UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
Faculdade de Farmácia
Trabalho de Conclusão de Curso de Farmácia

# MICROBIAL CONTAMINATION OF HERBAL MEDICINES: A REVIEW

Maria Laura Sunderhus Glória

Porto Alegre, novembro de 2020.

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Trabalho de Conclusão de Curso apresentado como requisito para obtenção de título de farmacêutico pelo Curso de Farmácia da Universidade Federal do Rio Grande do Sul.

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Porto Alegre, novembro de 2020.

Dedico este trabalho, ao meu amado pai, Luiz Otávio Cardoso Glória, que hoje é a estrelinha que me acompanha.

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## APRESENTAÇÃO

O presente trabalho apresenta-se sob a forma de artigo de revisão original, com o objetivo de ser submetido à publicação no periódico *Anais Academia Brasileira de Ciências*. As normas de instrução aos autores para preparação dos manuscritos encontram-se disponíveis ao final da apresentação (Anexo I) a fim de facilitar a análise pelos membros da Banca Examinadora.

HEALTH SCIENCES

**Microbial Contamination of Herbal Medicines: a review**

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## ABSTRACT

In this review, we deal with the safety of herbal medicines, regarding the levels of microbial contaminants and mycotoxins, which must be in accordance with the requirements present in the national and international official compendium. The high availability of these products without due compliance with the recommended quality standards, makes the use of these medicines an important public health problem that can, in more serious situations, lead to death of consumers. Thus, we have gathered several scientific articles that showed failures along the production process of herbal medicines, resulting in excessive contamination by, mainly, bacteria and fungi, but also by mycotoxins. We also demonstrated the different methods applied in the determination of microorganisms. Thus, we came to alert the population and the scientific community about the relevance of this problem, since the negligence during the manufacturing stages of herbal medicines may cause more damage than benefits to consumers.

**Key words:** Herbal medicines, determining contaminants, microbial contamination, mycotoxins, safety limits.

## INTRODUCTION

Herbal medicines refer to the general term covering herbs (raw plant material), materials, preparations and finished herbal products (WHO 2000; WHO 2007a). This concept has also been explored by the RDC (Resolução da Diretoria Colegiada) of ANVISA (Agência Nacional de Vigilância Sanitária) nº 26, of May 13, 2014. According to it, herbal medicines are those obtained with the exclusive use of active vegetable raw materials whose safety and efficacy had based on clinical evidence and that are characterized by the constancy of their quality. In this same RDC, the concept of traditional herbal products had approached, which are those obtained with the exclusive use of active vegetable raw materials whose safety and effectiveness had based on data of safe and effective use published in technical-scientific literature and which had designed to be used without the supervision of a physician for diagnostic, prescription or monitoring purposes. Both plant materials are subject to the presence of microorganisms, however the lower number of processing steps of traditional herbal products may provide them with a higher load of contaminants compared to herbal medicines. These products have been producing frequent around the world and several people show interest in using them in their health care. Thus, the safety of these products becomes an important public health issue (WHO 2004; Kosalec et al. 2009). Several factors can influence the quality, efficacy and safety of these drugs, from the stage of cultivation of medicinal herbs to the manufacture of the product. Among the main problems encountered are high contamination by bacteria, fungi, mycotoxins or a combination of both (Ghisleni et al. 2016).

Thinking about the manufacturing process as a whole, it can be seen that investments and improvements are needing in all production stages of a herbal medicine. First, it is essential that the workers working in the pre-cultivation process and harvest have been adequately trained. Then, adequate cleaning and extraction methods must be employed. To avoid the presence of adulterants and toxic compounds, the identification and standardization of active

principles must be performed. Drying should also be carried out, not forgetting to consider the thermostability of the products, as well as their levels of contaminants. If necessary, decontamination methods must be carefully applied. Finally, physical-chemical and microbiological quality control tests are necessary. Thus, with the planning of all these steps, together with the application and monitoring of good manufacturing practices, quality control and quality assurance standards, it will be easier to obtain quality phytotherapies that are safe and effective for their consumers (WHO 2007b; Ghisleni et al. 2016).

Considering the growing demand for the use of herbal medicines, especially the perpetuation of the idea that because they are "natural" they will be harmless, we know that many herbal medicines will be made available to consumers not meeting the necessary quality standards for consumption, especially regarding the levels of microbial contaminants. In this sense, this review aims to gather scientific studies that have shown failures during the production process of herbal medicines, resulting in excessive contamination by chemical agents (mycotoxins) and microbiological (bacteria and fungi). This will alert the population and the scientific community to this public health problem, since the carelessness during the manufacturing stages of herbal medicines can generate more damage than benefits to consumers.

## METHODOLOGY

The literature review has carried out through research in the PubMed database, using scientific articles published in the last 10 years, which addressed the evaluation of the microbiological quality of drugs of plant origin. The following keywords were applying in the research: "herbal medicines" and "microbial contamination". The references of the selected studies had explored to verify some other pertinent publication. Among the selection criteria, we defined:

1. Inclusion criteria: presence of contamination by bacteria, fungi or mycotoxins in drugs of plant origin with levels of contaminants above acceptable limits.

2. Exclusion criteria: Levels of microorganisms in accordance with the recommended parameters.

## DISCUSSION

### Microbial and mycotoxin contamination in herbal medicines

Herbal-based therapy has been using worldwide in developed or developing countries to treat various diseases. The biological activities observed in herbal therapies, the easy availability, combined with the low cost associated with these products, make them attractive therapeutic alternatives (Barakat et al. 2013; Turkson et al. 2020). Most distributors and consumers of herbal medicines consider these products safe for consumption, however, microbial contamination is a problem that deserves attention, since it represents a threat to their quality and to the health of consumers. The unhygienic conditions during the production process of these products are directly related to the high rates of contamination by pathogenic microorganisms (Oyetayo 2008; Nwoko & Mgbeahuruike 2011; Keter et al. 2016). In the following paragraphs, several studies that found the presence of microbiological contaminants in herbal medicines beyond acceptable limits will be demonstrated and discussed. The method of microbial identification as well as the results found in these studies are summarized in Table I.

Contamination of phytotherapeutic drugs refers to the undesired presence of impurities, whether chemical or microbiological contaminants or foreign particles, which were inserted during the handling of these products in the various production stages (WHO 2007a). This review focuses on chemical contaminants, more specifically mycotoxins and microbiological contaminants, bacteria and fungi.

All the articles in our review shows high contamination by bacteria, fungi, mycotoxins or both microorganisms. Aquino et al. (2010) obtained high counts of fungi, including toxigenics, in most samples. Ting et al. (2013) identified microbial cells in most samples. In the study of Noor et al. (2013) high rates of bacterial and fungal contamination had observed in liquid and semi-solid samples. Ezekwesili-ofili and collaborators (2014) demonstrated that the presence of microbial contaminants and aflatoxins is quite recurrent in herbal medicines. Dei-tutuwa et al. (2014) found that the growth of microorganisms was important in most samples. Pullirsch et al. (2014) observed a high bacterial count in the analyzed herbal medicine. Singh et al. (2015) found high contamination by *Cronobacter* spp. In the herbal materials. In the study of Ratajczak et al. (2015) high rates of contamination by bacteria and fungi had found in herbal medicines that would not undergo antimicrobial pre-treatment and in those that would undergo a hot extraction process. By the study of Walther et al. (2016) samples highly contaminated by fecal coliforms had been observing. Meissner et al. (2016) found bacterial levels too high in the yellow phenotype hypocotyles of Peruvian Maca. Yesuf et al. (2016) observed high contamination by aerobic bacteria and total coliforms in liquid and solid samples. Melchart et al. (2016) identified high bacterial contamination in their samples. According to Rajeshwari & Raveesha (2016) many herbal medicines showed high levels of fungi and aflatoxins. Zargaran et al. (2017) showed that samples prepared by traditional methods showed higher microbial contamination when compared to samples developed by modified techniques. Keter et al. (2017) observed samples highly contaminated by fungi and mycotoxins, among them aflatoxins and fumonisins. Lima et al. (2020) found excessive contamination by bacteria and fungi in home and commercial phytotherapics. Li et al. (2020) isolated many endophytic fungi, including toxigenics.

Many other studys have also highlighted the presence of chemical and microbiological contaminants with levels above acceptable limits. Some reported greater relevance for bacterial

contaminants (Nur et al. 2018; Shiaka et al. 2018; Bello et al. 2019; Uddin et al. 2019; Sornchaithawatwong et al. 2020; Dashen et al. 2020), others to fungi (Chandra et al. 2019), while some had high contamination by both microorganisms (Bernadin et al. 2018; Abualhasan et al. 2019; Shaikh et al. 2019; Shorifujjaman & Kabir 2019; Oladosu et al. 2020; Ameri et al. 2020). The presence of fungi along with mycotoxins was also quite significant (Korir et al. 2017; Chandra et al. 2019; Ikeagwulonu et al. 2020b).

However, other studies have reported the presence of these contaminants in accordance with the recommended safety parameters (Fernandes et al. 2018; Dutt et al. 2020; Ikeagwulonu et al. 2020a; Turkson et al. 2020; Uddin et al. 2020).

Regarding the most prominent fungal genders, we notice the constant presence of *Aspergillus* spp. (Ting et al. 2013; Ratajczak et al. 2015; Chandra et al. 2019; Oladosu et al. 2020) and *Penicillium* spp. or both in the same sample (Aquino et al. 2010; Ezekwesili-ofili et al. 2014; Rajeshwari & Raveesha 2016; Keter et al. 2017; Li et al. 2020). It has known that the presence of fungal contaminants in herbal medicines brings great alert, since they can degrade the active principles of these drugs, significantly reducing their efficacy, and in addition, they can provide contamination by mycotoxins (Matos 2000; Kneifel et al. 2002; Stevic et al. 2012). These toxins are toxic metabolites produced mainly by the fungal genera *Aspergillus*, *Penicillium* and *Fusarium* (Rizzo et al. 2004). There is concern about the ingestion, inhalation or dermal absorption of these fungi and their metabolites, since they can cause serious diseases, among them, allergies, respiratory and digestive diseases, high hepatotoxicity, which can lead to the death of humans and animals (Zain 2011; Wu et al. 2014).

Among the most evaluated and detected mycotoxins we highlight aflatoxins (Aquino et al. 2010; Ezekwesili-ofili et al. 2014; Melchart et al. 2016; Rajeshwari & Raveesha 2016; Keter et al. 2017; Korir et al. 2017; Ikeagwulonu et al. 2020b). Mycotoxins can cause several toxic results, among them, carcinogenic, immunotoxic, neurotoxic and teratogenic effects (Simsek et

al. 2002). It is understandable why aflatoxins have been the most prominent toxins, since they are quite toxic and carcinogenic. These can be of the most varied types, but the most important are the aflatoxins B1, B2, G1 and G2. Aflatoxin B1 is verified as the most toxic. The species *Aspergillus flavus* can produce the isoforms B1 and B2 while *Aspergillus parasiticus* can produce the 4 main isoforms (Bennett & Klich 2003).

Among the most commonly isolated bacterial, the genera *Bacillus* (Ting et al. 2013; Ezekwesili-ofili et al. 2014; Ratajczak et al. 2015; Yesuf et al. 2016; Bello et al. 2019; Chandra et al. 2019; Oladosu et al. 2020) and *Staphylococcus* are the main prevalent (Ting et al. 2013; Dei-tutuwa et al. 2014; Bernadin et al. 2018; Bello et al. 2019; Uddin et al. 2019; Shaikh et al. 2019; Shorifujjaman & Kabir 2019; Dashen et al. 2020; Lima et al. 2020; Oladosu et al. 2020). The high prevalence of *Bacillus* spp., endospore forming bacteria, is due to its wide distribution in nature, and its spores have high stability and resistance to unfavorable environmental conditions (humidity, temperature) that contribute to these bacteria remaining viable throughout the production process of herbal medicines, such as during drying processes, heating, among others (Araújo & Bauab 2012; EMA 2015). This bacterial genus causes, mainly, disorders of the gastrointestinal tract (diarrhea, severe inflammatory diseases), allergic reactions and in more serious situations, such as for ingestion by immunocompromised people, even bacteremia and septicaemia (Shahcheraghi et al. 2015). The detection of *Staphylococcus* spp., microorganism present in our normal microbiota (nose, throat, skin, among others), suggests negligence with good manufacturing practices and hygiene care in the production process of herbal medicines. It can cause staphylococcal gastroenteritis, scalded skin syndrome, folliculitis, among other diseases (Esimone et al. 2007).

Members of the *Enterobacteriaceae* family had also frequently detected (Noor et al. 2013; Ezekwesili-ofili et al. 2014; Dei-tutuwa et al. 2014; Pullirsch et al. 2014; Sing et al. 2015; Ratajczak et al. 2015; Walther et al. 2016; Yesuf et al. 2016; Melchart et al. 2016; Bernadin et

al. 2018; Nur et al. 2018; Shiaka et al. 2018; Bello et al. 2019; Uddin et al. 2019; Shorifujjaman & Kabir 2019; Ameri et al. 2020; Dashen et al. 2020; Lima et al. 2020; Oladosu et al. 2020). Although they can be evidenced in nature, they are large indicators of fecal contamination, since they inhabit the gastrointestinal tract of humans and animals (Esimone et al. 2007; Araújo & Bauab 2012). Thus, again it has been perceiving that the hygienic conditions of herbal medicines are precarious. The use of natural fertilizers can also lead to contamination by this group of bacteria (Dlugaszewska et al. 2019). They are often associated with respiratory and gastrointestinal tract infections (Trabulsi & Alterthum 2015).

**Safety limits**

The presence of contaminants can lead to decay in the quality of specialties and pharmaceutical inputs, causing physical and chemical changes in these products, and especially causing risks of infection and intoxication to its consumers. Thus, quality assurance and controls of production processes become essential so that contaminants are within safety limits. These limits must adapt to the multiple categories of existing products, where in our review we highlight the products of plant origin, to reflect the type of contamination most likely inserted during the manufacturing process, as well as the route of administration to be applied, its end user, among other factors (Brazil 2019). Depending on the route of administration to be used, the safety limits will present different concentrations. The contamination levels of products of vegetable origin, as for herbal medicines, must respect the safety limits pre-established by regulatory agencies. The World Health Organization (WHO) as well as the British Pharmacopoeia, European Pharmacopoeia, United States Pharmacopoeia, among others, establish the minimum quality requirements necessary for several health products, among them the drugs of vegetable origin.

As already discussed in the previous topic, we brought studies that obtained similar results to the articles we evaluated, presenting contaminants with levels above acceptable limits and others where these contaminants were in compliance with the established safety levels. Thus, in this topic we will bring the various safety limits applied, as well as the official guidelines on which they were based.

Among the studies that used the standardized regulation by the WHO, Aquino and collaborators (2010) showed the extrapolation of the maximum acceptable load of molds and yeasts of $1.0\times10^3$ CFU/g or mL (colony forming units per gram or mL), which was defined by the WHO in 1998. In this same study the presence of aflatoxins had evaluated, but the acceptable safety limits hadn't been reporting. Other studies also used the safety criteria recommended by the WHO official document (Fernandes et al. 2018; Uddin et al. 2020).

In the study of Lima et al. (2020) the WHO standards established in 2007 were used, being $10^5$ CFU/g or mL for aerobic bacteria, $10^3$ CFU/g or mL for fungi and enterobacteria, as well as the absence of *Salmonella* and *Escherichia coli* in 1g. In this study many samples exceeded the limits for bacterial and fungal counts, as well as the presence of *Salmonella* and *Escherichia coli*, which should be absent, could be observed. Several studies use the standards established by the WHO to ensure the microbiological safety of herbal medicines (Yesuf et al. 2016; Bernadin et al. 2018; Shiaka et al. 2018; Uddin et al. 2019; Chandra et al. 2019).

In the study of Noor et al. (2013), the pharmacopoeia requirements of the British Pharmacopoeia (2004) were applied, being $10^5$ CFU/g or mL for aerobic bacteria, $10^4$ CFU/g or mL for yeasts and moulds, $10^3$ CFU/g or mL for *Enterobacteriaceae* and other gram-negative organisms and absence of *Escherichia coli* and *Salmonella.* In this article some samples showed high contamination rates by bacteria and fungi, but *Salmonella* spp. is not isolated. Shorifujjaman & Kabir (2019) were also based on the British Pharmacopoeia criteria, but in

association with the WHO microbiological standards (2007). Thus, in this study it had observed that 20.83% of the samples did not meet the established microbiological criteria.

The microbiological standards established by the European Pharmacopoeia (EP) in 2007 were used in the study of Ezekwesili-ofili and collaborators (2014), where the maximum load of $10^5$ CFU/g or mL for total aerobic bacteria, $10^3$ CFU/g or mL for *Enterobacteriaceae* and other gram-negative organisms and absence of *Escherichia coli* and *Salmonella* must be respected, in addition to the application of WHO limits (2011). Aflatoxins B1, B2 and G1 were also tested in the samples, with the application of these same official documents, but the acceptable limits were not cited. The presence of these mycotoxins indicates, mainly, that Aspergillus flavus could produce aflatoxins. Korir et al. (2017) also had as safety parameter the EP (2007), and thus demonstrated the levels of mycotoxins, which should not exceed the limit of 4 ppb (µg/kg). Dashen et al. (2020) similarly applied the safety limits of EP (2007), but also the criteria of the National Agency for Food and Drugs Administration and Control (2000). This agency highlights that pathogenic bacteria must be absent in herbal medicines. Dashen and collaborators (2020) observed that all 45 herbal preparations analyzed had low bacteriological quality. Among the samples evaluated, 46.67% showed contamination by *Escherichia coli* and 13.33% were contaminated with *Salmonella.* In addition, bacterial counts had found around between $10^6/10^7$ CFU/mL and it was evident that the recommended standards hadn't been respected.

The Dei-tutuwa et al. (2014) study used the microbial standards established by the United States Pharmacopoeia (USP) in 2011 and by WHO (maximum bacterial count of $10^5$ CFU/mL) in 2007. Thus, a large part of the samples showed microbial growth, and the bacteria *Escherichia coli* and *Staphylococcus aureus* had detected. Turkson et al. (2020) and Dutt et al. (2020) similarly applied the WHO (2007) standards in their evaluations. In another study, the USP criteria had also used, but from the year 2017 (Abualhasan et al. 2019).

We observed that several studies use a set of forms that standardize acceptable microbial levels in plant samples. As in the study by Pullirsch et al. (2014), that applied the pharmacopeic criteria of EP (2001) and USP (2012). As a result, it was possible to verify that the analyzed sample exceeded the maximum tolerated load of $10^5$ CFU/g for total aerobic bacteria, as well as the permitted levels in the quantification of gram-negative biletolerant bacteria (maximum limit of $10^4$ CFU/g). Nur et al. (2018) showed that 13 of the 33 samples evaluated exceeded this same maximum tolerated load for aerobic bacteria as recommended by USP. It had also noted that 48.5% of the samples had *Escherichia coli.*

Walther et al. (2016) highlighted the non-conformity, in 87.6% of their samples, regarding the levels allowed for bacteria counting, with the results counting over $10^2$ CFU/mL. This criterion had derived from the safety limits recommended by USP (2003). *Klebsiella pneumoniae* and *Enterobacter aerogenes* were also frequently found. Sornchaithawatwong et al. (2020) made use of the USP criteria, but from the year 2014, together with the limits of the British Pharmacopoeia (2014). According to these criteria, only 14.5% of the analyzed phytotherapies had acceptable microbial quality, since 70% of the samples were contaminating with aerobic bacteria and 11% were contaminated with mold and yeast. The Thai Pharmacopoeia (2011) was highlighted as it is the official document of the country of the article in question, and from it, it was showing that only 9% of herbal remedies had contaminated with mold and yeast and less rigorous levels than those applied. The authors appeal to the Thai Food and Drug Administration (FDA) about the need for compliance with Good Manufacturing Practices, as well as the importance of applying stricter quality standards to herbal medicines.

The pharmacopeic guidelines of USP (2005) were addressed in the study by Keter et al. (2017), with a maximum limit of $10^5/10^4$ CFU/mL in the counting of moulds and yeasts, which were exceeded in 69% of the analyzed samples. Aflatoxins and fumonisins were evaluated in this same study, also by USP limits, but these were not mentioned. It had reported that only

31% of the phytotherapics met the recommended standards for mycotoxins. The WHO recommendations of the year 2007 were also used. Oladosu et al. (2020), similarly, evaluated their samples according to the acceptance criteria of USP (2018) and WHO of the year 2007.

Ratajczak et al. (2015) used the norms of EP (2010) for herbal medicines for oral use, having active ingredient that was not submitted to pre-treatment antimicrobial and those for oral use that will be submitted to hot extraction process (infusions, decoctions). The safety limits of the herbal products that have not been submitted to antimicrobial pre-treatment are: $10^4$ CFU/g for total aerobic bacteria, $10^2$ CFU/g for total moulds and yeasts, $10^2$ CFU/g for *Enterobacteriaceae* and other gram-negative bacteria. *Escherichia coli* and *Staphylococcus aureus* in 1g are also recommended, as well as *Salmonella* in 10g. The acceptance criteria for phytotherapeuticals that have undergone the hot process are: $10^7$ CFU/g for total aerobic bacteria, $10^5$ CFU/g for total moulds and yeasts and $10^3$ CFU/g for *Escherichia coli* in 1g. The absence of *Salmonella* in 1g is recommended. In this article, the great majority of the samples that did not comply with the recommended standards were the drugs of plant origin. Bello et al. (2019) used the same standards as EP (2010) regarding herbal medicines that would not undergo antimicrobial pre-treatment, but evaluated only the bacterial load and the detection of bacterial isolates.

Rajeshwari & Raveesha (2016) followed the 2001 WHO recommended limits (maximum fungal load $10^5$ CFU/g) and the EP (2011) recommended guidelines with a maximum limit for aflatoxin B1 of 2 μg/kg. According to the Food Safety and Standards Authority of India (2011), the safety limit for aflatoxin B1 should be 30 μg/kg. However, this limit should be applied to food items and not to herbal remedies. Ikeagwulonu et al. (2020a) also assessed the presence of aflatoxins, and used the safety limit of 20 ppb as defined by Nigeria and the European Commission (2006). Similarly, Ikeagwulonu et al. (2020b) in their analysis of mycotoxins (aflatoxins, ochratoxin A and fumonisins) applied the same criteria as

Nigeria and the European Commission (2006). In contrast, the presence of aflatoxins B1, B2, G1 and G2 was assessed in the study by Melchart et al. (2016), however, the safety limits of these mycotoxins hadn't reported. None of these aflatoxins were found.

The national standards of Iran (2008) were addressed in the study of Ameri et al. (2020) being that they do not exceed $10^5$ CFU/g for total aerobic bacteria, $10^3$ CFU/g for mold and yeast, $10^2$ CFU/g for *Bacillus cereus*, $10^3$ CFU/g for coliforms and absence of *Escherichia coli*. Differences in safety limits had observed between regulatory agencies, if we analyze the study of Shaikh et al. (2019) that used the Indian Pharmacopoeia (2010), for example, the maximum load of aerobic bacteria should be $10^3$ CFU/g or mL for water insoluble samples and $10^2$ CFU/g or mL for water-soluble samples. The maximum fungal load should be $10^2$ CFU/g or mL for water insoluble samples and $10^1$ CFU/g or mL for water-soluble samples. Thus, we note that there is a distinction between the concentrations of microorganisms that are acceptable in products of plant origin, depending on the country in which it was regulated.

Some articles evaluated, however, did not bring the safety limits applied (Ting et al. 2013; Sing et al. 2015; Meissner et al. 2016; Zargaran et al. 2017; Li et al. 2020), but they also did not report the presence of contaminants at acceptable levels, but rather that the samples had been contaminating.

In the research by Ting et al. (2013), the authors commented on the boiling process to significantly reduce the levels of microbial contaminants, but we know the specific recommendations for herbal remedies that will be subjected to the hot extraction process and these safety limits should have been applied, as demonstrated by Ratajczak et al. (2015). In the study by Singh et al. (2015) the acceptance criteria present in official compendia hadn't applied, since they researched in a very specific way the species *Cronobacter sakazakii* which is not usually investigated in official documents. By in this study, the bacterial isolates proved to be resistant to most of the antibiotics tested, with susceptibility only to phenicols and tetracyclines.

Meissner and collaborators (2016) did not bring the comparison of their results with the standards evidenced in the official guidelines, but by performing this comparison with the criteria reported by Walther et al. (2016) and Ratajczak et al. (2015) we can observe that the requirements had exceeded relating to the yellow hypocotyles of Peruvian Maca. Zargaran et al. (2017) aimed to demonstrate that the modified techniques proposed in the study bring improvements in the microbiological quality of chamomile oils compared to traditional techniques, thus bringing out the importance of care in the entire production process of herbal remedies, as we have already observed in Ghisleni et al. (2016) and WHO (2007b). Li et al. 2020 also take up this importance of care/good manufacturing practices, mainly regarding the processing and storage of *Coix lachrymal-jobi* grains.

**Methods for determining microbial contaminants**

**Quantification of microorganisms**

According to WHO (2007a) the counting of aerobic bacteria and fungi (moulds and yeasts) can be performed by 3 different methods. They are: membrane filtration, plate count or serial dilution. The Brazilian Pharmacopoeia (2019) also brings these 3 methods for the determination of the total number of mesophilic bacteria and fungi, but the serial dilution had named as multiple tube method. In this official guideline it is reported that, when possible, the membrane filtration technique should be employed and that the multiple tubes' method should be used as a last resort, when low load of microorganisms in the evaluated samples is expected. The preference for the membrane filtration method may be related to its higher sensitivity and analytical reliability, and can be applied to samples with high or low load of bacteria and fungi (Rompré et al. 2002; Wu 2008; Goldman & Green 2015). This technique, however, when compared to the plate count method, stands out for being more expensive, requiring a higher

amount of equipment, vacuum source and special membranes (Reasoner 2004). Few studies have applied membrane filtration to count their microorganisms.

Most studies used the plate count method for the quantification of aerobic bacteria and fungi (Aquino et al. 2010; Ting et al. 2013; Noor et al. 2013; Ezekwesili-ofili et al. 2014; Ratajcazk et al. 2015; Walther et al. 2016; Yesuf et al. 2016; Keter et al. 2017; Bernadin et al. 2018; Nur et al. 2018; Shiaka et al. 2018; Abualhasan et al. 2019; Bello et al. 2019; Chandra et al. 2019; Shaikh et al. 2019; Shorifujjaman & Kabir 2019; Uddin et al. 2019; Ameri et al. 2020; Dashen et al. 2020; Lima et al. 2020; Sornchaithawatwong et al. 2020). The plate count can be done by sowing in depth (*pour plate*) or in surface (*spread plate*). Surface seeding can stand out relates deep seeding since, by not using such high temperatures in their culture media, it does not provide so much cellular stress to microbial cells and thus triggers higher rates of recovery of microorganisms. It is also more advantageous regarding a better growth of the microorganisms investigated, since the microaerobiosis environment will not be formed. Nevertheless, deep seeding is simple, easy to use, inexpensive and can be applied in the quantification of microorganisms with the application of non-selective culture media and in the detection of specific microorganisms using selective culture media (Reasoner 2004).

However, some articles did not specify which determination method they applied. Dei-tutuwa et al. (2014) reported that the microbial count had done according to the method indicated by the United States Pharmacopoeia (USP) for non-sterile products. Pullirsch et al. (2014) mentioned that the microbiological analysis occurred according to the methodologies established by the European Pharmacopoeia and USP. Meissner et al. (2016) and Melchart et al. (2016) did not report how this analysis had performed. Singh et al. (2015) and Li et al. (2020) did not quantify the analyzed microorganisms.

Rajeshwari & Raveesha (2016) applied the technique of dilution in series or multiple tubes, in association with the filter paper method (*blotter test*). This test is adequate to verify

possible contaminations that bring hyphae, fruit or spores, being effective in the detection of many fungi that may contaminate any type of seed (Agarwal & Sinclair 1987; Peske et al. 2006). The identification had made by visualizing the morphology of the fungal colonies present in the filter paper. It is a basic and inexpensive method. Its principle is to raise the relative humidity, the luminosity and the temperature in adequate levels for better fungus development (Agarwal & Sinclair 1987).

**Detection of specific microorganisms**

According to the WHO (2007a) it becomes unnecessary to perform the determination of specific microorganisms when the herbal products reach the maximum levels allowed for their microbial counts. Instead, products should be rejected or more tests should be performed. Regarding this test, the Brazilian Pharmacopoeia (2019) also comments that even if the results of quantification of microorganisms are in accordance with the recommended limits, the analysis should not be excluded.

Regarding this analysis, most studies have used sowing in selective culture media (Aquino et al. 2010; Ting et al. 2013; Noor et al. 2013; Ezekwesili-ofili et al. 2014; Ratajcazk et al. 2015; Walther et al. 2016; Yesuf et al. 2016; Rajeshwari & Raveesha 2016; Keter et al. 2017; Bernadin et al. 2018; Nur et al. 2018; Shiaka et al. 2018; Bello et al. 2019; Chandra et al. 2019; Shaikh et al. 2019; Shorifujjaman & Kabir 2019; Uddin et al. 2019; Ameri et al. 2020; Dashen et al. 2020; Lima et al. 2020; Sornchaithawatwong et al. 2020).

Singh et al. (2015) identified *Cronobacter* spp. According to ISO 22964: 2006, along with the application of the Polymerase Chain Reaction (PCR) genus and species specific. Dei-tutuwa et al. (2014) also used PCR for the detection of *Escherichia coli*, *Staphylococcus aureus* and *Salmonella* spp. Li et al. (2020) combined the visualization of morphological aspects of fungal colonies with evaluation through PCR during fungal research. Jimenez et al. (2000) point

out that many microorganisms can be cultivated with the application of standard microbiological methods (morphological and biochemical), however, they show that these need much more time to provide results, between 6 and 8 days, while in PCR evaluation only 27 hours can be used. The speed in the analysis optimizes the time for subsequent processes (manufacturing, quality control and release of products). Jimenez et al. (2018) developed a real-time PCR assay for detection of *Burkholderia cepacia* bacteria in contaminated pharmaceutical products and this can be identified in only 30 hours. This bacterium is the main reason for the collection of non-sterile drugs due to microbial contamination in the USA. Famewo et al. (2016) identified several bacterial contaminants, *Bacillus, Enterobacter*, *Klebsiella*, among others, in herbal medicines with the use of PCR through the 16S rRNA gene.

Pullirsch et al. (2014), Meissner et al. (2016) and Melchart et al. (2016) did not specify how this evaluation had performed. Zargaran et al. (2017) applied the membrane filtration technique both for the quantification of bacteria, molds and yeasts and for specific detection of pathogenic microorganisms.

**Table I:** Microbial contaminants isolated in herbal medicine samples.

| Reference | Herbal medicines | Determining method | Results | Conclusion |
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| **Aquino et al. (2010)** | *Peumus boldus*, *Camellia sinensis*, *Maytenus ilicifolia* and *Cassia angustifolia.* | The 80 samples of medicinal plants were examining before and after 30 days of gamma radiation treatment (5 and 10 kGy). The quantification of fungi had performed by plate count of CFU/mL. Dichloran Glycerol Agar 18% was used along with macroscopic and microscopic criteria. The isolates of *Aspergillus flavus* had the evaluation of its toxigenic potential through the use of Coconut Agar (pH 7,0) and Thin-layer chromatography. Aflatoxins B1, B2, G1 and G2 were evaluated through the Extraction and Cleaning Method of samples with VICAM immunoaffinity columns (AflaTest kit) and Thin-layer chromatography. | Among the non-irradiated samples, 93,7% obtained high counts of fungi, including toxigenics, and this demonstrated that 75% of them did not comply with the requirements of the World Health Organization regarding the quantification of these microorganisms. The irradiation with 10 kGy could reduce the fungal counts in all samples after 30 days of storage. The importance of an adequate (hermetically sealed) packaging for the storage of medicinal plants is also highlighted. The most varied fungal genera had found in the non-irradiated samples: *Aspergillus* (toxigenic), *Penicillium* (toxigenic), *Cladosporium*, *Absidia*, *Fusarium* (toxigenic), *Mucor*, non-sporulated fungi, *Scopulariopsis*, *Phoma*, *Cephalosporium*, *Syncephalastrum* and *Wallimia. Aspergillus* and *Penicillium* were the most detected, but aflatoxins hadn't found. | Gamma radiation was efficient in decontaminating the samples, and revealed promising results to avoid the appearance of possible fungal contaminants during the storage of medicinal plants. This decontamination method provides better quality and safety to herbal medicines. |
| **Ting et al. (2013)** | "Eight Treasure Herbal Tea", "Herbal Tea", Xiyangshen (*Radix Panacis Quinquefolii*) and Dangshen (*Radix Codonopsis*). | Four Chinese herbal medicines were evaluated before and after boiling (100 °C for 1h). The quantification of microorganisms occurred by the plate count method. The plates of agar used were: Nutrient Agar, Hicrome™ Bacillus Agar, Mannitol Salt Agar, Reinforced Clostridial Agar and Potato Dextrose Agar. Lactophenol Cotton Blue had used for microscopic analysis. | Most samples had microbial cells, around 6 $log_{10}$CFU/mL, which were significantly reduced after boiling. The predominantly isolated bacteria were *Bacillus* spp., *Staphylococcus* spp. and *Clostridium* spp., all eliminated after boiling. Fungi has also been isolated and the presence of *Aspergillus* spp. had been identifying. The number of herb species (single or multiple) had no influence on the level of contamination observed. | The presence of microbiological contaminants in herbal medicines is not uncommon. Following quality control regulations significantly reduces this contamination. It was verified that the boiling process eliminated most of the pathogenic agents present in herbal medicines, guaranteeing |

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| | | | | the necessary safety for consumption after this process. |
| **Noor et al. (2013)** | Unani and Ayurvedic preparations. | The 125 samples of Unani and Ayurvedic (85 liquids and 40 semissolids) were evaluated. A pre-treatment of the samples with Polysorbate 80 (sterile inactivating agent) was necessary, due to their antimicrobial activity. The enumeration of bacteria, moulds and yeasts was performed by plate count. The bacteria have been investigated, in general, with the use of Soyabean Casein Digest Agar already the fungi were analyzed through the cultivation in Sabouraud Dextrose Agar. *Salmonella* spp. and *Shigella* spp. had been researched using Xylose Lysine Deoxycholate Agar and coliforms using MacConkey Agar, Eosin Methylene Blue Agar and Triple Sugar Iron Agar. | Among the liquid samples analyzed, 2 showed high rates of bacterial contamination, and 10 samples showed high contamination by mould and yeast. In 1 sample the presence of coliforms was verified, but *Salmonella* spp. and *Shigella* spp. bacteria were not isolated. Regarding the semissolid samples, 1 showed significant bacterial contamination and 5 excessive fungal contaminations. | It was found that aseptic manipulation during the production process of herbal preparations is of fundamental importance and necessity, since contaminated products can compromise the health of their users and can cause death. |
| **Ezekwesili-Ofili et al. (2014)** | Not specified. | The 210 samples of herbal preparations had evaluated. The pathogens were enumerated by plate count. Several plates of agar had used for the cultivation, namely, MacConkey Agar, Sorbitol MacConkey Agar, Blood Agar, Eosin Methylene Blue Agar and Potato Dextrose Agar with chloramphenicol. Gram staining, besides the motility and serological tests have also been applied. Lactophenol Cotton Blue had used for microscopic analysis of fungi. The presence of aflatoxins was verified by Thin-layer chromatography. | The presence of many bacteria (Enteropathogenic *E. coli* **-** EPEC, Enterohemorrhagic *E. coli* **-** EHEC, *Bacillus*, *Pseudomonas*, other coliforms, *Salmonella* and *Streptococcus*) and fungi (*Aspergillus, Cladosporium, Rhizopus, Penicillium, Mucor, Curvularia, Candida* and *Geotricum*) was verified. Among bacteria, the genus *Bacillus* was the most commonly isolated and among fungi, the genus *Aspergillus.* The presence of aflatoxins has been confirmed in 18,6% of the samples, being B1, B2 and G1. | It was observed that the presence of microbial contaminants and aflatoxins in herbal medicines used in the country in question, Nigeria, is recurrent. Neglect of basic hygiene care in the main production processes, including storage, demonstrates inefficiency in the segment of public health protocols in the production of herbal medicines, which exposes an endemic problem. As a result, the data gathered in this paper will be of great importance for the development of new public |

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| | | | | health standards in the safety and production process of herbal medicines in Nigeria. |
| **Dei-tutuwa et al. (2014)** | Not specified. | Seven herbal medicines were investigating. The microbial count occurred according to the method indicated in the United States Pharmacopoeia for non-sterile products. The methods of DNA extraction with tris-HCl EDTA buffer had used, by the boiling method and with the use of commercial kits – Gentra Puregene Yeast/Bact. Kit and DNeasy$^{TM}$ Tissue Kit. The extracted DNA's were applied in the Polymerase Chain Reaction (PCR) for identification of *Escherichia coli*, *Staphylococcus aureus* and *Salmonella* spp.. The gel electrophoresis allowed a good visualization of the results. | Microbial quantification ranged from 0 to $3x10^6$ CFU/mL and only 1 of the 7 phytotherapics analyzed showed no growth of microorganisms. Only the commercial kits could extract good quality bacterial DNA. PCR was effective in the direct detection of bacteria in samples. *Escherichia coli* could be detected at 10 CFU/mL, *Staphylococcus aureus* up to $10^3$ CFU/mL and *Salmonella* sp. couldn't be detected. | The detection of *E. coli* and *S. aureus* in herbal medicines was achieved through the PCR method. Due to its speed and reliability, the technique has the potential to be applied in the quality control of these drugs, as well as in clinical practice, however, it still needs to be further investigated. We consider that real-time PCR would be even more useful, since it can determine microorganisms qualitatively and quantitatively, but it also requires further exploration. |
| **Pullirsch et al. (2014)** | Not specified. | One phytotherapic drug had been analyzing, which was among 25 other drugs not approved, seized in the Austrian illegal market. The microbiological analysis took place according to the methodologies and acceptance criteria established by the European and United States Pharmacopoeias. Thus, tests had performed for total aerobic microbial count, quantification of total yeasts and moulds, measurement of anaerobic bacteria and enterobacteria, as well as research on pathogenic microorganisms (*Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella* spp. and *Staphylococcus aureus*). | It could be observed that the herbal medicine exceeded pharmacopoeia requirements both in the total aerobic microbial count, presenting 720.000 CFU/g, and in the measurement of bile tolerant gram-negative bacteria with counts over $10^4$ CFU/g. Among the species of bile tolerant gram-negative bacteria verified, *Klebsiella pneumoniae* was the most found. | It can be noticed that the non-approved drugs do not have the same care regarding their manufacturing method, especially when referring to sanitary conditions, when compared to the approved drugs. The consumption of non-approved drugs, often with microbial contaminants at excessively high levels, can bring great risks to the health of the population that uses them. |

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| **Singh et al. (2015)** | *Coriandrium sativum*, *Cuminum cymirium*, *Syzygium aromaticum*, among others and cumin, fenugreek, coriander, black pepper, clove, ginger and among others. | We explored 219 samples of medicinal plants, herbs and spices, food (dairy products, cereals and derivatives, fruits and vegetables), infant food formula, environmental and clinical samples. The *Cronobacter* spp, was identified according to ISO 22964: 2006 with the use of Chromogenic Agar for the isolation of *Enterobacter sakazakii* (*Cronobacter sakazakii*). This species was the target of the investigations, since it can cause severe infections in babies and newborns, as well as in elderly and immunocompromised people. For detection of the species *C. sakazakii* the use of citrate, methyl red, indol production and Voges Proskauer were performed, as well as confirmation of that isolates from the Polymerase Chain Reaction (PCR) genus and species specific. The 16S rRNA gene and the partial nucleotide internal transcript spacer (ITS) region had amplified by PCR. Agarose gel electrophoresis was also used. As for antimicrobial susceptibility analysis, the disc diffusion method was applied according to the National Committee for Clinical Laboratory Standards on Tryptic Soy Agar. The diameters of the inhibition zones had been measuring and interpreted according to the guidelines of this same Committee. The antibiotics used in this evaluation were: aminoglycosides, β-lactams, phenicols, glycopeptides, coumarin glycosides, macrolides, peptides and tetracycline. | We identified 45 isolates of *Cronobacter* spp. in the most varied products evaluated, and 3 of these isolates were found among the medicinal plants. Most of the biochemical tests presented as expected for the recognition of *C. sakazakii*, except for 2 isolates that were negative for the Voges Proskauer test. The PCR specimen-specific allowed the detection of 36 bacterial isolates such as *C. sakazakii.* The bacterial genus was most commonly found in herbal and spice materials (34%), demonstrating that this microorganism is commonly deposited in these products to protect against environmental stresses. Among the antibiotics evaluated, only susceptibility to phenicols and tetracyclines had found. | By knowing the materials in which the bacteria have a preference to allocate, it is possible to improve the screening and quality control of these products to prevent possible damage to consumers. It can be observed that combining confirmatory methods during the detection of *Cronobacter* spp. brings results of greater veracity. There should be caution in the use of antimicrobials, since the pathogen is increasingly resistant to commonly prescribed treatments. |

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| **Ratajczak et al. (2015)** | Not specified. | The 1.285 samples of non-sterile drugs had investigated and 267 of them were phytotherapeutic drugs (227 without antimicrobial pre-treatment and 40 submitted to hot extraction process). The quantification of total aerobic bacteria, moulds and yeasts was done by the CFU/mL plate count, using the Soy Casein Agar and the Sabouraud Dextrose Agar. Bile tolerant gram-negative bacteria (*Enterobacteriaceae*) were also quantified. For this, Soybean Casein Digest Broth with posterior sowing in Broth Mossel and Violet Red Glucose Agar had used. For isolation and detection of *Staphylococcus aureus*, *Pseudomonas aeruginosa, Escherichia coli* and *Salmonella* spp. the Tryptic Soy Broth with subsequent culture in selective media had used. The media used were: Mannitol Salt Agar, Cetrimide Agar, Broth and MacConkey Agar, Rappaport Vassiliadis Broth and Xylose Lysine Deoxycholate Agar. The VITEK® 2 system (bioMérieux) contributed to the recognition of microorganisms. | Most of the samples that did not meet the quality standards of the European Pharmacopoeia were medicinal products of plant origin (5,7%). Among the phytotherapics that would not suffer antimicrobial pre-treatment analyzed, 4 showed high rates of contamination by aerobic bacteria, and 7 samples showed high contamination by fungi. In 3 samples excessive contamination by bacteria of the *Enterobacteriaceae* family had observed, being them *Pantonea* spp., *Raoutella planticola* and *Citrobacter braakii.* The presence of *E. coli* was verified in 2 samples. Regarding the phytotherapics that would be submitted to a hot extraction process, 2 demonstrated high contamination by aerobic bacteria and 1 sample demonstrated high rate of fungal contamination. The aerobic bacteria analyzed belonged to the genera *Bacillus, Micrococcus* and *Enterococcus,* while the fungi belonged to the genera *Aspergillus, Rhizopus, Alternaria* and *Mucor.* The bacteria *S. aureus*, *P. aeruginosa* and *Salmonella* spp. as well as the yeast *Candida albicans* were not isolated in any group of drugs evaluated. | Microbiological quality to non-sterile drugs is required, whether at the registration, production or distribution stages of these drugs, according to Good Pharmaceutical Practices and pharmacopoeia standards (in this case, European Pharmacopoeia). The guidelines must be complied with so as not to bring risk to consumers, since many users already have their health harmed. |
| **Walther et al. (2016)** | Not specified. | The 109 samples of traditional liquid herbal medicines had been analyzing. The bacteria were measured by plate count. Blood Agar and MacConkey Agar had used for cultivation. To identify the bacterial species, the tests of lactose fermentation on MacConkey Agar were used, as well as the Triple Sugar Iron Agar, Sulfide Indole | Among the samples analyzed, 89 showed contamination with fecal coliforms and 87,6% of them exceeded the limits recommended by the United States Pharmacopoeia. The most commonly found bacterial species were *Klebsiella pneumoniae* (34,8%) and *Enterobacter aerogenes* | It was realized that multiple factors can contribute to the excessive contamination of herbal medicines, being some of them: low schooling of professionals involved with the preparation of products, lack of training, lack of registration with the Ministry of Health and Social |

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| | | Motility Agar, Citrate, Urease and Oxidase. | (29,2%). *Salmonella* spp. and *Shigella* spp. were not isolated. | Welfare, absence of proper packaging, inadequate storage, etc. This way, it becomes essentially a better qualification of the people involved with the preparation of these herbal medicines, as well as the implantation of regulations that aim at a greater safety to these products. |
| **Meissner et al. (2016)** | Peruvian Maca (*Lepidium peruvianum*). | Microbiological analyses of the different phenotypes of Peruvian Maca (*Lepidium peruvianum*) were performed, being them the phenotype yellow, black, red and purple. For this, 12 lots of hypocotyles of the plant with different phenotypes were used. The total viable bacterial quantification was performing and presented in the form of CFU/g and log10 CFU/g. | The highest contamination had observed for the yellow phenotype with a total viable bacterial load of $2{,}2x10^4$ CFU/g or 4,33 log10 CFU/g and gram-positive aerobic bacilli can be found. The purple hypocotyles obtained contaminants in intermediate levels with bacterial enumeration of $1{,}1x10^3$ CFU/g or 3,04 log10 CFU/g and it was possible to see gram-negative bacteria in rods. In the red and black phenotypes the contamination was of less importance. | Bacterial levels were too high, with statistically significant differences, for yellow hypocotyles, while black phenotypes showed much lower loads of microorganisms with only a few gram-positive cocci. |
| **Yesuf et al. (2016)** | Not specified. | We analyzed 55 samples of herbal medicines (13 in liquid form and 42 in solid form). The measurement of aerobic bacteria and total coliforms was performing by plate count of CFU/mL. The media used for cultivation were: Violet Red Bile Agar, Blood Agar, MacConkey Agar and *Salmonella Shigella* Agar. Gram staining had performed, as well as lactose and mannitol fermentation tests, urea hydrolysis test, decarboxylase lysine, H2S production, oxidase, indol, citrate, coagulase, catalase and novobiocin sensitivity test. A standard biochemical methodology had also been | The measurement of total aerobic bacteria ranged from zero to $2{,}41x10^9$ CFU/g with an average load of $1{,}99x10^8$ CFU/g or mL and in only one sample the limits recommended by the World Health Organization (WHO) were not exceeded. The quantification of total coliforms varied from zero to $2{,}1x10^9$ CFU/g with an average count of $1{,}05x10^8$ CFU/g or mL. 150 strains could be detected in the samples. Among the isolated bacteria, 34,7% were gram-positive and 65,3% gram-negative. 90,9% had pathogenic microorganisms or fecal contaminants. The genus *Bacillus* was the most frequently | It can be seen that the phytotherapics sold in several markets of Gondar (Ethiopia) presented too much contamination by pathogenic bacteria, with high counts of aerobic and coliform bacteria. Fecal contaminants could be verified in more than 40% of the analyzed products. Most of the bacterial isolates obtained multiple resistance to antibiotics. This high level of contamination can compromise the health of |

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| | | used for better identification of gram-negative and gram-positive bacteria. Regarding the susceptibility evaluation, the disc-diffusion method had used according to the Clinical & Laboratory Standards Institute guidelines. Several antimicrobials were evaluating, being: amoxicillin, ampicillin, ceftriaxone, chloramphenicol, cloxacillin, cotrimoxazol, erythromycin, gentamicin, nitrofurantoin, norfloxacin and penicillin. | found, followed by *Enterobacter*, *Shigella* and *Salmonella*. Among the isolated bacteria, 87,3% showed resistance to ampicillin, 63,3% to amoxicillin with clavulanic acid, 61,3% to amoxicillin, 57,7% to penicillin and 48,7% to nitrofurantoin. It was also observed that 83,3% of the microorganisms had resistance to 2 or more antimicrobials. | those who use these products. For this reason, a greater investment in qualification and training of phytotherapists is necessary, so that it is possible to supply products with higher quality and safety to consumers. |
| **Melchart et al. (2016)** | *Aurantii immaturus fructus (Zhishi), Citri reticulatae pericarpium (Chenpi), Lonicerae flos (Jin Yinhua)* and *Bupleuri radix (Chaihu).* | The 23 samples of herbal drugs from Traditional Chinese Medicine were examining. The European Pharmacopoeia supported the following evaluations: total aerobic microbial quantification, total fungi and yeast count, *Salmonella* spp. and *Escherichia coli* detection as well as aflatoxin B1, B2, G1 and G2 detection. | *Aurantii immaturus fructus* and *Bupleuri radix* exceeded acceptable limits for total aerobic microbial count and *Bupleuri radix* also showed higher levels of *E. coli*. Fungi, yeasts, *Salmonella* spp. and aflatoxins hadn't been isolating. Contaminated samples had rejected from hospital routine. | The pre-clinical quality control project implemented for all Chinese herbal medicines used at the Bad Kötzting hospital allowed low quality plant drugs to be excluded from routine administration. There is a constant quest to provide greater safety to patients, so tools that can anticipate, prevent or contain damage will always be desired. |
| **Rajeshwari & Raveesha (2016)** | *Acorus calamus*, *Cassia angustifolia*, *Centella asiatica*, *Myristica fragrans*, *Tinospora cordifolia* and *Withania somnifera.* | We evaluated 18 samples of 6 raw materials of phytotherapeutic drugs: *Acorus calamus*, *Cassia angustifolia*, *Centella asiatica*, *Myristica fragrans*, *Tinospora cordifolia* and *Withania somnifera*. For analysis of the fungal contaminants the following methods have been used: Filter paper method and Dilution in series with sowing in Czapek Dox Agar. Staining with Lactophenol Cotton Blue have also been used. *Aspergillus flavus* isolates had their toxigenic potential (aflatoxin B1) evaluated, through the use of Liquid | Fungal contaminants have been observing in all plant raw materials examined. It can be verified that all samples of *A. calamus*, *M. fragrans* and *T. cordifolia,* besides two samples of *C. asiatica,* as well as one sample of *C. angustifolia* had fungal levels above the limits recommended by the World Health Organization. The 302 fungal isolates were detected and these belonged to 42 species of 17 different genera. *Aspergillus* and *Penicillium* were the most commonly found genera. Regarding the toxigenic potential analysis, 61,53% of *A. flavus* isolates could | The proof of the microbiological quality of the raw materials that compose the herbal medicines is of extreme importance, since the consumption of these medicines with high levels of fungi and mycotoxins brings great risk to the consumers. Relates too much contamination by AB1, there are already known severe effects that this toxin can cause, some of them being mutagenic, carcinogenic, neurotoxic, |

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| | | Medium composed of Sucrose, Magnesium Sulphate, Potassium Nitrate, Yeast Extract and Distilled Water (SMKY). Aflatoxin B1 (AB1) was quantified in samples with high levels of contaminants through Thin-layer chromatography with some modifications and Spectrophotometry. To confirm the presence of this aflatoxin the analysis have also performed by High Performance Liquid Chromatography coupled with mass spectrometry. | produce AB1 and the highest yield of this was found from fungal isolates found in *T. cordifolia.* Among the 6 raw materials of phytotherapics investigated, half of them demonstrated AB1. Regarding the recommended levels for AB1, *T. cordifolia* exceeded both the European Pharmacopoeia and the Food Safety and Standards Authority of India. *A. calamus* and *M. fragrans* exceeded only the limits allowed by the European Pharmacopoeia. | nephrotoxic, etc. The processing and storage systems must be improved to obtain a safer environment. It is also necessary to invest in the qualification of the manipulators of these raw materials, so that in this way, the contaminations can be significantly reduced. |
| **Zargaran et al. (2017)** | Chamomile Oil (*Matricaria chamomila*). | We evaluated 6 types of chamomile oils, 4 prepared with direct heat, being one of them a traditional method of Persian medicine and 2 produced with indirect heat. The "non-traditional" methods had modified techniques proposed in this study (Clevenger appliance, modified Clevenger appliance, soxhlet and microwave), aiming to improve the final product. The total count of microorganisms of each of the 6 oils had done through the membrane filtration technique. The membrane applied was mixed cellulose ester and the tryptic soy agar have been used. The samples have also been investigated for the presence of pathogenic microorganisms, being investigated *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella* spp. and *Candida albicans*. The following culture media had used in the research of pathogens: Baird-Parker Agar, Mannitol Salt Agar, Lactose Broth, Eosin Methylene Blue Agar, MacConkey Agar, Cetrimide Agar, Selenite Cystine Broth, Tetrathionate Broth, Xylose Lysine Deoxycholate Agar, | Among the samples produced by direct heat, it can be seen that the modified techniques of Clevenger apparatus and modified Clevenger apparatus obtained bacterial colony counts at lower levels, both with less than 10 CFU/g. For oils prepared by indirect heat, it was possible to notice that the modified microwave method showed lower bacterial counts when compared to the traditional Persian medicine method with only 20 CFU/g. Regarding pathogen research, it can be verified that the samples elaborated through the modified Clevenger apparatus and the modified Clevenger apparatus did not present any of the microorganisms investigated. | The use of modified techniques for the production of chamomile oils (modified Clevenger and microwave) really brought improvements to the final product, and among these benefits we highlight the lower levels of bacterial contaminants. |

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| | | Brilliant Green Agar and Yeast Extract with chloramphenicol. | | |
| **Keter et al. (2017)** | Not specified. | It was examined 100 products based on plants. The fungal load has been determined by the plate count method with the use of Sabouraud Dextrose Agar with Chloramphenicol. To verify the various fungus species, the Potato Dextrose Agar with Chloramphenicol and Potato Dextrose Glucose at 20% were used. Lactophenol Cotton Blue had used for microscopic analysis. Aflatoxins and fumonisins were quantified through QuickToxTM Kit and this tool is similar to the Enzyme-linked Immunosorbent Assay (ELISA). | Among the products analyzed, 69% exceeded the fungal limits required by the United States Pharmacopoeia (USP). The fungal quantification ranged from 1 CFU/g to more than 1000 CFU/g. It was possible to detect many fungal genders: *Aspergillus*, *Penicillium*, *Saccharomyces*, *Rhizopus*, *Rhodotorula*, *Cryptococcus*, *Basidiobolus*, *Mucor*, *Malbranchea*, *Absidia*, *Trichophyton*, *Scedosporium*, *Fusarium* and *Candida*. *Aspergillus* was the most found genus, followed by *Penicillium.* The aflatoxins were between 1 and 24 parts per billion (ppb) and the fumonisins between 1 and more than 20 ppb observing that only 31% of the samples met the limits recommended by USP. | The microbiological quality of herbal medicines can be improved if there is greater care and standardization during the production stages of these products (harvest, processing, storage and marketing). It has been suggested that a policy should be implemented so that herbal products have greater regulation, since these products are widely accepting worldwide, as also occurs in Kenya. |
| **Lima et al. (2020)** | Not specified. | We analyzed 132 home and commercial herbal medicines. The load of bacteria and fungus had performed by plate count. In the bacterial analysis, the Tryptic Soy Agar had used and in the fungus the Sabouraud Dextrose Agar. For the isolation and identification of *Escherichia coli*, *Salmonella* spp., *Pseudomonas aeruginosa* and *Staphylococcus aureus* the Tryptone Soy Broth or the Lactose Broth with posterior sowing in more selective media have been used (MacConkey Agar, Cetrimide Agar, Eosin Methylene Blue Agar, Mannitol Salt Agar, Brilliant Green Agar and Triple Sugar Iron Agar). Gram | Regarding aerobic bacteria, 31,8% of the samples exceeded the limits recommended by the World Health Organization (WHO), where 15,1% corresponded to comercial herbal medicines and 16,7% to home herbal medicines. Likewise, 23,5% of the samples exceeded the permitted fungal count. The most frequently isolated bacteria were *S. aureus* (49,2%), followed by *Salmonella* spp. (34,8%), *E. coli* (25,8%) and *P. aeruginosa* (14,4%). | The absence of microbiological quality standards, either in the production process or in the sale of herbal therapies, brings great risk to the health of the elderly, since they often use these therapies. Bacterial and fungal counts above the recommended limits have been observed, as well as the presence of pathogenic microorganisms. A greater control of the commercialization of these products is necessary, to avoid that medicines that do not meet |

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| | | staining, oxidase research, gas production and catalase test has also been performing. | | the quality standards are consumed. |
| **Li et al. (2020)** | Six *Coix lachrymal-jobi* cultivars. | Grains from 6 cultivars of *Coix lachrymal-jobi* were examined. Before the analysis the materials had sterilized with the use of 75% alcohol, sterile distilled water and application of Mercury chloride. Endophytic fungi have been researched in the plant materials by visualizing morphological aspects of the fungal colonies, as well as by evaluating the sequence of the Internal Fungal Spacer (ITS) which was amplified by the Polymerase Chain Reactin (PCR). The culture media used were MS (Murashige & Skoog) and Potato Dextrose Agar with penicillin and streptomycin. Regarding the fungal DNA, it was extracted using the commercial DNeasy Plant Mini Kit and the amplified products generated were applied to agarose gel electrophoresis for later analysis with the GoldView™ system and sequencing with the use of ABI Prism 310. ITS sequences had added to GenBank and a phylogenetic tree was built using MEGA X. | Eight endophytic fungi isolates were detected and these belonged to five species (*Penicillium expansum*, *Penicillium polonicum*, *Cladosporium cladosporioides*, *Alternaria alternata* and *Aspergillus flavus*) and two main genera (*Aspergillus* e *Fusarium*). It can be observed that the fungi *P. expansum*, *Aspergillus oryzae* and *C. cladosporioides* can produce lipases, amylases, celulases with useful functions in food science, fermentation processes, among others, but this issue still needs to be further explored. The fungi *P. polonicum, A. flavus, Fusarium* spp., *C. cladosporioides* and *A. alternata* are potentially toxigenic and can bring harm to severa living beings. | The use of especific antimicrobials as well as better care during post-harvest processing and storage can prevent these grains from being contaminated with various microorganisms. |

## CONCLUSION

In this review, we gathered numerous scientific articles that demonstrated a high load of microbial contaminants in herbal medicines. This fact is worrying, since the population consuming these products grow daily, and can be exposed to infections and intoxications that compromise their health. Relates the methods of microbial determination, there was emphasis on plate count (quantification of microorganisms) and sowing in selective media (evaluation of specific pathogens). The microorganisms isolated most frequently in these products were *Aspergillus* spp., *Penicillium* spp., aflatoxins, *Bacillus* spp., *Staphylococcus* spp. and bacteria of the *Enterobacteriaceae* family. We observed that the obtaining of phytotherapics of better quality, security and effectiveness had been strongly related to the greater care with the manufacturing process in general, the fulfillment of the good manufacturing practices and the most efficient application of the quality control and guarantee.

## AUTHOR CONTRIBUTIONS

Author Maria Laura Sunderhus Gloria was responsible for writing the manuscript and authors Priscilla Maciel Quatrin and Alexandre Meneghello Fuentefria critically reviewed it and approved its final version.

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**ANNEX I**

**Preparation of manuscripts**

All parts of the manuscript should be double-spaced throughout. After acceptance, no changes will be made in the manuscript so that proofs require only corrections of typographical errors. The authors should send their manuscript in electronic version only.

**Length of manuscript**

While papers may be of any length required for the concise presentation and discussion of the data, succinct and carefully prepared papers had favored both in terms of impact and in readability. They must not, however, exceed 50 pages, including all items (figures, tables, references, etc…), unless previously agreed with the Editor-in-Chief.

**Title page**

The title page of the manuscript should present the following items: 1. Title of the article (the title should be up to 150 characters including spaces, and informative to a broad scientific community); do not include abbreviations in the title. 2. Full name(s) of all author(s); use superscript numbers right after each author name to indicate the affiliation; 3. Professional address and ORCid of all authors, including Department and Institution name, street name and number, ZIP/Postal code, City, State and Country; 4. Keywords (four to six in alphabetical order separated by commas); 5. Running title (a short version of the title, up to 50 characters including spaces); 6. Academy Section to which the content of the work belongs; 7. Name, address, phone number, e-mail of the correspondent author, including to whom all correspondence and proofs should be sent to (please indicate the corresponding author with an * after the name). Should any of these requirements do not be met, we may unsubmit your paper and ask for corrections.

**Abstract**

The abstract must contain no more than 200 words and present the main one finding of the article, including a brief introduction, the objectives of the work and a conclusion based on had presented findings. If the authors are submitting an invited/authorized review, the abstract must introduce the main theme of the review and explicit the contribution of the revision to the field. References should not be included in the abstract.

**Manuscript text**

All text should be written in double-space using 12-point Times New Roman or equivalent typeface. Please organize, whenever possible, the text into the following parts: 1. Title Page; 2. Abstract (written on a separate page, 200 words orfewers, no abbreviations); 3. Introduction; 4. Materials and Methods; 5. Results; 6. Discussion; 7. Acknowledgments, if applicable; 8. Author contributions, when there is more than one author, explaining briefly how each author has contributed for the paper 9. References. 10. Figure and table legends, if applicable.

Articles from some areas such as Mathematical Sciences should follow their usual format. In some cases it may be advisable to omit part (4) and to merge parts (5) and (6). Whenever applicable, the Materials and Methods section should indicate the Ethics Committee that evaluated the procedures for human studies or the norms followed for the maintenance and experimental treatments of animals. All procedures must be described in detail. Use American English style to write the text. Chemical names should be provided according to IUPAC, and strains of organisms should be specified. Provide names of reagents and equipment suppliers. Use units and symbols according to Bureau International des Poids et Mesures (SI) symbols whenever possible.

## Acknowledgments

These should be included at the end of the text. Personal acknowledgments should precede those of institutions or agencies. Footnotes should be avoided; when necessary they must be numbered. Acknowledgments to grants and scholarships, and of indebtedness to colleagues as well as mention to the origin of an article (e.g. thesis) should be added to the Acknowledgments section. Include the full name of the funding agency, country, and funded project number (if applicable).

## Abbreviations

These should be defined at their first occurrence in the text, except for official, standard abbreviations. Units and their symbols should conform to those approved by the Bureau International des Poids et Mesures (SI).

## Figure Legends

This information must be provided at the end of the manuscript, after the abbreviations. All figures must contain a descriptive legend. The legend must contain an introductory sentence that describes the main findings. All panels (if applicable) must be identified in the figure legend by lower case letters (1a, 2a, 2b, 3c, 3d, etc.). When presenting error bars, please inform if a number that follows the ± sign is a standard error of mean (SEM) or a standard deviation of mean (SD). Or include in the legend if the presented result is representative of N individual experiments.

## Tables

Each table should have a brief title above it. Table footnotes should be placed below the table. Tables have to be cited in the paper in Roman numerals (Table I, Table II, Tables IV and

V, etc.). Tables must be submitted as separate files in editable format, preferably as *.doc or *docx file.

**Figures**

Only high-quality figures will be accepted (minimum of 300 dpi). All illustrations will be considered figures including drawings, graphs, maps, photographs, etc. Their tentative placement in the text should be indicated and all figures must be cited with their respective number along the text. Figures should be sent according to the following specifications: 1. Drawings and illustrations should be in formats.PS/.EPS or .CDR (PostScript or Corel Draw) and never be inserted in text; 2. Images or figures in grayscale should be in formats .TIF and never be inserted in text; 3. Each figure should be saved and sent in a separate file; 4. Figures should, theoretically, be submitted at the size they are to appear in the journal, i.e., 8 cm (one column) or 16.2 cm (two columns) wide, with maximal height for each figure and respective legend smaller than or equal to 22 cm.

The legends to the figures should be sent double-spaced on a separate page. Each linear dimension of the smallest characters and symbols should not be less than 2 mm after reduction. Colored figures had accepted just as much as b/w ones, but up to 5 black and white figures are free of charge, while every colored figure will be charged, due communication will be made in the production phase (after the evaluation process), should the author want them colored as well in the printed version. For counting black and white figures, tables occupying two thirds of the page or having more than 12 columns or 24 rows will be considered b/w figures; 5. Manuscripts on Mathematics, Physics or Chemistry may be typesetter in TEX, AMS-TEX or LaTEX; 6. Manuscripts without mathematical formulae may be sent in .RTF or doc/docx for Windows.

**References**

Authors are responsible for the accuracy of the References. Published articles and those in press may have included. Personal communications (Smith, personal communication) must be authorized in writing by those involved. References to thesis, meeting abstracts (not published in indexed journals) and manuscripts in preparation or submitted, but not yet accepted, should be cited in the text as (Smith et al., unpublished data) and should NOT be included in the list of references.

The references should be cited in the text as, for example, 'Smith 2004', 'Smith & Wesson 2005' or, for three or more authors, 'Smith et al. 2006'. Two or more papers by the same author(s) in the same year should be distinguished by letters, e.g. 'Smith 2004a', 'Smith 2004b' etc. Letters should also distinguish papers by three or more authors with identical first author and year of publication. References should be listed according to the alphabetical order of the first author, always in the order SURNAME XY in which X and Y are initials. If there are more than ten authors, use et al. after the first author. References must contain the title of the article. Names of the journals should be abbreviated without dots or commas. For the correct abbreviations, refer to lists of the major databases in which the journal is indexed or consult the World List of Scientific Periodicals. The abbreviation to be used for the Anais da Academia Brasileira de Ciências is An Acad Bras Cienc. The following examples are to be considered as guidelines for the References.

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