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 aerobic 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 aerobic 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), anti-inflammatory, 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
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 Pharmacopoeia.

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 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 Pharmacopoeia delimited 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>
<thead>
<tr>
<th>Microbiological indicator</th>
<th>Dried plant</th>
<th>Pretreated extracts 1 (treatments which can lead to the decrease of microbial load)</th>
<th>Pretreated extracts 2 (treatments which can't lead to the decrease of microbial load)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of mesophilic aerobe bacteria</td>
<td>10^7</td>
<td>10^4</td>
<td>10^6</td>
</tr>
<tr>
<td>Fungi</td>
<td>10^3</td>
<td>10^2</td>
<td>10^4</td>
</tr>
<tr>
<td>E.coli</td>
<td>Absent (in 1 g or 1 ml)</td>
<td>Absent (in 25 g or 25 ml)</td>
<td></td>
</tr>
<tr>
<td>Salmonella spp.</td>
<td>Absent (in 25 g)</td>
<td>Absent (in 25 g or 25 ml)</td>
<td></td>
</tr>
</tbody>
</table>

The media used in the testing are the one recommended by European Pharmacopoeia, 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 microorganisms recovery. Supplementary decimal dilutions (10^3 to 10^5) 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 was 35°C/24-48 hours, while for the fungi 28°C/2-3 days.

RESULTS

For the herbal products registration there are some regulatory challenges to be faced, including the microbiological loads in respect 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)

<table>
<thead>
<tr>
<th>Medicinal plant</th>
<th>Mesophilic aerob bacteria</th>
<th>Fungi</th>
<th><em>E. coli</em></th>
<th><em>Salmonella</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Eupatorium spp.</td>
<td>5.2x10⁷</td>
<td>1.5x10⁷</td>
<td>0.1x 10⁷</td>
<td>-</td>
</tr>
<tr>
<td>Helleborus spp.</td>
<td>TNBC*</td>
<td>5.0x10⁷</td>
<td>0.8 x 10⁷</td>
<td>-</td>
</tr>
<tr>
<td>Inula spp.</td>
<td>2.2x10⁷</td>
<td>2.5x10⁴</td>
<td>0.6 x 10⁷</td>
<td>-</td>
</tr>
<tr>
<td>Mix product</td>
<td>2.8x10⁷</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*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 phytopharmacutical 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 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.

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FOOD
BIOTECHNOLOGY
EFFECT OF COLD STORAGE ON ANTIOXIDANTS FROM MINIMALLY PROCESSED HERBS

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Abstract

Vitamin C and total polyphenols are quality markers used to assess the effect of treatments and storage on foodstuffs. Although, the effect of other conventional shelf-extension methods is well established, refrigeration was taken for granted and rather neglected. The present study aims to describe its influence on three minimally processed herbs stored at 4°C for 12 days: parsley (Petroselinum crispum), dill (Anethum graveolens) and lovage (Levisticum officinale). The content of ascorbic acid and total polyphenols was determined on methanolic extracts. Ascorbic acid was separated, identified and dosed using HPLC coupled with an UV–VIS detector. Total polyphenols were determined spectrophotometrically, following Folin-Ciocalteu method. On the first day of storage, the content of vitamin C was above 170 mg/100 FW for the three herbs: dill had the highest content, followed by parsley andLovage, statistically similar. During the 12 days of storage, the content of vitamin C decreased by 18% for parsley, by 8% for lovage and by 3% for dill. At the beginning of the study, lovage had the highest content of total phenols followed by parsley and dill. On day 5 of storage, the content increased, reaching the maximum values for the three herbs and then it decreased below the levels of the first day. It was noted that during the 12 days of study, the evolution of total polyphenols at refrigeration temperature was given by a function of second degree. Thus, the present study confirms that vitamin C can be successfully used as a quality marker for herbs due to its low stability during storage. The evolution of total phenols is polynomial, reaching its peak during the shelf-life of herbs.

Key words: ascorbic acid, dill, lovage, parsley, phenols.

INTRODUCTION

Consumers are becoming more literate about the benefits of fresh products containing bioactive compounds (Parfitt et al., 2010). Aromatic herbs are believed to provide antioxidant compounds; vitamins, phenolic acids, flavonoids, sterols and coumarins are the compounds with functional properties in herbs (Charles, 2012; Santos et al., 2014). But, the continuous race against time of modern society has led to an increasing demand for ready-to-eat products. This generated a steady increase of minimal processing industry of fruit and vegetables (Parfitt et al., 2010). The commodities are fresh-like containing all the valuable phytochemicals and consumers perceive them as convenient, of high quality, less wasteful and with a reasonable price (Alvarez et al., 2013).

Although minimal processing keeps the products alive, every operation in the production chain promotes senescence processes (ethylene production, respiration and browning) and reduces resistance to microorganisms (Alvarez et al., 2013). This lowers quality, shortens shelf-life and enhances microbial contamination. Hydrosoluble vitamins, such as vitamin C, are the compounds with the highest variation during the storage of minimally processed products, because the changes are more intense in the water fraction (Santos et al., 2014). Thus, vitamin C and total polyphenols are quality markers traditionally used to assess the effect of treatments and storage on vegetables. Although, the effect of other conventional shelf-extension methods is well established (Śledź et al., 2013; Mezeyová et al., 2016), refrigeration was taken for granted and rather neglected. Herbs from Lamiaceae family (basil, lemongrass, marjoram, mint, oregano, rosemary, thyme) are thoroughly studied (Blasa et al., 2010; Śledź et al., 2013; Curutchet et al., 2014; Santos et al., 2014), while Apiaceae family (coriander, dill, lovage, parsley) has...