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MICROBIOLOGICAL QUALITY CONTROL OF A NEW PLANTS MIX EXTRACT FOR VETERINARY USE

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Abstract

The microbiological control of veterinary products needs an integrated approach, being part of the quality assurance in the pharmaceutical industry. During the production of a phytopharmaceutical product of veterinary use it is compulsory to have a standardized method for the quantification of the microbial charges (fungi and bacteria) from the raw vegetal material to the final product. Our work has been focused on the microbial charges of a new phytoimmunomodulator veterinary product based on Inula sp., Eupatorium sp. and Helleborus sp. For the product standardisation several attempts have been done and one part of the work was related to microbiological criteria fulfilment. The microbial charges have been quantified according to adapted method developed by the authors and correlated to limits recommended by European Pharmacopeia. In the case of the raw dried and grounded plants the total mesophilic aerobe bacteria load is much higher than the recommended limits, while the fungal load has reached almost the maximum recommended limits. Acceptable contents of coliforms and no traces of Salmonella have been detected in the final product. The phytopharmaceutical company to patent and produce the new veterinary product, should make efforts especially in the raw material procurements, as long as their actual sources comes with a much more higher content in aerobe bacteria than the recommended limits. Supplementary measures should be taken to avoid in this context the cross-contamination.

***Key words**: good manufacturing practices, microbial charge, veterinary phytopharmaceutical product.*

INTRODUCTION

The medicinal plants represents, by centuries, the main raw material for the old but always new phytopharmacy. The medicinal plants' extracts have been demonstrated to have different effects on human, but also on animal health, like antimicrobial and antioxidant activity, resistance against toxins or stimulate the enzymatic activity and nitrogen absorption (Viegi et al., 2003, Burcea et al. 2007).
A special attention have been given in the last decades to develop mix products made of different plants with medicinal effects for a better prevention or cure of human and animal diseases. Because our work has taken into account a mix made of *Inula*, *Eupatorium* and *Helleborus*, these plants will be shortly presented for their phytopharmaceutical potential.
Relatively recently, the studies have demonstrated that *Inula* sp. shows different positive biologic activities, respectively: anticancerigenic (Dorn et al., 2006), antimicrobial (Cohen et al. 2002, Diguta et al., 2014; Zhao et al., 2010), hepato-protector or anti-inflammatory. Empirically, dried roots of *Inula* were used for the cows for a better and safety milk production of for the sheep and pigs to keep away their illness (Khuroo et al., 2007, Davidovic et al., 2012).
Also, the literature reported *Eupatorium* having different pharmacological effects such as antimicrobial (Purcaru et al., 2015), antiinflammatory, immunoregulatory, liver damage protection, blood glucose decrease (Kazuo, et al.: 1979; Xu et al., 1998; Yan et al., 2003). Moreover, extracts of *Eupatorium lindleyanum* are proposed to be used as food additive (Li et al, 2008), while essential oil of *Eupatorium cannabinum* can be employed during food storage against *Aspergillus* development and aflatoxin formation (Kumar et al, 2007).
Meanwhile, in the case of *Helleborus* have been proven its antibacterial activity (Puglisi et

al, 2009) or antineoplasmic properties (Wang et al, 2004).
The microbiological control of veterinary products needs an integrated approach, being part of the quality assurance in the pharmaceutical industry. The quality control must be applied along the whole technological flux, from the raw material reception to the final product, ready to be delivered. Each phytopharmaceutical company should elaborate internal procedures according to the recommendation of the *Pharmacopoeias*.
During the technological process it is necessary to minimize as much as possible the microbial contamination by following the principles of *Good Manufacturing Practices (GMP)*. It is very important to be taken into account the fact that most of the physical or chemical interventions taken for the microbial load reduction may affect negatively the active principles of the plants.
The main contamination source is even the plant as raw material and the initial microbiological control is a must, as well as after a preliminary processing to be able to avoid the cross-contaminations and the final product to have a minimal microbial load, under the recommended limits. In the case of non-aqueous products, the contamination issue is diminished because the microorganisms can't survive in environments with low water activity (aw).
In the case of phytopharmaceutical products, including the one of veterinary use, the European *Pharmacopeia* delimits the following microbiological quality indicators: total number of aerobe mesophilic bacteria and fungi, as well as the presence of specific pathogens, like *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* or *Salmonella* (Table 1).

Table 1 – Acceptability limits for microbial loads in phytopharmaceutical products in CFU /g or CFU /ml (source: *European Pharmacopeia* 8.0)

Microbiological indicator	**Dried plant**	**Pretreated extracts 1** (treatments which can lead to the decrease of microbial load)	**Pretreated extracts 2** (treatments which can't lead to the decrease of microbial load)
Total nuber of mehsophilic aerobe bacteria	10^7	10^4	10^5
Fungi	10^5	10^2	10^4
E.coli	10^3	Absent (in 1 g or 1 ml)	Absent (in 1 g or 1 ml)
Salmonella spp.	Absent (in 25 g)	Absent (in 25 g or 25 ml)	Absent (in 25 g or 25 ml)

The final phytopharmaceutical product of veterinary use is proposed to be made of three different plants, as described above (*Inula, Eupatorium* and *Helleborus*), this is why the microbiological indicators have been analysed for the plants as raw materials, as well as for the final mix product, according to the *European Pharmacopeia*.

MATERIALS AND METHODS

The **biologic material** consisted in dried and grounded parts of *Inula, Eupatorium* and *Helleborus* plants cultivated under ecological conditions in Brasov county, Bod area. Also, the analysis have been applied to a mix of these plants extracted (0.25 g/ml) in 20% ethanol (v/v). The recipe of the product is subject to a patent and data can't be disclosed.

The **media** used in the testing are the one recommended by *European Pharmacopeia*, respectively, for the bacterial counting has been used nutrient agar, for the fungal load YGP (Yeast Extract-Glucose-Peptone), for the coliforms both BBLV and GEAM Levine media.

Sample preparation: 5 g of each sample (plants and mix) have been suspended in 45 ml of buffered peptone water followed by an agitation at 100 rpm/30 min for a total micro-organisms recovery. Supplementary decimal dilutions (10^{-3} to 10^{-7}) have been applied to the samples, according to former preliminary results. For the analysis last two dilutions have been employed and made it in triplicate.
In the case of aerobe bacteria and fungi counting has been used the classical

inoculation method, respectively plate spreading technique. The **incubation** temperature for bacteria the was 35^{o}C/24-48 hours, while for the fungi 28^{o}C/2-3 days.
After the incubation the colony-forming units have been counted and the results have been compared to the recommended limits.
In the case of *E.coli* analysis the samples have been inoculated in triplicate in fermentation tubes with BBLV medium and incubated at 37^{o}C/48 hours; positive samples (forming gas) are laid into plate with GEAM medium at 37^{o}C/24 hours for the confirmation.
For *Salmonella* spp. presence classical steps have been followed: pre-enrichemnt in buffered peptone water at 35˚C/6 hours, enrichment in Muller-Kaufmann at 35˚C/24 hours, isolation by plating on deoxycolate medium and incubated at 35˚C/48 hours; if positive colonies would appear the confirmation is made by plating on TSI (Triple Sugar Agar) medium at 35˚C/48 hours.
In the case of *E.coli* and *Salmonella* spp., have been used test microorganisms, respectively *Escherichia coli* ATCC 8739 and *Salmonella typhimurium* ATCC14028.

RESULTS

For the herbal products registration there are some regulatory challenges to be faced, including the microbiological loads in respected to the recommended limits.
In the recent past years have been developed a veterinary immunomodulatory product (under patent) made of three plants mix, respectively *Inula, Eupatorium* and *Helleborus.*
For the product standardisation several attempts have been done and one part of the work was related to microbiological criteria fulfilment.
In this regard, microbiological analysis have been performed for the raw materials and for the final mix and compared with limits recommended by the *European Pharmacopeia*.
The results are presented as average of three different analysis and can be followed in table 2. In the case of the raw dried and grounded plants the total mesophilic aerobe bacteria load is much higher than the recommended limits, while the fungal load has reached almost the maximum recommended limits.

Table 2-Microbial load of raw materials and plant mix of a phytoimmunomodulator veterinary new product (CFU/g)

Medicinal plant	**Mesophilic aerob bacteria**	**Fungi**	***E. coli***	***Salmonella***
Eupatorium spp.	5.2×10^{7}	1.5×10^{4}	0.1×10^{3}	-
Helleborus spp.	TNBC*	5.0×10^{4}	0.8×10^{3}	-
Inula spp.	2.2×10^{7}	2.5×10^{4}	0.6×10^{3}	-
Mix product	2.8×10^{3}	-	-	-

*TNBC - too numerous to be counted
- not detected

No thermal process have been applied to the raw plants, but in the mix have been added ethanol (20% v/v); in this context, the total mesophilic aerobe bacterial and fungal loads have registered values under the *European Pharmacopeia* recommended limits.
In the case of all the samples the coliforms loads are positively under the recommended limits, while the presence of *Salmonella* has not been registered in any of those.
In terms of microbial diversity, all three medicinal plants have shown moderate diversity; for instance have been isolated four different bacterial species (to be identified) and two main fungal species of which one was macroscopically identified as *Aspergillus* sp. (figure 1). The presence of fungi should be carefully investigated and/or monitored, since some common species produce toxins, especially aflatoxins. Aflatoxins in herbal drugs can be dangerous to health even if they are absorbed in minute amounts.

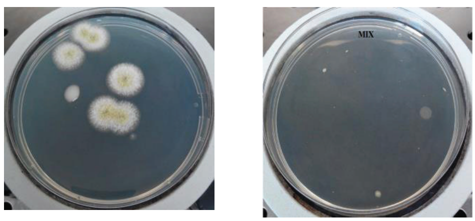

Fig.1 Aspects regarding the fungal load of dried medicinal plant (left) and of the plants' mix (right)

CONCLUSIONS

The microbiological control of veterinary products needs an integrated approach, being part of the quality assurance in the pharmaceutical industry and must be applied from the raw material reception to the final product. Medicinal plants may be associated with a broad variety of microbial contaminants, represented by bacteria, fungi, and viruses. Inevitably, this microbiological background depends on several environmental factors and exerts an important impact on the overall quality of herbal products and preparations. Herbal drugs normally carry a number of bacteria and molds, often originating in the soil. Poor methods of harvesting, cleaning, drying, handling, and storage may also cause additional contamination, as may be the case with *Escherichia coli* or *Salmonella* spp.

In the case of the new veterinary immune-modulator product made of three plants *Inula, Eupatorium* and *Helleborus*, same principle should be followed, in line with the appropriate *Pharmacopeia*.

According to our results, the phytopharmaceutical company to patent and produce the new veterinary product, should make efforts especially in the raw material procurements, as long as their actual sources comes with a much more higher content in aerobe bacteria than the recommended limits. Supplementary measures should be taken to avoid in this context the cross-contamination. However, as a positive aspect, the company is conducted by the principles of GMP which has lead to acceptable contents of coliforms and no traces of *Salmonella* spp. in the final product.

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