

CONTRIBUTIONS TO THE STUDY OF THE PHARMACEUTICAL QUALITY OF SOME CHAMOMILE COMERCIAL SAMPLES. NOTE II. THE MICROBIAL CONTAMINATION

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Abstract

Dried plant products used for therapeutic purposes or as seasonings are frequently highly microbially contaminated, the thermal and chemo-resistant spores being the most problematic. They are threatening the health of the consumer directly or through their proliferation by concentrating in the final products. Given that within the European Community there are regulations for *Chamomillae flos*, requiring strict control of the manufacturer of the raw material whether it comes from spontaneous flora or culture, we proposed an evaluation in terms of medicinal teas from the Romanian pharmaceutical market network. For a complete image of the quality we analyzed ten chamomile samples in accordance to the requirements of the 5th European Pharmacopoeia (in force at the time the products were marketed) including the microbial contamination. We hereby present the results obtained regarding the microbial and fungal contamination investigation. Among the microbial strains found in the investigated samples of *Matricariae flos* were: *Aspergillus niger*, *Rhizopus nigricans*, *Mucor mucedo*, *M. racemosus*, *Penicilium sp.* Total microbial count (TMC) values for all samples were within the imposed limits, but 80% of the chamomile samples did not comply regarding TYMC (total yeast and mould count) to actual requirements.

Rezumat

Produsele vegetale uscate utilizate în scopuri terapeutice sau drept condiment sunt frecvent contaminate microbiologic, cei mai problematici fiind sporii microorganismelor termo- și chemorezistenți. Aceștia pun în pericol sănătatea consumatorului, în mod direct sau datorită proliferării lor prin concentrarea în produsele finale. Având în vedere faptul că pe plan european, pentru *Chamomillae flos* există reglementări care solicită producătorului un control riguros al materiei prime indiferent dacă aceasta provine din flora spontană sau din cultură, ne-am propus evaluarea ceaiurilor medicinale comercializate în rețeaua farmaceutică românească. Pentru o imagine completă a calității am analizat zece probe de mușetel, în conformitate cu cerințele Farmacopeei Europene ediția a 5-a (în vigoare la data la care produsele au fost comercializate), inclusiv contaminarea microbiană. În cele ce urmează vom prezenta rezultatele obținute în cadrul studiului de contaminare microbiană și fungică. Dintre tulpinile microbiene găsite în probele de *Matricariae flos* investigate menționăm: *Aspergillus niger*, *Rhizopus nigricans*, *Mucor mucedo*, *M. racemosus*, *Penicilium sp.* Am stabilit că pentru șarjele analizate numărul total de germeni (TMC) înregistrat pentru toate probele s-a încadrat în limitele în vigoare până în 2007, dar astăzi 80% din probele de mușetel nu ar corespunde în ceea ce privește numărul total de drojdii și mușegai (TYMC).

Keywords: chamomile, European provisions, microbial/fungal contamination

Introduction

The therapeutic importance of chamomile flowers is illustrated by the presence of three European Pharmacopoeia monographs: one for the drug itself, *Matricariae flos*, a second which refers to the fluid extract, *Matricariae extractum fluidum* and a monograph for the volatile oil (*Matricariae aetheroleum*) [1,2].

In many European countries, the plant material from the spontaneous flora is marketed in supermarkets, but even here it is considered part of the sorts of cheap tea, just because of the impossibility of making predictions in terms of quality and effectiveness [5,6,14]. Chamomile pharmaceutical crops are obtained only from controlled cultures, which use a few recognized clones because of the rich content in pharmacologically active principles [13].

Our research was focused on the assessment of the pharmaceutical quality according to pharmacopoeia requirements, for 9 commercial samples of *Chamomillae flos* marketed by Romanian pharmacies and one control sample exclusively produced by Sidroga for the pharmaceutical network. Using FR X [3] and 5th European Pharmacopoeia [1] guidelines we analysed the vegetal material from the point of view of the macro- and microscopic aspect, the microbial contamination, the chemical composition (HPLC, GC/MS, spectrophotometry, TLC methods) and the biological activity of chamomile extracts (infusions, tinctures, volatile fractions) [8,9]. This study intends to be a guideline for the pharmaceutical quality assesment for the Romanian producers that wish to supply the pharmaceutical network with a medicinal tea that complies with pharmacopoeial provisions.

In this paper, we present the results obtained for the microbial and fungal contamination of both chamomile samples and infusions for which we pursued: total microbial count (TMC), the assessment of the number of colony forming units of *Staphylococcus aureus*, *Escherichia coli*, *Salmonella sp.*, as well as the number of fungal colony forming units.

Materials and Methods

In 2005 we undertook for research 10 different *Chamomillae flos* samples as follows: **a control** (C0) bought from Sidroga - Germany, sachet packed sample that complies with the European Pharmacopoeia requirements, and was exclusively found in the pharmacies; as **test samples**, 2 were from Belin (C1) and Celmar (C2) (both tea bags) packed in Romania, but for which the vegetal material was imported from Poland (assumed to be cultivated), and other 3 consisting of inflorescences certainly harvested from Romania, but practically

of unknown origin, conditioned as tea bags provided by Fares (C3), Ciprod (C4) and Hofigal (C5). We also selected 4 „bulk” samples probably harvested from the indigenous wild flora commercialized by Digitalis (C6), Cyani (C7), Stef-Mar (C8) and Plafar Botosani (C9).

The assessment of the microbial contamination of samples consisted in bringing the germs on the surface of plant material in aqueous suspension and seeding it on regular or special culture media. Since chamomile flowers were designed for medicinal tea, the same culture media were sowed with the chamomile infusions obtained as described by FR X (3%, filtration after brewing for 30 minutes), corresponding plates being read and interpreted in the same way [3].

Of the 10 samples we prepared a camomile extract with peptone-phosphate buffer that has been inoculated on Sabouraud (assessing the TMC, Figures 1, 2), Chapman (for *Staphylococcus sp.* and *Penicilium sp.*, Figures 1, 2) and MacConkey medium cultures. For this purpose 10 g of each sample were weighed under aseptic environment into 200mL Erlenmeyer sterile flasks, adding 100 mL of phosphate-peptone buffer (pH 7). After intense agitation and rest for 10 minutes, the supernatant was separated and then diluted with phosphate-peptone buffer in a ratio of 1/10, 1/100, 1/1000, 1/10000, in conditions of perfect sterility [4,12]. On the other hand, for the infusions, there were prepared the same dilutions using sterile distilled water. Samples consisting of 1 mL suspension (dilution of supernatant in phosphate-peptone) and infusions (water dilution) were seeded on Petri dishes that were brought in 15 mL culture medium, which was chosen depending on the nature of the contaminant pursued. The content of Petri dishes was homogenized by shaking on the surface of the rotating work table, then they were left to stand for solidification.

Once inoculated the Petri plates were incubated: for 5 days, at 30-35°C, when bacteria were grown; for 7 days, at 20-25°C, when molds and yeasts proliferation was stimulated. Depending on the sought parameter, seeded culture media were different as follows: the Sabouraud for TMC count, Chapman agar for *Staphylococcus aureus* identification and McConkey agar for *Escherichia coli* assesment [7,17,21].

All chemical and reagents were of analytical grade and were purchased from Sigma Aldrich (Seelze, Germany) or Fluka (Buchs, Switzerland).

Results and Discussion

Total microbial count assesment

Samples sown on Sabouraud and Chapman cultures were evaluated and photographed after 5 days (microbial assessment) and 7 days (fungal assessment)

of plates incubation at 35°C. During that period of time on the surface of media it occurred white-yellowish, light pink and even yellow individual or cluster aggregates, depending on the degree of contamination of each sample. Using a Colyte Supercount automated colony counter we selected the appropriate dilution plates with less than 300 bacterial colonies and less than 100 fungal colonies [10,11]. Taking into account the dilution we calculated the number of colony forming units (CFU) per gram of plant product. The general aspect of the incubated Petri plates is presented in figures 1 and 2.

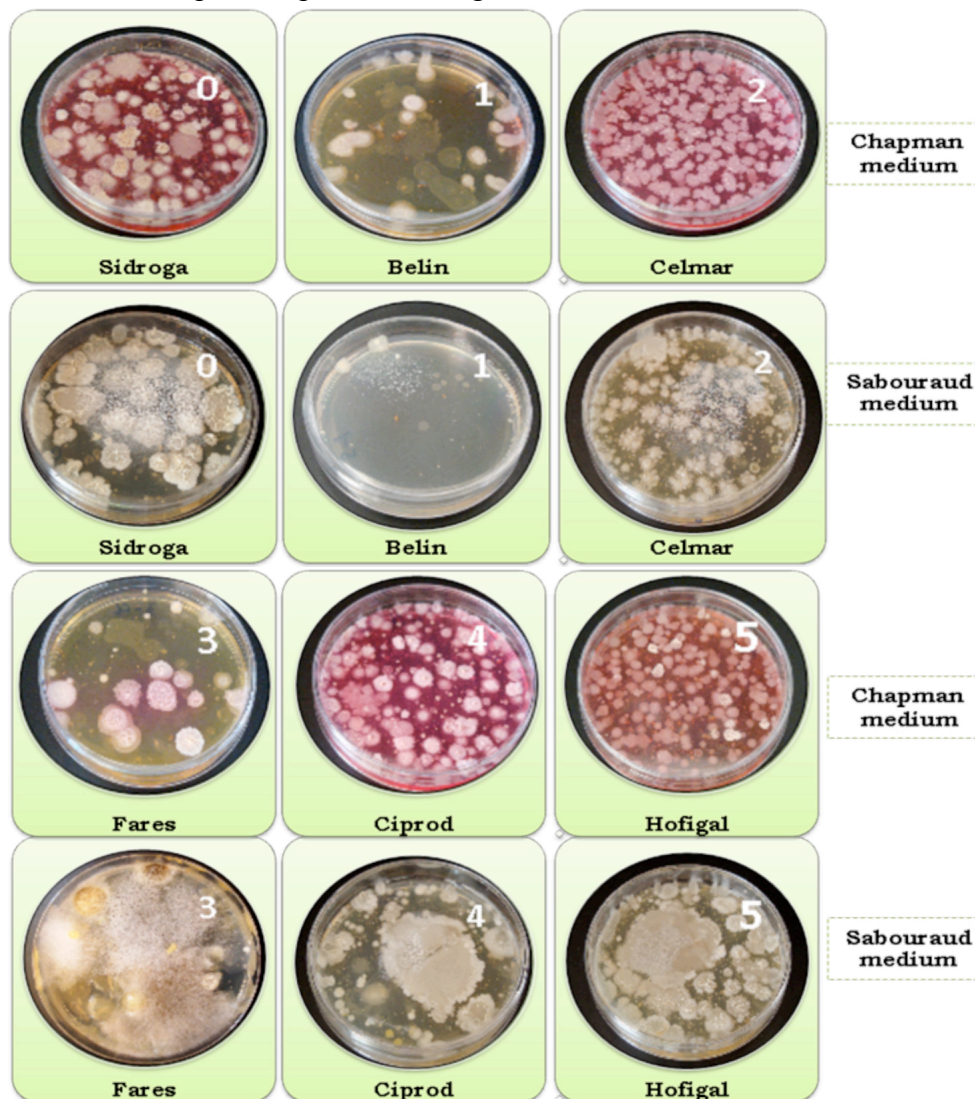


Figure 1

Petri dishes incubated for 5 days at 35°C for the samples packed as tea-bags

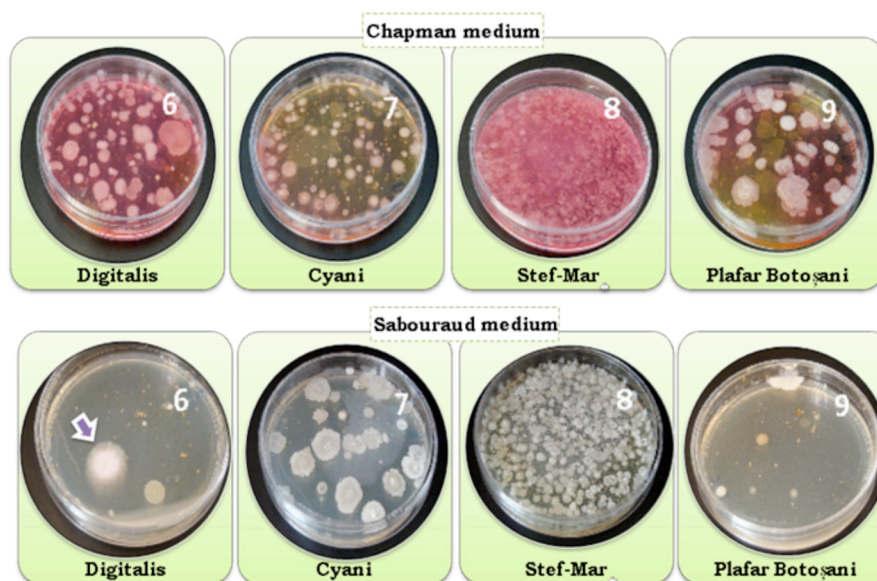


Figure 2

Petri dishes incubated for 5 days at 35°C for the bulk samples

Microbial and fungal strains found as contaminants are presented in table I.

Table I

Microbial strains found in the investigated samples of *Matricariae flos*

Microbial strains	Samples									
	C0	C1	C2	C3	C4	C5	C6	C7	C8	C9
Bacilli										
<i>Escherichia coli</i>	○	○	+	○	○	○	○	○	○	○
Fungi										
<i>Aspergillus sp.</i>	○	+	○	○	○	○	○	○	○	○
<i>Aspergillus niger</i>	○	○	○	+	○	○	○	○	○	○
<i>Rhizopus nigricans</i>	○	○	○	+	○	○	○	○	○	○
<i>Mucor mucedo</i>	○	○	○	+	○	○	○	○	○	○
<i>Mucor racemosus</i>	○	○	○	+	○	○	○	○	○	○
<i>Penicilium sp.</i>	○	○	○	+	○	○	+	○	○	○
Yeasts	+	+	+	+	+	+	+	+	+	+

⊕ = present; ○ = absent

The results were interpreted according to the stated rules and the values that express the number of colony forming units (CFU) per gram identified for each sample are listed in Table II.

Table II

TMC/g vegetal material from *Chamomillae flos* samples

Code	TMC (CFU/g)	<i>S. aureus</i> (CFU/g)	<i>E. coli</i> (CFU/g)	Fungi (CFU/g)	Provision compliance
C0	2000	abs	abs	500	✓
C1	1500	abs	abs	80	✓
C2	5000	abs	40	200	✓
C3	2000	abs	abs	600	✓
C4	5800	abs	abs	2000	✓
C5	6300	abs	abs	1500	✓
C6	2800	abs	abs	60	✓
C7	3000	abs	abs	400	✓
C8	9800	abs	abs	6000	✓
C9	1200	abs	abs	1500	✓

abs = absent

As it can be seen, C8 (bulk), C5 (tea-bags) and C4 (tea-bags) samples have the highest microbial contamination. Nevertheless, all samples of *Chamomillae flos* fulfill the requirements of both FR X (valid at the time when samples were marketed 2005) and the 5th European Pharmacopoeia, on the total aerobic plate count and colibacili (*E. coli* less than 10^2 CFU/g) admitted. We observed the presence of *Escherichia coli* just for the chamomile from (C2) Celmar, but in this case the number of CFU/g has not exceeded the imposed limit. No bacteria was isolated from the Chapman agar, indicating the absence of *Staphylococcus aureus* strains [4].

Since chamomile flowers are used as tea, they were prepared as described in the *Solutiones extractivae aquosae* monograph FR X, and not according the manufacturer's prescriptions, which are inconsistent, leading to results that can not be compared with each other. After brewing for 30 minutes, samples of 1 mL of infusions were seeded on agar for microbial quality assesment by the method described above. The results are presented in table III, the calculation taking into account that the infusion is 3%, which means that after processing, TMC corresponding to 1g chamomile flowers can be found in 33 mL infusion.

Table III
Microbial quality of brewed sample

Code	TMC (CFU/g)	Fungi (CFU/g)	Provision compliance
C0	500	100	✓
C1	0	0	✓
C2	0	0	✓
C3	110	20	✓
C4	1400	700	✓
C5	2000	300	✓
C6	0	0	✓
C7	0	0	✓
C8	4500	700	✓
C9	400	0	✓

(TMC/33 mL infusion = 1g chamomile)

All samples fulfill the requirements of FR X and 5th Ph.Eur. regarding the microbiological and fungal contamination, being for the most samples below the acceptable limits. Also by infusing for about half of the samples, bacterial and fungal load decreased to zero (For TCM, the proportion is 4 of 9, i.e. less than half (50%), but close to it; Because results are expressed in CFUs, the use of percentage (0%) is not justified).

Fungal contamination assesment

After 7 days from the inoculation of the samples on Sabouraud agar (kept at room temperature), we performed the macroscopic analysis of Petri dishes prepared to assess the extent of contamination by fungal strains. For the correct identification of fungal strains we observed colony characteristics such as: the color of mycelium, vegetative conidia heads and sclerotia (assessed both on the surface and the back of Petri plate), the colony edge (which can be defined or diffuse, smooth or lobate, with the same thickness or thinner than the colony), the colony type (air or partially submerged), the colony form (round, smooth, star, arched), the colony surface (smooth or presenting different groove forms) and aspect (soft, fluffy, woolly, granular or shrub shape), colony zoning (spores on the whole surface or only in certain areas of the colony), the smell (mold, fruit or edible mushroom flavor), the presence or absence of exudate, and if it exists, what form (drops or diffuse) and color develops [10,15].

The development of molds was noted only in the case of samples C1, C3 and C6. Fragments of mycelium from each fungal colony were isolated and were microscopically analyzed (WF 10 X Optika microscope, Canon PS A540 fotocamera) in order to identify the strain [4,15]. Thus, we have established the following: sample C1 (Belin) was contaminated with *Aspergillus niger* (fig. 3A), sample C3 (Fares) contained *Aspergillus niger* (fig. 3A), *Rhizopus nigricans* (fig. 3B), *Mucor mucedo* (fig. 3C), *Mucor racemosus* (fig. 3D), *Penicillium sp.*, whereas sample C6 (Digitalis) was contaminated with *Penicillium sp.*

Also, the presence of yeasts was noted in all samples, but all registered values were within the limits allowed by the European Pharmacopoeia.

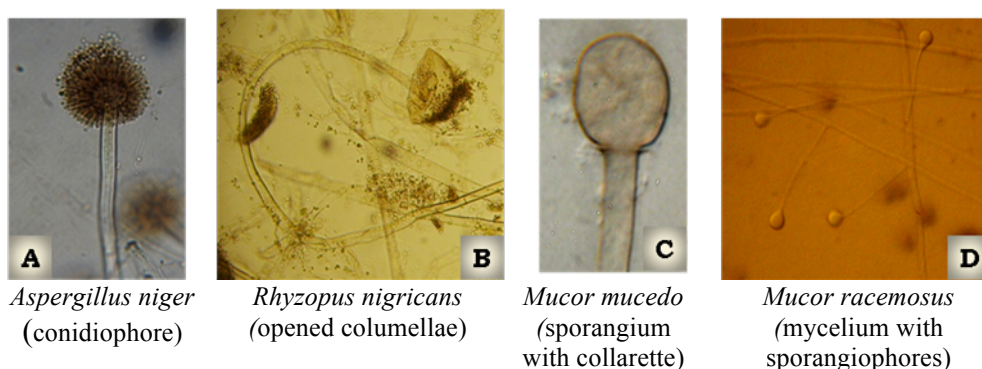


Figure 3

Microscopic features of the molds found in the chamomile samples

When referring to the fungal load samples Ciprod (C4), Hofigal (C5), Stef-Mar (C8) and Plafar Botosani (C9) had the highest values, but they did not exceed 10^5 fungi per gram product.

Conclusions

We concluded that there are no differences between the packing categories (bulk or tea-bags), moreover higher TMC values are to be found for the excessively grounded products (such as C5) or containing stem parts in large quantities (C2, C4) presented as tea-bags (hence indicating excessive handling).

TMC (total microbial count) values for all samples were within the imposed limits, but compared to the actual European Pharmacopoeia [2] 80% of the chamomile samples would not comply regarding the TYMC (total yeast and mould count). On the other hand, it is clear that by brewing,

the microbial and fungal load will reduce; the infusion should be made according to pharmacopoeia provisions.

Due to the fact that frequently, both with community framework and within the hospitals, once opened, a package of tea is not fully consumed immediately. It may be assumed that the plant material, regardless of packaging, can be exposed to humidity and temperatures exceeding recommendations for conservation, which could, in theory, lead to proliferation of both the aerobic germs as those of fungi and yeasts, high enzyme-producing and possibly mycotoxins synthesizers [16,18,19,20].

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