ORIGINAL ARTICLE

EVALUATION OF THE MICROBIOLOGICAL QUALITY OF NATURAL PROCESSED PRODUCTS FOR MEDICINAL USE MARKETED IN QUITO, ECUADOR

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ABSTRACT

Objectives: To determine the microbiological quality of samples from processed natural products used for medicinal purposes and marketed in Quito, Ecuador. **Materials and methods:** Aerobic microorganisms, molds and yeasts were counted by conventional standardized techniques, according to the United States Pharmacopoeia (USP), in samples from 83 products. The microorganisms found were identified and their antimicrobial sensitivity was determined using the agar diffusion method. **Results:** The total aerobic microorganism count exceeded the specified limits in 17.0% of syrups, 27.0% of topical products and 43.0% of oral solids; the molds and yeasts count exceeded the limit in 33.0% of syrups, 7.0% of topical products and 36.0% of oral solids. Products for eye use did not pass the sterility test. The most frequently isolated bacterial genus was *Bacillus*, followed by *Escherichia coli, Klebsiella* and *Enterobacter*. *Salmonella* and *Staphylococcus aureus* were not found in any product, but potentially pathogenic microorganisms such as Pseudomonas were isolated in 40.0% of the eye drops. Enterobacter and Escherichia coli showed resistance to multiple compounds and Pseudomonas was not resistant to any antibiotic. **Conclusions:** The microbiological quality of the products examined was not adequate. Potentially pathogenic and antibiotic resistant microorganisms were isolated from the samples. These products may not be suitable for distribution and consumption, even though many of them have sanitary registration. Control and regulation by the corresponding authorities is essential.

Keywords: Herbal Products; Bacteria; Fungi; Microbial Drug Resistance (source: MeSH NLM).

INTRODUCTION

Natural processed products include a variety of herbal preparations purchased without prescription, such as food, nutritional supplements, cosmetics, and herbal products for medicinal use [1]. People use these products because of their low cost and the belief that they have fewer side effects than synthetic products. Their use is increasingly common, not only in developing countries but also in industrialized countries, however, they may not be safe if contaminated with microorganisms or toxins [2].

Studies have reported the contamination of these products with potentially pathogenic microorganisms, such as Salmonella and *Staphylococcus aureus* [3,4]. Others have shown the presence of intestinal and environmental bacteria, suggesting scarce microbiological control in the process of elaboration and an inadequate application of good manufacturing practices (GMP). Thus, users could be exposed to a variety of microorganisms, many of them may cause diseases. Some of these products could be contaminated with antibiotic-resistant bacteria, such as methicillin-resistant *Staphylococcus aureus* [5], which implies a potential dissemination of drug-resistant microorganisms in the community.

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Manufacture and sale of natural processed products for medicinal use should be controlled from the earlier stages and throughout their life cycle, to ensure quality and safety. In this context, it should be understood that pharmaceutical quality is the basis for patients and consumers to have confidence in the safety and effectiveness of medicines [6]. In fact, quality is fundamental for the reproducible efficacy and safety of natural products, which implies the absence of contaminants and residues that can cause harm to consumers [7].

In Ecuador, health authorities have defined a processed natural product for medicinal use as the finished and labeled medicinal product whose active ingredients are composed by any part of the natural resources for medicinal use or their combinations as a raw drug, standardized extract or in a recognized pharmaceutical form, which is used for therapeutic purposes [8]. The sale and post-registration control are regulated by the National Agency of Regulation, Control and Sanitary Surveillance (ARCSA). However, locally manufactured and imported products are sold in naturist stores without the corresponding certification or permits. Besides, the national companies where natural products are manufactured and commercialized have managed to delay the obligatory compliance with GMP and still have until 2021 to implement it [9]. In the meantime, these products continue to be commercialized and consumed in the country.

Data on the microbiological quality of products sold without restriction in markets and health food stores in Ecuador's capital are scarce. This study aims to determine the microbiological quality of a sample of natural products: syrups, creams, solutions, eye drops, capsules and tablets; to quantify the microbial load; to identify the microorganisms present; and to determine sensitivity to antibiotics.

KEY MESSAGES

Motivation for the study: In Ecuador, studies on the microbiological quality of processed natural products for medicinal and over-the-counter use are scarce, and it has not been reported whether they contain pathogenic and drug-resistant microorganisms.

Main findings: The products analyzed are not microbiologically safe and exceed the criteria for aerobic microorganisms and for molds and yeasts. *Enterobacter* and *Escherichia coli* isolated from solid compounds were shown to be resistant to multiple antibiotics.

Implications: Control measures are needed in the manufacture and sale of natural processed medicinal products, as well as the implementation of good manufacturing practices.

MATERIALS AND METHODS

Design and sample

An observational, descriptive, cross-sectional study was conducted in three sectors (north, center and south) of Quito. Ten naturist centers were conveniently selected from some representative and highly commercial areas. The products were acquired from each naturist store, a total 83 products of local or foreign manufacture were collected: 24 syrups, 15 products of topical use (creams and solutions), 30 eye drops (solutions) and 14 oral solids (tablets and capsules). The execution phase of the study was from April 2018 to July 2019.

We documented the information stated on products labels: composition, sanitary registration, manufacture and expiry dates, and whether they included a leaflet. The products were transported in sealed plastic cases to the General and Pharmaceutical Microbiology Laboratory of the Chemical Sciences Faculty of the Universidad Central del Ecuador and were processed within 4 hours after being purchased.

Verification of counting methods

To demonstrate the validity of the counting methods, a sufficient volume of microbial suspension was added to the diluted products and to a control (not including the sample) to obtain an inoculum of no more than 100 colony-forming units (cfu) of standardized strains of microorganisms as specified in the United States Pharmacopoeia (USP) [10]. The method was considered suitable if the number of cfu recovered from the products did not differ by more than a factor of 2 from the control value.

Counting microorganisms in syrups, topical products, and oral solids

We used the methodology described in the USP 42 [10]. Briefly, an aliquot of 1 mL or 1 g of each sample was placed in 9 mL of TSB broth; Tween 80 was added in creams at 0.1% to achieve complete dispersion. In some cases, more successive dilutions were necessary to obtain an adequate count of microorganisms. The dilutions were seeded in duplicate, both by extension and by pouring, on trypticase soy agar (TSA) for total aerobic microorganism count (TAMC) and on sabouraud dextrose agar (SDA) for total mold and yeast count (TMYC). TSA plates were incubated during 24 hours at 37 °C; and SDA plates, during 5 days at 25 °C. After incubation, the colonies were counted to calculate the colony forming units per milliliter or gram of product.

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Detection of microbial contamination in eye drops

Based on the USP 42 [10] sterility test, the eye drops were analyzed as follows. The entire contents of the container were filtered through a 0.45 µm mixed cellulose ester membrane, this process was carried out in a Biobase A2 Class II biosafety cabinet. The membranes were grown in both thioglycolate broth and TSB. Both media were incubated for 14 days: thioglycolate broth at 37 °C and TSB at 22.5 °C. The tubes were examined daily and visually for turbidity. The contents from positive thioglycolate tubes were passed to TSA, MacConkey, mannitol, and cetrimide agars, and from TSB tubes to TSA and SDA.

Identification of microorganisms

Bacterial isolates were identified by their morphology and by Gram staining, on selective and differential media: salted mannitol agar, MacConkey agar, cetrimide agar, XLDA agar and egg yolk mannitol agar. The genera and species of the isolated microorganisms were confirmed by means of specific biochemical tests according to the type of microorganism [11].

Fungi were identified by macroscopic growth observation on SDA agar and by microscopic characteristics in lactophenol blue staining. *Candida* identification was made by simple staining with violet crystal, sowing on chromogenic *Candida* agar, germ tube test, sugar fermentation and urease test [12].

Antimicrobial sensitivity

The agar diffusion technique was performed according to the standards of the Clinical and Laboratory Standards Institute (CLSI) 2019 [13]. The response of 8 *Enterobacter*, 4 *Escherichia coli* and 1 *Klebsiella* isolates to different antibiotics was evaluated. The tested antibiotics (Bioanalyse) were ertapenem (10 µg), cefoxitin (30 µg), cefotaxime (30 µg), trimetroprim/sulfamethoxazole (1.25/23.75 µg), streptomycin (10 µg), nitrofurantoin (300 µg), amoxicillin/clavulanic acid (20/10 ug), chloramphenicol (30 µg), gentamicin (10 µg), azithromycin (15 µg), phosphomycin (50 µg), ciprofloxacin (5 µg), tetracycline (30 µg), amikacin (30 µg), penicillin (10 U), cefazolin (30 ug) nalidixic acid (30 µg), piperacillin/tazobactam (100/10 µg), imipenem (10 U), ampicillin (10 µg), levofloxacin (5 µg) and ceftriaxione (30 µg).

Microbiological criteria according to USP

According to USP 42 [10], oral aqueous preparations should have counts equal to or less than 102 cfu/mL for TAMC and 101 cfu/mL for TMYC; for oral solids, values equal to or less than 103 cfu/g for TAMC and 102 cfu/g for TMYC. The criteria for topical products are the same as for aqueous orals. Aqueous and non-aqueous oral products should be free of *Escherichia coli* and topical products, of *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Since they are considered sterile products, the eye drops must be free of any microorganism.

Data analysis

The analysis of this study was descriptive. Initially, types of the studied natural products were presented, showing the percentage of each one of them compared to the sample total. The variables that indicate quantity of microorganisms were transformed from their quantitative nature into categories to define the fulfillment of the microbiological criterion or the lack of it, according to the values of the USP 42. The percentages of products that do not meet this criterion were calculated in relation to the total of the products analyzed and for each type of product.

For each identified microorganism, the percentage of products with the presence of each germ in relation to the total of analyzed products and for each type of product was calculated. The sensitivity/antibiotic resistance was qualitatively catalogued based on CLSI 2019 [13]. The percentage of sensitive, intermediate sensitive and resistant strains was calculated in relation to each identified bacterial genus.

Ethical aspects

The databases used in this study do not provide sensitive information such as the brand names of the products analyzed, nor the sales location.

RESULTS

The analysis was carried out in 83 natural product units of 38 different brands, most of them were eye drops (n = 30; 36.1%), followed by syrups (n = 24; 28.9%), products for topical use (n = 15; 18.1%), and solids for oral use (n = 14; 16.9%). A total of 17 products (20.5%) had no Ecuadorian sanitary registration. In relation to the type of product, 20.0% of eye drops, 36.0% of oral products, and 40.0% of topical products did not have this commercialization requirement.

The microbiological criteria for total aerobic microorganism count were not met by 17.0% of syrups, 27.0% of topical products, 43.0% of oral solids and 80.0% of eye drops. Approximately 33.0% of syrups, 7.0% of topical products, 36.0% of oral solids and 20.0% of eye drops did not meet the microbiological criteria for molds and yeasts (Table 1).

The most frequently isolated bacterial genera were *Bacillus* and *Pseudomonas*; within the former, B. *subtilis*

was more prevalent than B. *cereus*. Oral solids and eye drops were the ones most contaminated by B. subtilis, while syrups and oral solids were the most contaminated by B. cereus. Only 15.0% of the products were contaminated by *Pseudomonas*, most of them were eye drops. Other genera, such as coagulase negative *Staphylococcus* and bacteria belonging to the *Enterobacteriaceae* family were isolated in less than 5.0% of the samples and were found mainly in oral solids, with the exception of a single *Escherichia coli* isolate from an eye drop sample. No *Staphylococcus aureus* or *Salmonella spp.* were identified in any of the samples (Table 2).

Regarding isolated molds and yeasts, *Aspergillus* and *Candida* were isolated in approximately 13.0% of the products. *Aspergillus* was isolated from syrups and oral solids; *Candida* was isolated from eye drops, syrups, and topical products. Penicillium was isolated in 5.0% of products, all were oral solids. *Cladosporium* and *Absidia* (fungi) were isolated from a sample of tablets each (Table 3).

All *Enterobacter* (n = 8), *Escherichia coli* (n = 4), and *Klebsiella* (n = 1) isolates were resistant to penicillin. The only strain isolated from *Klebsiella* was also resistant to piperacillin/tazobactam and imipenem and sensitive to the other antibiotics. Of the 4 strains of *Escherichia coli*, 1 was resistant to trimetroprim/sulfamethoxazole, chloramphenicol, gentamicin, phosphomycin, piperacillin/tazobactam and ampicillin. Of the 8 *Enterobacter* isolates, 7 were resistant to ampicillin, 3 to cefoxitin and amoxicillin plus clavulanic acid, 2 to nitrofurantoin, cefazolin, and piperacillin, and 1 to cefotaxime, trimetroprim/sulfamethoxazole, and nalidixic acid. All 3 microorganisms were sensitive to ertapenem, streptomycin, ciprofloxacin, tetracycline, azithromycin, amikacin, levofloxacin, and ceftriaxone. Five strains of Pseudomonas were tested against 5 antibiotics and were sensitive to all of them: ciprofloxacin amikacin, piperacillin, imipenem, and levofloxacin.

DISCUSSION

The microbiological quality of the products analyzed was generally not acceptable. It was found that 46.0% of the products exceeded the limits for the count of aerobic microorganisms and 24.0% for molds and yeasts. The study by Saeed El-Houssieny *et al.* [14] also analyzed non sterile products, such as syrups, suspensions, oral solids, creams, ointments, lotions and gels marketed in Egypt, and documented that their microbiological quality was adequate, with the exception of a few cases, such as 3.3% of tablets that presented counts greater than 2000 cfu/g for bacteria and greater than 20 cfu/g for molds and yeasts. The 4.2% of syrups also presented increased infections in molds and yeasts.

The genus *Bacillus* was the most predominant in our study, these bacteria are usually frequent in pharmaceutical production environments and in raw materials, due to their ability to form endospores and resist conditions of dryness and lack of nutrients. Their presence has been frequently reported [15,16] and could indicate a lack of environmental control in the manufacturing, conditioning, storage, or distribution site. Only 20.0% of eye drops were contaminated by *Bacillus cereus*, and 37.0% by *Bacillus subtilis*, these microorganisms could cause serious infections at eye level. A study in Cairo-Egypt [17] also analyzed eye drops and of the 58 samples positive for the presence of microorganisms, 55.1% were contaminated with *Bacillus spp*.

One finding related to poor hygiene practices in product manufacturing was the discovery of enterobacteria, in-

Table 1. Compliance with microbiological criteria for the total count of aerobic microorganisms, and molds and yeasts in the natural products analyzed

Microbiological criteria	Syrups	Topical Products	Oral Solids	Eye drops *
Aerobic microorganisms	≤100 ufc/mL n (%)	≤100 cfu/mL n (%)	≤1000ufc/mL n (%)	Absent n (%)
No	4 (16.7)	4 (26.7)	6 (42.9)	24 (80.0)
Yes	20 (83.3)	11 (73.3)	8 (57.1)	6 (20.0)
Molds and yeasts	≤10 cfu/mL n (%)	≤10 ufc/mL n (%)	≤100 ufc/mL n (%)	Absent n (%)
No	8 (33.3)	1 (6.7)	5 (35.7)	6 (20.0)
Yes	16 (66.7)	14 (93.3)	9 (64.3)	24 (80.0)

*In eye drops the compliance criterion is presence or absence

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Table 2. Identification of bacterial germs in the natural products analyzed

Microorganism	Total (83) n (%)	Syrups (24) n (%)	Eye drops (30)	Topical products n (%)	Oral Solids (14) n (%)
Coagulase-negative Staphylococcus	2 (2.3)	n (%)	0	0	2 (14.3)
Staphylococcus aureus	0	n (%)	0	0	0
Escherichia coli	3 (3.5)	0	1 (3.3)	0	2 (14.3)
Enterobacter	4 (4.6)	0	0	0	4 (28,6)
Klebsiella pneumoniae	1 (1.2)	0	0	0	1 (7.1)
Salmonella	0	0	0	0	0
Pseudomonas	13 (15.1)	0	12 (40.0)	0	1 (7.1)
Bacillus cereus	26 (31.3)	12 (50.0)	6 (20.0)	3 (20.0)	5 (35.7)
Bacillus subtilis	31 (37.3)	7 (29.2)	11 (36.7)	5 (33.3)	8 (57.1)

cluding *Escherichia coli*, present in 3.3% of eye drops and 14.3% of oral solids. *Escherichia coli* is a Gram-negative bacillus that belongs to the intestinal flora of mammals and other animals. Its presence in water, food and pharmaceutical products is a strong indication of fecal contamination. Obi *et al.* [18] analyzed tablets (natural and synthetic) sold in Nigeria and determined the presence of *Escherichia coli* in all products collected from pharmacies, hospitals, and health food stores in Abia State.

Contamination of the raw material with enterobacteria is also possible. Ratajczak *et al.* [19] analyzed the microbiological quality of non-sterile pharmaceutical products, and in those containing raw materials of natural origin, *Escherichia coli* was isolated. The findings were attributed to the lack of pre-treatment to reduce the microbial load of the raw material, as well as to the poor quality of the cultured water and soil.

Other enterobacteria isolated in the oral solids of our study were *Klebsiella* and *Enterobacter,* which supports the fact of a possible inadequate treatment of the raw material as well as deficient agricultural practices. The fecal contamination of the soil and the crops may be due to the use of natural fertilizers based on animal feces, where enterobacteria are the main microorganisms. None of the analyzed products were contaminated by *Salmonella*, which coincides with what is reported by Shaqra *et al.* [15], but differs from the findings of Rauf *et al.* [20] that reports finding this pathogen in suspensions, syrups and tablets in a study carried out in non-sterile preparations in Pakistan.

Pseudomonas, a potentially pathogenic bacteria, which causes multiple antibiotic resistant keratitis [21], was found in 40.0% of eye drops, products that according to USP should be sterile. This microorganism tends to form biofilms, and its presence may be due to inappropriate distribution systems and storage of purified water for pharmaceutical use [22].

Aspergillus and *Penicillium* were the most frequently isolated genera of molds, which could indicate inadequate control of environmental conditions, inappropriate humidity levels and temperature. These microorganisms, in addition to deteriorating the products, could affect the health of users, especially those with a weakened immune system, because they can produce toxins as a result of their metabolism [23].

Yeasts like *Candida* were isolated in 16.7% of the syrups, 20.0% of the eye drops, and 7.0% of the topicals. Their presence may be due to inadequate hygienic practices by the operators since this microorganism is a member of the intestinal and urogenital microbiota. The isolation of *Candida* could be consid-

Table 3. Identification of molds and yeasts in the natural products analyzed

Microorganism	Total (83) n (%)	Syrups (24) n (%)	Eye drops (30) n (%)	Topical products (15) (%)	Oral Solids(14) n (%)
Aspergillus	11 (13.2)	7 (29.2)	0	0	4 (28.6)
Candida	11 (13.2)	4 (16.7)	6 (20.0)	1 (6.7)	0
Cladosporium	1 (1.2)	0	0	0	1 (7.1)
Absidia	1 (1.2)	0	0	0	1 (7.1)
Penicillium	4 (4.8)	0	0	0	4 (28.6)

ered a public health problem since it is one of the main causes of associated infections in patients with HIV; and strains resistant to antibiotics, such as fluconazole have been detected [24].

No *Staphylococcus aureus* was isolated in the samples used for this study, which contrasts with what was found by Stanley *et al.* [25] in Nigeria, who analyzed solid and liquid natural products, and Staphylococcus aureus was the most prevalent bacterium, which could indicate incorrect handling of the products by the personnel.

Regarding the response of Gram-negative bacilli, such as *Enterobacter* and *Escherichia coli*, to different antibiotics, it was observed that the products analyzed may be a source of drug-resistant bacteria. In the study by Daniyan and Sangodere [26], where they evaluated syrups in Minna Metropolis in Nigeria, the pattern of antimicrobial susceptibility of *Escherichia coli*, *Staphylococcus aureus* and *Bacillus subtilis* was analyzed, and it was found that they also had a wide resistance to antibiotics.

The type and amount of microbial contamination found in this study is a strong indication of poor application of GMP, poor control of the manufacturing environment, poor quality of raw materials and water used, as well as inadequate hygiene of the personnel in charge of the processes. Therefore, it is mandatory for establishments where natural processed products for medicinal use are manufactured, stored, distributed, and marketed to comply with the implementation of GMP, otherwise the health of users may be at risk. GMP for natural products in first-world countries and regions, such as China and the European Union, and to a lesser extent the United States, focus on the assessment and management of risk, rather than the quality assurance and control that, in an outdated way, is still recommended by the WHO [27]. In Ecuador, the first approach should be prioritized because it does not only consider the multiple factors involved in drug quality, but also makes it possible to rationalize and concentrate the usually scarce resources on the aspects that have the greatest impact on drug safety and efficacy.

A limitation of this study was the fact that the results obtained are applicable exclusively to the products and brands analyzed, since other natural products could vary in origin and manufacture. It is difficult to apply the results to other natural products, because the selection of the samples was not random. Similarly, we do not know if the products studied really contain natural ingredients, however, regardless of the origin, our results show the deficit in the microbiological quality of these products of free-market sale in the city.

The strengths of our analysis consist in investigating a variety of pharmaceutical forms for different administration routes and of diverse composition and physicochemical characteristics, which include liquids, semi-solids and solids, sterile and non-sterile, national and imported, which represents an overview of the microbiological quality of the products marketed in Quito. Likewise, the counting and identification methods applied in this study are included in the official bibliography (USP).

In conclusion, this microbiological analysis of natural processed products for medicinal use shows that 46.0% of these present unacceptable quantities of aerobic microorganisms, and 24.0% exceed the molds and yeasts acceptable quantity. There were isolated microorganisms that indicate a deficient control of the manufacturing or storage environment, deficient hygiene practices, especially regarding oral solids, potentially pathogenic and pharmaco-resistant bacteria were also observed. These findings imply that the products may not be suitable for distribution and consumption, even though many of them have sanitary registration. Control and regulation are indispensable and the application of standards by the regulatory entities must not be postponed.

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