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Drug Resistance in Pathogenic Micro-organisms Isolated from Oral Herbal Medicinal Products and a Survey on the Usage of Herbal Medicine in Bangladesh

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Abstract

Objectives: The present study was conducted to assess the condition of usage of herbal medicine from a survey study and also the drug resistance of pathogenic microorganisms present in oral herbal medicines in Bangladesh popularly used for different therapeutic purposes. At the consumer level, herbal drugs could be contaminated in various ways and antibiogram testing results can also vary.

Methods: The antibacterial potential of six herbal medicines randomly selected were tested to assess their level of contamination. Minimal Inhibitory Concentration (MIC) was performed to determine the lowest concentration of each of the herbal medicine against specific microorganisms. Moreover, among the microorganisms isolated from the tested herbal medicines, *Klebsiella* spp. was tested for measuring the antibiotic susceptibility for different kind of antibiotics.

Results: Number of isolated pathogenic bacteria and the percentage of resistance and susceptibility of isolated pathogens are not negligible for public health.

Conclusion: Authorities need to be more careful when inspecting freely sold herbal medicines in order to avoid possible outbreaks due to contamination of those medicines.

Keywords: Pathogenic microorganisms; Drug resistance; Antibacterial; Antibiotic susceptibility; Minimal inhibitory concentration; Public health; Survey

Introduction

Natural products have long been used as sources for the formulation of useful drugs. Archaeological evidence indicates that the use of medicinal plants dates at least to the Paleolithic, approximately 60,000 years ago. There are many forms in which herbs can be administered, the most common of which is in the form of herbal teas and plant extracts [1]. Whole herb consumption is also practiced either direct or in dried form.

The World Health Organization estimates that about 80% of the populations of some Asian and African countries use herbal medicines in primary health care [2]. Over five hundred medicinal plants have so far been enlisted for which pharmacological evaluation and ethnomedicinal survey have been conducted; however, in many cases, their microbiological aspects have not been checked out [3,4]. Herbal medicines play an important role in healthcare being used in the treatment of ulcers, diabetes and even in cancer therapy in many countries people rely more and more on herbal drugs as the prices of synthetic medicines increases and their possible detrimental effects are better known [5]. Unfortunately, the number of reports of people experiencing negative effects caused by the use of herbal drugs has also been increasing. Among the various reasons for this, is the often poor quality of herbal medicines. We can conclude that little or no attention has been given to the quality assurance and management of herbal medicines.

Microbial presence can vary at the different stages of drug preparation, from the collecting throughout their consumption. Similarly, found contaminants can have different antimicrobial activity. Being a developing country, accessibility, cost usefulness and accurateness of medication are important for the overall public health management in Bangladesh [6]. Antimicrobial agents of natural origin can provide the means to treat infections caused by microorganisms thus saving millions of lives. On the other hand, during the last decades, microbial resistance has increased emphasizing the need for supplementary natural products as alternatives to existing therapies. The present study was conducted to assess the condition of using herbal medicine in the capital of Bangladesh, to assess the minimum inhibitory concentration of several herbal medicines against specific clinically pathogenic microorganisms and to determine the antibiotic resistance or susceptibility of pathogens isolated from herbal medicines commonly used in Bangladesh. Results can be used to

advise authorities concerning the quality of herbal medicinal products available in Bangladesh.

Materials and Methods

Sampling and sample processing

The experiment was performed in six categories of oral herbal drugs in liquid formulations. Manufacturing and expiration date varied between January 2013 and February 2014. Samples were randomly collected from different registered, private drug stores in Dhaka city, Bangladesh according to the standard sampling method [7]. All samples were aseptically processed followed by homogenization of 10 mL of each with 90 mL normal saline solution and dilution of up to 10-6 for the isolation and quantification of pathogenic bacteria. 100 mL of sterile saline solution was used as a control. Samples were then kept at 37°C. MIC was performed for determining the *in vitro* antibacterial activity of tested medicines. Antibiotic susceptibility testing was performed after the results of cultivated bacterial isolates from culture plate method experiment to check the resistance against common antibiotic.

Determination of microorganisms in the samples

0.1 mL of each 10^{-3} and 10^{-5}, dilutions were used and spread respectively onto a MacConkey agar plate (Hi-Media Laboratories Pvt. Ltd., India) for the triplicate enumeration of total fecal coliforms (TFC), and coliforms (especially, *Escherichia coli* and *Klebsiella* spp.), respectively. After that, the plates were incubated for 24 hr at 44.5°C and 37°C for fecal coliforms and coliforms, respectively to observe microbial growth and isolate the different organisms. *Staphylococcus* spp. was isolated onto Mannitol Salt Agar (Hi-Media Laboratories Pvt. Ltd., India), by adding 0.1 mL of each dilution and incubation at 37°C for 24 hr. Selenite Cysteine Broth (SCB) was used to enrich *Salmonella* spp and *Shigella* spp growth *in vivo* and Alkaline Peptone Water (APW), for *Vibrio* spp. For the enrichment, two 10 mL volume of each sample was transferred into 90 mL of SCB and into 90 mL of APW respectively and incubated at 37°C for 6 hr. After incubation, samples were diluted up to 10^{-6} and 0.1 mL of each dilution concentration were spread onto *Salmonella-Shigella* agar (Hi-Media Laboratories Pvt. Ltd., India) and thiosulfate citrate bile salt sucrose agar (Hi-Media Laboratories Pvt. Ltd., India) for the isolation of *Salmonella* spp. and *Shigella* spp., and *Vibrio* spp., respectively. The plates were incubated at 37°C for 48 hr to detect typical colonies. Finally, all isolates were biochemically examined following standard procedures [8,9]. All experiments were done in triplicate.

Observation of *in-vitro* antibacterial activity

For this, the Minimal Inhibitory Concentration (MIC) or broth micro-dilution assay was performed to determine the lowest concentration of each tested herbal medicine capable of inhibit the viability of the tested bacteria [10]. Two-fold serial broth dilution methods were used following the recommendations of the Clinical and Laboratory Standards Institute (2006). 100 µL aliquot of the overnight (~12 hr) culture of each test bacteria was added to sterile tubes containing Mueller Hinton (MH) broth [11] with the turbidity adjusted with 0.5 McFarland standard. Seven different volumes (32 µL, 64 µL, 128 µL, 256 µL, 512 µL, 1024 µL and 2048 µL) of herbal medicine samples were added to make a total volume of 3 mL. The residual or extract concentration of the herbal medicines for each of the above mentioned volumes of the aqueous samples used in the MIC assay was determined. All the tubes were incubated at 37°C for 24 hr. In order to know the minimum concentration (mg/mL) of each sample extract able to delay the reproduction of the tested bacteria we visually checked the lack of turbidity in essays using the McFarland standard method.

Antibiotic susceptibility test (AST) of microorganisms present in the tested herbal medicines

Antibiotic Susceptibility Test (AST) analyses the susceptibility of bacteria to antibiotics. It is usually performed by the Kirby-Bauer method [12] to find the most appropriate antibiotic for a given pathogen. In this study, the AST were done using the disc dispersion assay on Mueller-Hinton agar (MHA, Difco, Detroit, MI) and commonly used antibiotics following the standard protocol. For this assessment, commercially available laboratory grade antibiotic discs of Penicillin G, Gentamicin, Amoxicillin, Erythromycin, Ciprofloxacin, Trimethoprim sulfamethoxazole, Azithromycin and Ampicillin were aseptically placed in Mueller-Hinton agar plates previously inoculated with the bacterial suspensions obtained earlier and prescribed turbidity as compared to that of the McFarland standard of 0.5. Should be noted that the bacteria was allowed to develop prior to the antibiotic inoculation. For this experiment, *Klebsiella spp.* was chosen randomly among the isolated bacteria from the herbal medicine to get the preliminary idea about the resistance of herbal medicine's bacteria.

Survey study

The survey has been conducted among the university student and the results were transferred to the excel sheet to make the column chart (Figure 1).

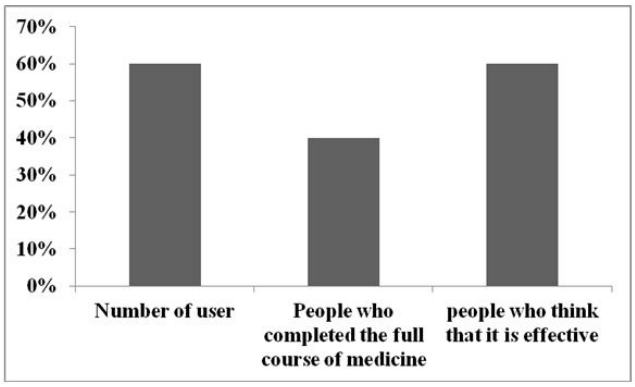

Figure 1: Survey study on the usage of herbal medicine among the university student in Bangladesh.

Statstical Aanalysis

All measurements were entered in Excel and then a single factorial analysis of variance (ANOVA) was used to test the two types of extracts (water and methanol) against specific bacterial strains. Values of $P<0.05$ were taken as significant [16].

Results and Discussion

Like in Bangladesh, many countries use conventional herbal medicinal systems. Unfortunately, not all of them practice a good routine of monitoring the hygiene and the anti-bacterial potency of these drugs, which is essential to guarantee a sustainable health administration system. To our knowledge, such examination of herbal medicinal samples is not that frequent in Bangladesh as well as posing possible adverse effects upon usage of these medicines. Our survey results were conducted among the university student to know the fate of herbal medicine use in the educated and young generation. The result showed that a certain number (60%) of student use herbal medicine and the same number also believe that it is effective. But most of them do not complete the full course of the medicine. It could be a reason to develop the drug resistance or bacterial resistance. From the microbiological study, it has found that all tested samples were found to be contaminated with bacteria (10^2-10^5 cfu/mL); although they were initially sealed (Table 1). The study continued afterwards once the cap was opened in order to demonstrate further microbial growth, which in turn might focus on the contamination from the environment or due to improper handling. Then the bacterial growth in all samples was also found to increase along with time up to 10^6 cfu/mL. However, contamination with particular pathogenic bacterium such as, *Staphylococcus* aureus (10^3-10^5 cfu/mL) in all samples indicates contamination though mismanagement throughout harvesting, handing out, manufacturing, delivery or storage of the samples. All samples were found to be contaminated with *Klebsiella* spp. (10^2-10^6 cfu/mL), which possibly might have occurred during the harvesting process or from the farming (i.e. Manure) due to incorrect handling [13]. Sample no. 2 was contaminated with *Salmonella* spp. and no.4 had fecal coliforms (Table 1). All samples were contaminated with *Vibrio* spp (10^2-10^3 cfu/mL).

Samples	FCC	*Staphylococcus* spp.	*Klebsiella* spp.	*Salmonella* spp.	*Shigella* spp.	*Vibrio* spp.
1 (N=5)	0	3.9×10^4	2.5×10^3	0	0	3.6×10^2
2 (N=5)	0	4.0×10^3	1.0×10^6	1.0×10^2	0	4.4×10^2
3 (N=5)	0	2.6×10^3	1.2×10^2	0	0	3.6×10^3
4 (N=5)	1.0×10^3	7.0×10^3	3.0×10^5	0	0	1.0×10^2
5 (N=5)	0	2.9×10^5	5.0×10^5	0	0	1.5×10^2
6 (N=5)	0	1.9×10^2	4.5×10^5	0	0	2.0×10^2

Table 1: Microbiological contamination of the studied samples (cfu/mL). The average load has been shown in the table. Total aerobic bacteria 10^6 cfu/mL, [Microbial limits (World health Organization 2007), *Shigella* spp was completely absent].

Stipulation of the *in vitro* anti-bacterial activity of the herbal medicine samples

The study showed that most samples exhibited *in vitro* antibacterial activity against the tested bacteria used in this study. It is the first time that this is shown for these common oral herbal medicines (Table 2). Sample no. one possessed the activity against five tested bacteria successfully, sample no. two was found to be effective against two tested bacteria, while sample no. three possessed the antibacterial activity against just only one bacterium species. No antibacterial activity was found in sample no. 4-6. Additionally, the *in vitro* anti-bacterial goings-on of the samples was promote supported by observing the result of MIC (Table 2). Three samples were found to show anti-bacterial activity against *E.coli*, *Pseudomonas* spp., *Bacillus* spp., *Salmonella* spp., *Staphylococcus* spp., *Vibrio* spp. and Listeria spp. The highest MIC value was found at 40 mg/ mL (*Bacillus spp.*) and the lowest MIC value was found at 1 mg/mL (*Klebsiella* spp.) (Table 2). Sample no. 1 showed MIC values between 20-40 mg/mL, thus being the weakest. Sample no. 2 was the most potent against all tested microorganisms and shoed a MIC in the range of 8 mg/mL (Table 2). However, agar well diffusion and disc diffusion tests may not be the most appropriate to determine the antibacterial activity of natural compounds. The rate of diffusion of natural antimicrobials can be strongly affected by the polarity, the concentration or the molecular size of the compounds. That is why this time this experiment (MIC) was performed. No significant difference was found from the statistical analysis (Table 2).

Sample	Name of the microorganisms							
	E. coli	*Klebsiella spp.*	*Staphylococcus spp.*	*Pseudomonas spp.*	*Salmonella spp.*	*Vibrio spp.*	*Bacillus spp.*	Anova (F)
1	20 mg/mL	38 mg/mL	30 mg/mL	40 mg/mL	20 mg/mL	20 mg/mL	40 mg/mL	0.10
2	3 mg/mL	1 mg/mL	8 mg/mL	8 mg/mL	8 mg/mL	5 mg/mL	3 mg/mL	0.20
3	22 mg/mL	22 mg/mL	30 mg/mL	25 mg/mL	22 mg/mL	22 mg/mL	8 mg/mL	0.15

Table 2: Minimum Inhibitory Concentration (MIC) of the samples. Samples 4, 5 and 6 exhibited no MIC value, [The enduring concentrations of the herbal medicines have been provided. Among the five times separately performed experiment data, one data has been shown here.]

Tests of the drug-resistance in pathogens isolated from the studied herbal medicines

Nowadays, many drug-resistant bacteria are isolating from food, water and also from the synthetic drugs as well which could be alarming in the near future [14].

In this study, one of the pathogens isolated from the studied samples was used for Antibiotic susceptibility testing. *Klebsiella* spp. exhibited up to 69% resistance and it was found to be highly resistant to penicillin G and gentamicin (Table 3). Drug-resistant pathogenic isolate from all of the samples studied may create a further danger to the overall public health remedies in the course of the application of these medicines. So our advice is to do the routine inspection of herbal medicines for the presence of drug-resistant bacteria is thus necessary.

Klebsiella spp.	A	C	E	G	P	Tr	Az
(S-31% and R-69%)	11 mm	22 mm	6 mm	0 mm	0 mm	25 mm	9 mm

Table 3: Determination of resistance or susceptibility (in mm) of *Klebsiella* spp. to commonly used antibiotics. Data indicates the frequency of resistance or sensitivity of *Klebsiella* spp isolates compared with *Staphylococcus* spp. P=Penicillin G (10 µg), G=Gentamicin (10 µg), A=Amoxicillin (30 µg), E=Erythromycin (15 µg), C=Ciprofloxacin (5 µg), Tr=Trimethoprim-sulfamethoxazole (25 µg), Az= Azithromycin (15 µg), A=Ampicillin (10 µg). R=Resistant, S=Sensitive.

Conclusions

In general, the development of microorganisms in the tested samples with minor anti-bacterial activity demands better formulation as well as proper handling of oral herbal medicines. The presence of bacteria in the tested herbal medicines reveals inappropriate hygiene and maintenance during manufacturing and processing phases. The growth and proliferation of pathogenic bacteria will lead to danger during the usage of these medicines. Besides, the complete absence of anti-bacterial properties in the majority of the tested samples shows their ineffectiveness against pathogenic infections. Taken together, a careful formulation of the herbal medicines, good manufacturing practices along with good and tested quality management is recommended for improved medication against diseases.

About 2,000 medicinal plants are presently included in the traditional medicine practices. Over 500 of them have so far been enlisted as growing in Bangladesh. Although a huge pharmacological evaluation and ethnomedicinal survey of medicinal plants have been conducted in the country, there is no microbiological survey of these popular medicines in spite of the wide research on microbiological assessment of pharmaceutical products [15-18]. In Bangladesh, over 80% of the rural population depends on traditional remedies for common ailments such as a cough, cold, fever, headache and dysentery. Neem (*Azadirachta indica*), for example, is used to treat some skin disease and in cosmetics. Turmeric is used as an anti-inflammatory, to treat digestive disorders and skin diseases, and in wound healing. Despite this, and the presence of more than 400 companies producing herbal medicines, more than 90% of the plants and products needed to meet demand are imported from India, Nepal, and Pakistan. Plants such as garlic, mint, turmeric and neem could boost Bangladesh's economy if planted on a larger scale, even if it is just in villagers' backyards. Although Bangladesh has no official policy or regulations about growing, conserving and marketing of medicinal plants, some universities and non-governmental organizations are collaborating to boost the country's production of these plants.

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Conflict of Interest

All authors revised the manuscript and there is no conflict of interest.

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