [20]. Cadmium toxicity may cause kidney and skeletal damage, epigenetic changes in DNA expression, hypertension, diabetes, apoptosis, and insulin resistance [21,8]. Lead can form complex with different biomolecules and affect their functions. Furthermore, various complications like reproductive defects, hearing and vision problem, brain and kidneys damage and poor muscle coordinations can also occur with excess lead exposure [8].Mercury is toxic in its all forms and mostly shows its toxicity in the gastrointestinal tract, nervous system and in the kidney [22].Inorganic arsenic is acutely toxic, and there is an increased risk of mortality from lung, bladder and kidney cancer with the people who exposed to arsenic via drinking water. Skin-related complications like skin cancer and lesions in the skin are also increased with highly arsenic exposed persons [23]. Previusly the presence of lead, cadmium, mercury and arsenic in medicines was reported in different countries including England, China, Malaysia, Mexico, Nigeria, India etc. [5, 24,25]. Our current study reveals that all the tested samples of pharmaceutical and CAM preparations for cadmium, lead, mercury and arsenic levels were below the detection limit of the equipment, indicating their safe use.

It has been reported that for different biochemical and physiological functions some other heavy metals such as cobalt (Co), copper (Cu), chromium (Cr), iron (Fe), manganese (Mn), nickel (Ni) and zinc (Zn) play vital roles. They are actually essential nutrients, and variety of deficiency diseases or syndromes can be seen if they are inadequate in our body. However, cellular and tissue damage can occur if these metals are in an excess amount which ultimately leads to toxicities and responsible for various human diseases. Some carcinogenic metals like nickel and chromium can cause DNA damage through base pair mutation, deletion or free radical attack on DNA [22]. Toxicities and harmful effects with other metals like copper [22] cobalt [26], manganese [27], iron [28] and zinc [29] were also reported. Earlier, different pharmaceutical products were investigated in Nigeria of their country made as well as imported from other countries like India, England, Ireland, France and Egypt. They found that most of the products contain one/more heavy metals including chromium, nickel and manganese [6]. In the present study, the concentrations of Cr, Cu, Mn, Fe, Ni and Zn in all the tested samples of both pharmaceutical and CAM preparations, were within permissible range of USP, ICH and EMEA regulatory guidelines refers their safe use (Table 1 and 2). But about 27.78%

Table 1. Heavy metal contents in Pharmaceutical products and comparison of their daily exposure level for a child with reference standards

Heavy metals	No. of products with detectable levels of the metal (% of total No. of products)	Concentration range (ppm)	Manufacturers recommended intake range level (µg/day)	USP Oral PDE[a](µg/day)	ICH Oral PDE[b] (µg/day)	EMEA Oral PDE[c](µg/day)
Pb	0 (0)	<0.1	NA	2.05	2.05	NE
Cd	0 (0)	<0.1	NA	10.25	2.05	NE
Cr	18 (100)	0.030 – 1.480	1.000– 118.400	NE	4510	512.5
Co	18 (100)	0.030– 0.400	0.750– 32.000	NE	20.50	NE
Cu	1 (5.56)	<0.1 – 0.140	NA – 1.400	410	1230	1025
Mn	3 (16.67)	<0.1 – 0.290	NA – 8.800	NE	NE	2050
Fe	18 (100)	4.780– 7.870	53.600 –629.600	NE	NE	6150
Ni	18 (100)	0.220– 1.100	4.000– 71.200	205	82	410
Zn	18 (100)	0.211– 0.790	1.632– 56.881	NE	NE	5125
As	0 (0)	<0.1	NA	0.615	6.15	NE
Hg	0 (0)	<0.03	NA	6.15	12.3	NE

NE – Not established; NA= Not applicable; The results are presented as Mean ± SD (n=2)

estimated maximum Permissible Daily Exposure (PDE) for a 20.5 kg child calculated from USP (United States Pharmacopoeia) references doses based on a 50-kg person; USP Revision Bulletin (2013) ‹232› ELEMENTAL IMPURITIES http://www.usp.org/sites/default/files/usp_pdf/EN/USPNF/key-issues/c232_final.pdf Accessed 7 October 2016

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Table 2. Heavy metal contents in CAM products and comparison of their daily exposure level for a child with reference standards

Heavy metals	No. of products with detectable levels of the metal (% of total No. of products)	Concentration range (ppm)	Manufacturers recommended intake range level (µg/day)	USP oral PDE[a] (µg/day)	ICH oral PDE[b] (µg/day)	EMEA oral PDE[c] (µg/day)
Pb	0 (0)	<0.1	NA	2.05	2.05	NE
Cd	0 (0)	<0.1	NA	10.25	2.05	NE
Cr	10 (83.33)	<0.1 – 0.700	NA – 28.000	NE	4510	512.5
Co	12 (100)	0.100– 0.580	4.200– 17.400	NE	20.50	NE
Cu	7 (58.33)	<0.1 – 0.200	NA – 7.200	410	1230	1025
Mn	9 (75)	<0.1 – 3.370	NA – 84.150	NE	NE	2050
Fe	12 (100)	4.780– 13.850	66.40– 623.25	NE	NE	6150
Ni	12 (100)	0.120– 0.760	4.300– 22.800	205	82	410
Zn	12 (100)	0.278– 1.863	2.717– 40.143	NE	NE	5125

Heavy metals	No. of products with detectable levels of the metal (% of total No. of products)	Concentration range (ppm)	Manufacturers recommended intake range level (µg/day)	USP oral PDE[a] (µg/day)	ICH oral PDE[b] (µg/day)	EMEA oral PDE[c] (µg/day)
As	0 (0)	<0.1	NA	0.615	6.15	NE
Hg	0 (0)	<0.03	NA	6.15	12.3	NE

NE – Not established; NA= Not applicable; The results are presented as Mean ± SD (n=2)

estimated maximum Permissible Daily Exposure (PDE) for a 20.5 kg child calculated from USP (United States Pharmacopoeia) references doses based on a 50-kg person; USP Revision Bulletin (2013) ‹232› ELEMENTAL IMPURITIES http://www.usp.org/sites/default/files/usp_pdf/EN/USPNF/key-issues/c232_final.pdf Accessed 7 October 2016

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Table 3. Results of microbial load of Pharmaceutical products

Product code	TAMC (CFU/mL)	Gram-negative bacterial count (CFU/mL)	*E. coli/ Enterobacter* (CFU/mL)	*Salmonella* and *Shigella*spp. (CFU/mL)	*Staphylococcus* spp. (CFU/mL)	TYMC (CFU/mL or CFU/g)
A-01	1.5×10^2	-	-	-	-	-
A-02	-	-	-	-	-	-
A-03	9×10^2	-	-	-	-	1×10^2
A-04	5×10^3	-	-	-	-	-
A-05	3×10^2	-	-	-	2×10^2	-
A-06	3.7×10^4	-	-	-	-	-
A-07	2.3×10^6	1.2×10^3	1×10^2	-	-	1.8×10^3
A-08	4.7×10^4	-	-	-	1.7×10^3	-
A-09	4×10^2	-	-	-	-	-
A-10	-	-	-	-	-	-
A-11	2.5×10^3	-	-	-	-	-
A-12	1.2×10^5	-	-	-	2.6×10^3	-
A-13	3×10^3	-	-	-	-	-
A-14	7×10^3	-	-	-	-	2.0×10^2
A-15	-	-	-	-	-	-
A-16	3.2×10^4	-	-	-	-	-
A-17	6.5×10^5	-	-	-	-	-
A-18	5.2×10^4	-	-	-	-	-

A= Pharmaceutical products; n = 2, The values are presented as mean only; (-) Absence of microorganism;TAMC = Total aerobic microbial count; TYMC= Total combined yeast and mould count

Table 4. Results of microbial profile of CAM products

Product Code	TAMC (CFU/mL)	Gram-negative bacterial count (CFU/mL)	*E. coli*/ *Enterobacter* (CFU/mL)	*Salmonella* and *Shigella* sp. (CFU/mL)	*Staphylococcus* spp. (CFU/mL)	TYMC (CFU/mL or CFU/g)
B-01	1×10^2	-	-	-	-	-
B-02	2.1×10^4	-	-	-	3.8×10^3	-
C-01	3×10^5	-	-	-	3×10^2	-
C-02	7.2×10^5	-	-	-	-	-
D-01	-	-	-	-	-	-
D-02	3.3×10^4	-	-	-	6×10^2	2×10^2
D-03	2×10^2	-	-	-	-	-
D-04	9.2×10^3	-	-	-	-	-
D-05	6×10^4	-	-	-	-	-
D-06	4.8×10^8	3.5×10^4	2.3×10^3	-	1.6×10^3	2.3×10^4
D-07	2.4×10^3	-	-	-	-	-
D-08	5.3×10^7	2×10^2	1.5×10^2	-	-	9×10^2

B= Herbal products; C= Ayurvedic products; D= Unani products; n = 2, The values are presented as mean only; (-) Absence of microorganism
TAMC = Total aerobic microbial count; TYMC= Total combined yeast and mould count

Table 5. Recommended acceptance criteria for microbiological quality of non-sterile pharmaceutical dosage forms (aqueous preparations for oral use)

Reference guideline	USP[a]	BP[b]	WHO[c]
Total aerobic microbial count (CFU/mL or CFU/g)	10^2	10^2	10^2
Bile-tolerant Gram-negative Bacteria	NA	NA	NA
E. coli	Absent in 1 g or 1 mL	Absent in 1 g or 1 mL	Absent in 1 g or 1 mL
Salmonella spp.	NA	NA	NA
Staphylococcus aureus (CFU/mL or CFU/g)	NA	NA	NA
Shigella	NA	NA	NA
Total combined yeast and mould count (CFU/mL or CFU/g)	10^1	10^1	10^1

[a]*United States Pharmacopoeia Convention, Inc. United States Pharmacopoeia 36-National Formulary 31. Chapters <61>, <62>, <610>, <1111>, <1112>, <1163>, <1191>. Rockville, MD: US Pharmacopoeial Convention, Inc.; 2013.; British Pharmacopoeia Volume IV.Appendix XVI D. Microbiological Quality of Pharmaceutical Preparations.http://www.uspbpep.com/bp2008/data/841.asp Accessed 7 October 2016; World Health Organization. Supplementary information, S.3.7 Microbiological quality of non-sterile products: recommended acceptance criteria for pharmaceutical preparations: Final text for revision of The International Pharmacopoeia (April 2012).;NA – Not Assigned*

Table 6. Recommended acceptance criteria for microbiological quality of non-sterile dosage forms (aqueous preparations for oral use)

Reference Guideline	USP[a]	BP[b]	WHO[c]
Product	Containing botanical ingredients	Containing raw materials of natural (animal, vegetal or mineral) origin	Herbal materials for internal use
Total aerobic microbial count (CFU/mL or CFU/g)	10^4	10^4	10^5
Bile-tolerant Gram-negative Bacteria (cfu/mL or CFU/g)	NA	10^2	10^3 (other than *E. coli*)
E. coli	Absence in 10 g	Absence in 1 g or 1 mL	10 in 1 g
Salmonella spp.	Absence in 10 g	Absence in 10 g or 10 mL	Absence in 1 g
Staphylococcus aureus	NA	Absence in 1 g or 1 mL	NA
Shigella	NA	NA	Absence in 1 g
Total combined yeast and mould count (CFU/mL or CFU/g)	10^3	10^2	10^3

USP – United States Pharmacopeial Convention, USP-NF 37-32, 2014.

British Pharmacopoeia Volume IV.Appendix XVI D. Microbiological Quality of Pharmaceutical Preparations.http://www.uspbpep.com/bp2008/data/841.asp Accessed 7 October 2016

[c]WHO – World Health Organization, WHO Guidelines for Assessing Quality of Herbal Medicines concerning Contaminants and Residues, 2007

NA – Not Assigned

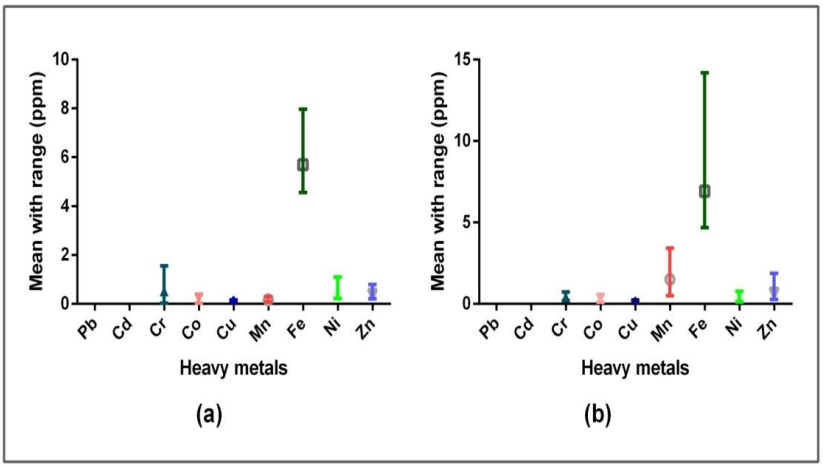

Fig. 1. (a) The range of heavy metal contents with mean in Pharmaceutical products (b) The range of heavy metal contents with mean in CAM products

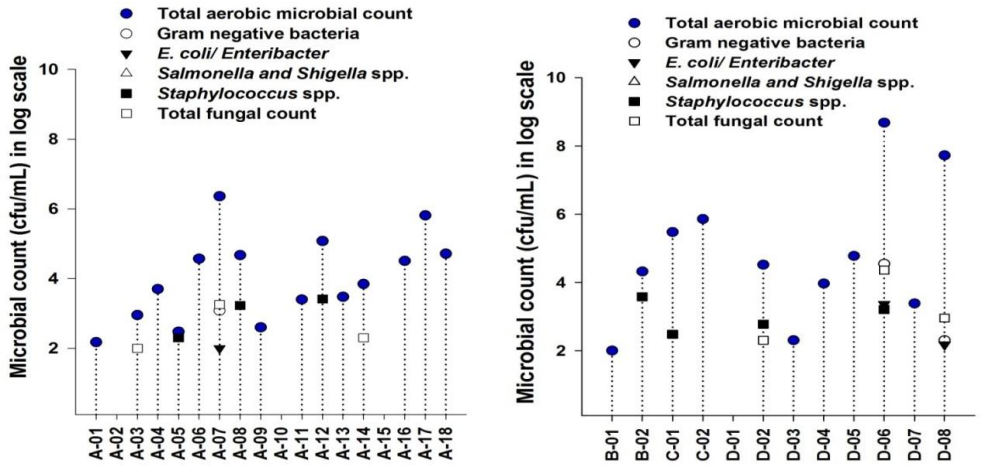

Fig. 2. (a) A plot showing the number of microorganisms contained in pharmaceutical products (b) A plot showing the number of microorganisms contained in CAM products

pharmaceutical products failed to comply with the safety limit of cobalt compared with the ICH guideline (Table 1). Hence these pharmaceutical products are not safe to use due to the presence of high Co level. However, the cobalt contents in all the CAM products were within the permissible range of aforesaid different regulatory guidelines (Table 2).

Inorganic impurities such as heavy metals may be derived from the manufacturing processes used for bulk drugs, and the sources are, water used and the reactors where acid hydrolysis takes place. Metal catalysts and reagents used in the synthesis of pharmaceutical products can potentially result in trace levels of metals in the final product that can be toxic to human life [30]. By using demineralized water and glass-lined reactors, heavy metal impurities can be easily avoided [31]. Usually, CAM products need to pass through multiple stages before reaching to the patient from which the products might be contaminated with toxic metals. Raw materials for CAM products often come from different sources like water, air and soil. The plant may absorb toxic compounds from soil, water and air which ultimately reach to the final products [5]. Transport of products creates possible routes for toxicant exposure. For instances, exhaust pollutants may reach into CAM ingredients in open-bed trucks. Substandard factory conditions where raw materials are processed contribute to

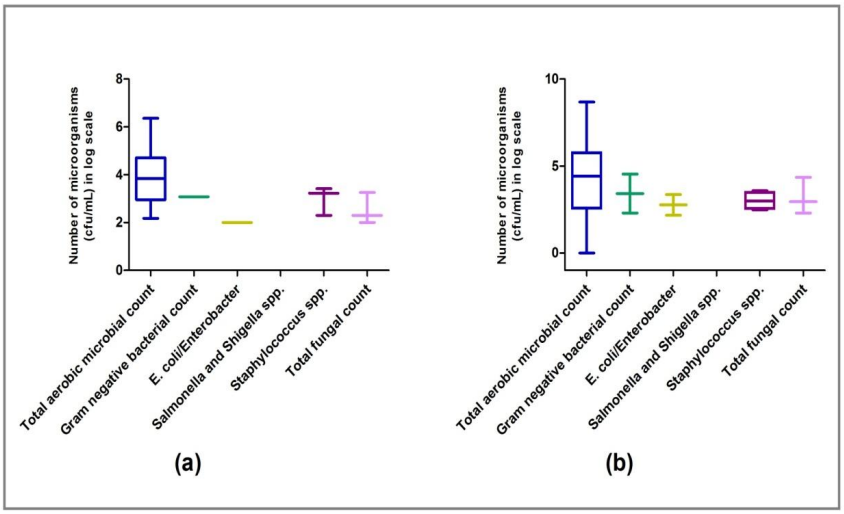

Fig. 3. (a) Distribution of microorganisms in pharmaceutical products (b) Distribution of microorganisms in CAM products

the contaminations of CAM products. Moreover, some dishonest people can contaminate products by adulteration.Finally, intentional additives to supplements may be introduced for perceived therapeutic value [5].

4.2 Evaluation of Microbial Profile

The use of contaminated pharmaceutical preparations has proved hazardous to the health of the users. There have been reports of drug-borne human infections worldwide [15]. Various pathogens like bacteria, fungi, yeasts, or moulds can contaminate the pharmaceutical products resulting in chemical, physical or organoleptic degradation and make it unsuitable and ail for use. Therefore microbial presence must remain free or within the acceptable limit because patients consume medicines when they become sick, so quite possibly more vulnerable to infection.

According to the USP and WHO guideline the maximum TAMC in the pharmaceutical product is $\leq 10^2$ CFU/mL. In this study, it was found that about 77.78% of the pharmaceutical products have crossed both USP and WHO acceptable limit. According to the BP guidelines, 83.33% pharmaceutical products exceeded acceptable TAMC limit (Table 3 and Table 5). In case of CAM products, 58.33% preparations exceeded the safety limit according to USP and BP guideline. WHO guideline for TAMC is more relaxed than USP or BP but still 41.66% CAM preparations exceed this guideline (Table 4 and 6). For the gram-negative bacterial count of pharmaceutical products, there is no USP, BP and WHO guideline. Only for CAM products BP ($\leq 10^2$ CFU/mL) and WHO ($\leq 10^3$ CFU/mL) has the guideline. About 16.67% and 8.33% of the CAM products crossed the BP and WHO guideline respectively (Table 4 and 6). According to the USP, BP *and WHO* guidelines, *E. coli* must be absent in 1g or 1 mL of pharmaceutical products but unfortunately, one pharmaceutical preparation (A-07) was contaminated with *E. coli* (Table 3). In case of CAM two products (D-06, D-08) has exceeded the safety limit of USP, BP *and WHO* guidelines (Table 4 and 6). *E. coli* is a well-known enteropathogen and is the most common causative agent of childhood diarrhoea of bacterial origin [32]. *E. coli* is also used as a marker of faecal contamination in standard assays of water and food [33,34]. Detection of *E. coli* in collected herbal samples actually indicates faecal contamination, which is directly related to unsanitary conditions. This organism grows at 44^0C and produces acid and gas from lactose and indole from tryptophan that will help in biochemical identification tests for isolated organisms from collected samples. The most common causes of this contamination are inadequate processing and cross contamination [16]. Heat treatment during production of herbal medicine products may cause a significant reduction in the counts of *E. coli* [35]. Staphylococci are the most common forms of skin organisms. According to the BP, *S. aureus* must be absent in 1g or 1 mL of sample in case of CAM products. However, the present

investigation showed that in 33.33% (4 samples out of 12) of the products were above the BP and WHO acceptable limit for *S. aureus* (Table 4 and 6). There is no USP, BP or WHO guideline for pharmaceutical preparations (Table 5). Frequently, human handling is involved during the collection, washing, assembling, drying, packing and dispensing of medicinal plants. In herbal medicine products the presence of *S. aureus* is mostly related to the unsanitary human handling processes. During the life of a product if at any time the levels of *S. aureus* exceed 10^5cfu/g or mL, bacterial enterotoxin may cause illness and this enterotoxin will remain in the product. Any product with catalase-positive Staphylococci levels in excess of 10^3 CFU/g should be observed with suspicion and further inquiry is necessary [16]. However the presence of *S. aureus* in the oral preparations may not necessarily constitute a potential hazard to users since not all strains of *S. aureus* produce the enterotoxin that causes poisoning and in any case, the organism would have to grow to a density of several million cells/g for its toxin to constitute a problem [36].

In TYMC 16.67% pharmaceutical products were beyond the USP, BP and WHO acceptable guideline (Table 3 and 5). About 8.33% CAM products crossed both USP and WHO acceptance guideline and 25% were crossed BP limits (Table 3 and 5). Fungal contamination of herbal medicine products mostly happens during postharvest storage if relative humidity is high and during a slow or inappropriate drying process, and temperatures are suitable enough for fungal growth [37]. The fungal contaminated herbal preparations may be responsible for fungal infections. Besides, some fungi like *Aspergillus parasiticus* and *Aspergillus flavus*can produce mycotoxin which may cause serious health complications to the patients [37]. Several studies had already established the presence of mycotoxins in botanical preparations [38,39]. The presence of certain moulds causes rapid deterioration of the product by the toxins produced by them [40].

Raw materials, ingredients, unhygienic environmental condition and lack of aseptic handling would be the main factors for the observed microbial growths in the samples studies [41]. Microorganisms can also be introduced into the products from the packaging materials used. Herbal medicinal products are prepared from natural ingredients, therefore, prone to microbial contamination. The microbial quality of herbal medicine products may be influenced by harvesting, drying, storage conditions, improper handling, inappropriate manufacturing environment and quality of raw materials used during preparation [42]. The therapeutic activity of the herbal medicine products may be decreased or even the products may become inactive due to the presence of microorganisms in the products [43]. In pharmaceutical preparations raw materials, environmental factors, personnel, equipment etc. may act as the potential source of microorganisms.

From our current study, it was observed that CAM products showed more pathogenic microbial (*E. coli*) as well as fungal count than pharmaceuticals. The possible reasons behind this may be the pharmaceutical manufacturer's better adherence to Current Good Manufacturing Practice (CGMP). There are less or no strict regulations of Directorate General of Drug Administration (DGDA) in Bangladesh for the herbal drug manufacturers due to a shortage of inspecting manpower. Well-equipped quality control (QC) lab, microbiology lab and proper in-process quality test (IPC Test) opportunities are available in most of the pharmaceutical companies, but unfortunately, these facilities are not available in the majority of the CAM manufacturers [16]. To reduce the microbial count within the acceptance range of USP, BP or other guidelines both pharmaceutical and CAM manufacturers should strictly follow the CGMP guideline. Also, the regulatory authorities should monitor the quality of both categories of products on a regular basis.

5. CONCLUSION

Concern over the toxicity of heavy metals in medicine is increasing day by day as toxic metals can accumulate into the body and can cause serious harm to the children. This study demonstrates, however, in all the pharmaceuticals and CAM products heavy metal contents are within the permissible limit of standard guideline other than Cobalt. A small percentage of products contain pathogenic microorganisms. Although most of the products were free from pathogen but the majority of them have total aerobic microbial counts above the USP, BP and WHO standards. This excess microbial presence may degrade the product and consumption of these degraded products can cause great harm to the children. As these pediatric preparations are widely used, so it is

necessary to ensure the quality of the pediatric products for the safety of the children. By strictly following the CGMP and other standard guidelines both Pharmaceutical and CAM products manufacturers can produce quality medicine. Therefore, strict adherence and implementation of these guidelines are strongly recommended.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

ACKNOWLEDGMENTS

This work was supported by National Science and Technology (NST) fellowship (Number-39.012.002.01.03.021.2014-07), Ministry of Science and Technology, Bangladesh. The authors are grateful to Md. Ali Hasan Masud, Laboratory technical officer, Department of Microbiology, Jahangirnagar University and Md. Azizul Maksud, Md. Shahidur Rahman, and Lutfun Naher Lutfa, Experimental officer, Atomic Energy Centre, Dhaka for their kind cooperation.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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