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MICROBIOLOGICAL QUALITY ASSESSMENT OF HERBAL MEDICINAL PRODUCTS AND ANTIBIOTIC RESISTANCE PROFILE OF BACTERIA

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Abstract

The microbiological quality of some herbal medicinal products available in Dhaka, Bangladesh was evaluated. In the studied samples, the total aerobic bacterial count (TABC) varied from 6.20×10^2 to 2.97×10^5 cfu /ml, total yeast and mold count ranged from 0 to 1.80×10^4 cfu /ml, total coliform count varied from 0 to 2.80×10^3 cfu/ml and total staphylococcal count (TSC) ranged from 0 to 2.30×10^4 cfu/ml. A strong positive correlation (r = 0.849) was observed between TABC and TSC. Out of 24 herbal medicinal samples examined, 5 (20.83%) did not comply with the microbiological criteria set by internationally recognized organizations. The study revealed that 100% of the *Escherichia coli* isolates were resistant to amoxicillin and penicillin G, 85.71% were resistant to nitrofurantoin and 71.43% were resistant to ceftriaxone and cefuroxime: and 57.14% were resistant to erythromycin. Among the *Staphylococcus aureus* isolates tested, 100% were resistant to amoxicillin, nalidixic acid and Penicillin G; whereas 33.33% showed resistance to cefuroxime.

Introduction

Herbal medicine is a plant-derived material or preparation with therapeutic or other human health benefits which contains either raw or processed ingredients from one or more plants (WHO 2000). Herbal medicines are also referred to as herbal remedies, herbal products, herbal medicinal products, phytotherapeutic agents, phytopharmaceuticals and phytomedicines (Barnes *et al.* 2007). Many people throughout the world, particularly from the developing countries, rely on herbal medicinal products to get relief from different diseases or health related problems. Herbal medicines have gained its popularity among the people of different ages because of presumed low side-effect, low cost and a high level of acceptance by patients (Wilt *et al*. 2000). With the increasing use of herbal medicinal products and the worldwide expansion of the herbal medicinal market, quality and safety have become the concern for both health authorities and the public in many countries.

Microbial contamination of herbal preparations with microorganisms irrespective of being pathogenic or nonpathogenic can bring changes in their physical, chemical and organoleptic properties. The presence of certain microorganisms in non-sterile herbal products may adversely affect the therapeutic potential of the product or even make the product harmful for the patient. Several studies (Ali *et al*. 2005, Govender *et al*. 2006, Okunlola *et al*. 2007, Khurana *et al.* 2011, Onyambu *et al.* 2013) have showed that in some developing countries herbal medicinal products are released to the market without adequate supervision. Hence, high microbial contamination may occur in these products (Ali *et al.* 2005, Sharafati-chaleshtori *et al*. 2011). Studies have reported contamination of herbal products with disease causing organisms such as *Bacillus* spp., *Salmonella* spp. and *Staphylococcus aureus* (Govender *et al*. 2006).

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Antibiotic resistance is one of the most pressing public health problems (WHO 2014). Almost every type of bacteria is becoming stronger and less sensitive to antibiotic treatment when it is really needed. These antibiotic-resistant bacteria can quickly spread to family members, co-workers and schoolmates threatening the whole community with a new strain of infectious disease that is more difficult to cure and more expensive to treat. Further exacerbating the problem, pharmaceutical companies are developing fewer new antibiotics to replace those that are no longer effective (Silbergeld *et al*. 2008).

In Bangladesh, many people rely upon the herbal medicinal products ignoring the microbial hazards associated with them. To fulfill the growing demand, several registered and unregistered local companies are producing herbal medicines. Some studies have been done on the chemical quality of herbal medicinal products manufactured in Bangladesh but study on the microbiological quality and antibiotic resistance profile of the bacteria isolated from those products is limited (Shoeb *et al*. 2011). Presence of unacceptable level of microbes in oral liquid herbal medicinal products can be harmful for the consumer particularly for the children, elderly people and the immunocompromised persons. Thus, the present study was conducted to evaluate the microbial quality of oral liquid herbal medicinal products available in Bangladesh and to determine the antibiotic resistance profile of some isolated bacteria.

Materials and Methods

A total of 24 liquid herbal medicinal products representing 8 different brands were randomly collected from different drug stores located in Dhaka, Bangladesh. The samples were transported to the Department of Botany, Jahangirnagar University for microbiological analysis. The volume, date of manufacture, date of expiry and therapeutic indications were recorded. The density of the samples was determined according to Okunlola *et al*. (2007). The microbiological analysis of samples were done before the expiry date. One milliliter sample was withdrawn aseptically and transferred to 9 ml sterile normal saline (0.9%) for serial dilution (Mugoyela and Mwambete 2010). One hundred microlitre of original or ten-folds serially diluted samples were spread aseptically onto Petri plates containing different medium. Nutrient agar (NA), MacConkey agar and mannitol salt agar (MSA) media (Hi-Media) were used for the enumeration of total aerobic bacteria, total coliform and total Staphylococci, respectively. The plates were incubated at 37ºC for 24 hrs. Characteristic colonies that formed onto the MacConkey agar and MSA plates were counted and average number of colony forming unit (cfu) was determined for each ml of the liquid herbal samples. The mesophilic spore forming bacteria was enumerated according to Lopamudra and Kuila (2005). For the enumeration of total yeast and mold, potato dextrose agar (PDA) medium supplemented with antibiotic was used. Inoculated PDA plates were kept at room temperature for 5 days for the growth of yeast and mold. Colonies developed on the plates were counted and expressed as colony forming unit (cfu) /ml after proper calculation.

Bacterial colonies developed on the culture plates were randomly picked for pure culture. The purified bacterial isolates were identified based on the colony morphology, growth on selective media, Gram staining, spore staining, catalase test, coagulase test, indole production, methyl red (MR) test, Voges-Proskauer (VP) test, sugar fermentation tests, mannitol fermentation test, gas production, β hemolytic activity and novobiocin susceptibility (Finegold and Martin 1982, Cappuccino and Sherman 1996, Brooks *et al*. 2007).

Susceptibility of the *E. coli* and *S. aureus* isolated from herbal sample to antibiotics was determined *in vitro* by employing disc diffusion method (Bauer *et al.* 1966). Susceptibility of the isolates was tested against 10 antibiotics. A portion of *E. coli* or *S. aureus* colony was inoculated into the Mueller Hinton (MH) broth and incubated at 37°C to obtain a young culture. A sterile

cotton swab was dipped in the suspension and streaked over the surface of MH agar medium. Antibiotic impregnated discs were then aseptically placed on the surface of the MH agar medium with the help of a pair of sterile forceps. The plates were incubated at 37°C. After 24 hrs incubation, the plates were examined and the diameter of the zones of inhibition was measured to the nearest whole millimeter. The *S. aureus* and *E. coli* isolates were classified as sensitive (S) and resistant (R) to a particular antibiotic based on the diameter of zone of inhibition (CLSI 2006).

Results and Discussion

Microbial load in the herbal medicinal samples was determined and the data were compared with the standards mentioned in the British Pharmacopoeia (2004) and WHO (2007). On the label of all the samples, the date of manufacture, date of expiry, volume and therapeutic indications were clearly mentioned (Table 1).

Table 1. Basic information about the studied samples.

Sample code	Volume (ml)	Colour	Density (g/ml)	DOM	DOE	Therapeutic indication
AS1	450	Dark brown	1.00	Oct 2014	Oct 2016	Arthritis, epilepsy, insanity, insomnia, leanness, memory disorder, nervous debility and syncope.
AS2	450	"	1.13	Jun 2015	Jun 2017	
AS3	450	"	1.00	Jan 2016	Jan 2018	
BA1	450	"	1.00	Dec 2014	Dec 2016	Anorexia, general debility, loss of weight, malnutrition and rheumatism.
BA2	450	"	1.03	Jul 2015	Jul 2017	
BA3	450	"	1.10	Nov 2015	Nov 2017	
CA1	100	"	0.90	Dec 2014	Dec 2016	Abdominal pain, anorexia, constipation, digestive and carminative to correct hyperacidity, flatulence, indigestion, etc.
CA2	100	"	1.30	Jan 2015	Jan 2017	
CA3	100	"	0.90	Jan 2016	Jan 2018	
EN1	200	"	1.07	Jun 2015	May 2017	Debility, strength and energy booster, tonic for asthmatic patient, etc.
EN2	100	"	1.30	Feb 2015	Jan 2017	
EN3	200	"	1.13	Sep 2015	Aug 2017	
HE1	100	Off white	1.00	Mar 2015	Mar 2017	Abdominal pain and symptoms during teething, diarrhea, flatulence, indigestion.
HE2	100	"	0.93	Jul 2015	Jul 2017	
HE3	100	"	1.00	Aug 2015	Aug 2017	
NA1	100	Light orange	0.93	Dec 2013	Dec 2015	Anemia, deficiency of vitamin A and C, fatigue, general weakness, nervous debility, weakness of stomach and liver.
NA2	100	"	0.93	Aug 2014	Aug 2016	
NA3	100	"	1.00	Jun 2015	Jun 2017	
SA1	100	Dark brown	1.27	Aug 2015	Aug 2018	Constipation, depression, indigestion, nose bleeding, skin diseases, etc.
SA2	100	"	1.00	Aug 2015	Aug 2018	
SA3	100	"	1.20	Feb 2016	Feb 2019	
VI1	100	Yellow	1.20	Jun 2015	Jun 2017	Anemia, depression, fatigue, general weakness, nervous debility, vitamin A and C deficiency, weakness of stomach and liver.
VI2	100	"	1.03	Jul 2015	Jul 2017	
VI3	100	"	1.03	Jan 2016	Jan 2018	

DOM= Date of manufacture, DOE= Date of expiry.

The presence of aerobic bacteria and spore forming bacteria were observed in all the samples tested in this study (Table 2). However, the bacterial counts varied widely among the brands. The highest aerobic bacterial count was found in CA2 (2.97×10^5 cfu/ml) and the lowest aerobic bacterial count was found in HE1 (6.20×10^2 cfu/ml). Presence of high level of aerobic bacteria in herbal medicines was also reported in several studies conducted in different countries (Alwakeel 2008, Khurana *et al*. 2011, Onyambu *et al*. 2013). The plants used in the preparation of herbal medicine are rich in organic compounds and minerals which provided the nutrition to the microorganisms associated with the herbal raw material and might have facilitated the multiplication of the microorganisms found in this study. These microbes may lead to the deterioration and variation in composition of the final product which may give rise to inferior quality of herbal products. Use of such substandard herbal medicinal products might not provide the claimed therapeutic benefits (Table 1).

Table 2. Microbial load in various herbal medicines.

Sample code	TABC (cfu/ml)	TCC (cfu/ml)	TSC (cfu/ml)	MSFB (cfu/ml)	TYMC (cfu/ml)
AS1	1.60×10^5	ND	1.30×10^3	2.60×10^2	1.10×10^3
AS2	3.50×10^3	ND	ND	2.20×10^2	30
AS3	6.90×10^3	ND	2.00×10^2	3.10×10^2	80
BA1	1.20×10^5	ND	5.00×10^2	2.10×10^2	1.10×10^2
BA2	1.70×10^3	ND	40	1.40×10^2	ND
BA3	6.40×10^3	ND	2.40×10^2	4.00×10^2	2.10×10^2
CA1	2.05×10^4	2.05×10^3	1.85×10^3	1.30×10^2	5.00×10^2
CA2	2.97×10^5	2.80×10^3	2.30×10^4	55	1.30×10^3
CA3	8.00×10^3	7.70×10^2	30	4.00×10^2	1.60×10^2
EN1	1.66×10^4	ND	50	90	2.00×10^2
EN2	4.60×10^3	ND	40	90	2.80×10^2
EN3	7.40×10^3	ND	1.00×10^2	2.40×10^2	ND
HE1	6.20×10^2	ND	30	1.90×10^2	1.00×10^2
HE2	3.10×10^3	ND	ND	1.40×10^2	1.20×10^2
HE 3	8.80×10^3	3.60×10^2	1.90×10^2	2.00×10^2	ND
NA1	1.45×10^5	ND	1.70×10^4	1.40×10^2	1.80×10^4
NA2	1.86×10^4	ND	5.00×10^1	2.60×10^2	20
NA3	8.00×10^3	ND	90	3.40×10^2	2.10×10^2
SA1	4.60×10^3	ND	40	40	50
SA2	1.04×10^4	ND	ND	60	1.70×10^2
SA3	1.30×10^4	6.50×10^2	1.50×10^2	90	1.10×10^2
VI1	1.29×10^4	ND	30	1.00×10^2	10
VI2	2.70×10^4	ND	ND	1.25×10^2	20
VI3	8.50×10^3	8.10×10^2	1.30×10^2	2.30×10^2	10

TABC = Total aerobic bacterial count, TCC = Total coliform count, TSC= Total staphylococcal count, TYMC = Total yeast-mold count, MSFB = Mesophilic spore forming bacteria, cfu = Colony forming unit, ml = Milliliter and ND= Not detected.

The highest coliform bacterial count (2.80×10^3 cfu/ml) was found in CA2 (Table 2). The water of the manufacturing plant might be the source of this type of bacteria. A moderate positive correlation was observed between TABC and coliform count (Table 3). The presence of *Staphylococcus* spp. were observed in 20 (83.33%) samples. The highest *Staphylococcus* spp. were also found in CA2 (2.30×10^4 cfu/ml). A significant strong positive correlation was observed between TABC and TSC (Table 3). The mesophilic spore forming bacteria was found in all of the samples ranging from 40 to 4.00×10^2 cfu/ml. The presence of yeast or mold was observed in 21 (87.5%) samples. The highest yeast and mold count was found in NA1 (1.80×10^4 cfu/ml). Presence of high level of yeast and mold in some commercial herbal drugs was also reported by Khurana *et al*. (2011). Among the fungal isolates, *Aspergillus* spp., *Penicillium* spp., *Rhizopus* spp. and *Saccharomyces* spp. were predominant.

Table 3. Pearson correlation coefficient (r) among the microbial counts.

	TABC	TCC	TSC	MSFB	TYMC
TABC	1				
TCC	0.5291**	1			
TSC	0.8488**	0.5904**	1		
MSFB	−0.1897	−0.1982	−0.2718	1	
TYMC	0.3914	−0.037	0.6219**	−0.1035	1

** Correlation is significant at the $p < 0.01$ level.

A total of 37 bacterial isolates were randomly selected and purified for identification. On the basis of cultural characteristics and biochemical tests, 12 (32.43%) were identified as *Bacillus* spp., 7 (18.91%) were identified as *E. coli*, 4 (10.81%) were identified as *Klebsiella* spp., 4 (10.81%) were identified as *S. epidermidis*, 3 (8.10%) were identified as *S. aureus*, 3 (8.10%) were identified as *Proteus* spp., 1 (2.70%) was identified as *E. aerogenes* and 3 (8.10%) isolates were unidentified. *Bacillus* spp. was the most frequently found in these herbal medicinal products because they are widely distributed in the soil, dust and air. Bacteria of this genus produce spores which are resistant to harsh processing, elevated heat and dry conditions. Therefore, they can survive for a long period in the product in a dormant state (Kunene *et al*. 1999).

In some studied samples *S. epidermidis* and *S. aureus* were found and those might have originated from personnel involved in production process. In a previous study, it was reported that approximately 2300 microorganisms are present per square centimeter of human skin and thus people are generally considered as the major source of contamination of drugs (Yerima *et al*. 2012). The skin flakes that make up most of the dust act as rafts for these organisms. Since these rafts are very light, they are carried by air current surrounding the personnel involved in formulation, filling and packaging, thus bringing about contamination of the medicinal products. The presence of potentially pathogenic opportunistic microbes, including *S. aureus*, is a matter of serious concern because they may cause a significant deterioration in the health status of patients, particularly those who are immunocompromised or have an immature immune system. The microbiological data obtained in this study were compared with the acceptance criteria mentioned in well-recognized international monographs or standards (British Pharmacopoeia 2004, WHO 2007). Out of the 24 samples, 5 (20.83%) did not comply with the acceptance criteria stipulated in British Pharmacopoeia (2004) and WHO (2007) standards.

Antibiotics are important arsenal to combat with infectious disease causing bacteria. However, widespread misuse of antibiotics in health and poultry sector has made many bacterial species resistant to several antibiotics. Antibiotic susceptibility test was carried out for the 7 selected *E. coli* isolates and 3 selected *S. aureus* isolates after being confirmed by morphological and biochemical tests. The diameters of zone of inhibition produced by the selected antibiotics against each of the isolates are presented in Table 4. The isolates produced diverse type of zone of inhibition against the antibiotics tested. Antibiotic resistance profile of *E. coli* isolates is presented in Fig.1. The study revealed that all the *E. coli* isolates (100%) were resistant to amoxicillin and penicillin G. Out of 7 isolates 6 (85.71%) were resistant to nitrofurantoin while 5 (71.43%) were

Table 4. Zone of inhibition (mm) produced by *E. coli* and *S. aureus* isolates against some selected antibiotics.

Antibiotics (disc potency)	Zone of inhibition (mm)									
	Bacterial isolate									
	EC_1	EC_2	EC_3	EC_4	EC_5	EC_6	EC_7	SA_1	SA_2	SA_3
Amoxicillin (10 µg)	14	8	7	8	7	21	8	11	17	11
Ceftriaxone (30 µg)	45	8	10	10	11	13	23	16	37	42
Cefuroxime (30 µg)	41	6	6	6	6	6	22	9	29	26
Ciprofloxacin (5 µg)	37	34	28	26	30	33	35	37	30	42
Erythromycin (15 µg)	32	36	37	10	16	21	29	40	34	31
Gentamicin (10 µg)	29	29	30	28	29	36	38	36	32	31
Imipenem (10 µg)	52	40	37	36	38	40	29	41	46	55
Nalidixic acid (30 µg)	30	25	18	18	23	28	20	20	19	20
Nitrofurantoin (300 µg)	21	22	20	23	22	19	21	25	27	16
Penicillin G (10 units)	11	7	6	6	6	11	7	9	16	12

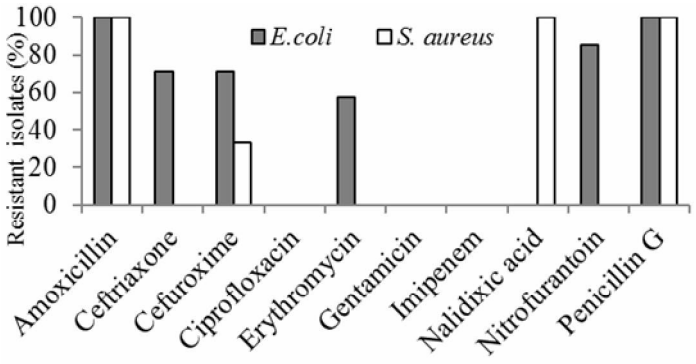

Fig. 1. Antibiotic resistance profile of the *E. coli* and *S. aureus* isolates.

resistant to ceftriaxone and cefuroxime. Four isolates (57.14%) demonstrated resistance to erythromycin. However, ciprofloxacin, gentamicin, imipenem and nalidixic acid were found effective against the *E. coli* isolates tested (Fig.1). The study showed that all of the *S. aureus*

isolates (100%) were resistant to amoxicillin, nalidixic acid, penicillin G. Only one of the *S. aureus* isolates was resistant to cefuroxime. Esimone *et al.* (2007) also reported the presence of cefuroxime and nitrofurantoin resistant bacteria in herbal medicine. However, ceftriaxone, ciprofloxacin, erythromycin, gentamicin, imipenem and nitrofurantoin were found effective against *S. aureus* isolates (Fig. 1). The presence of antibiotic-resistant bacteria in herbal medicine is highly objectionable because these bacteria may facilitate the transfer of antibiotic resistant genes. The study revealed that some of the herbal medicinal products contain microorganisms above the acceptable limit stipulated in British Pharmacopoeia (2004) and WHO (2007) guidelines. The presence of large number of microbial contaminants including the antibiotic resistant bacteria in some herbal samples may reduce or destroy the therapeutic potential of the medicinal product and may also cause the onset of another disease to the patient. The manufacturers of herbal products must properly follow current good manufacturing practice to ensure the microbial quality and safety of the herbal medicinal products.

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