MilkQua

Quality along the Dairy Chain for a Safe and Sustainable MILK
PRIMA S2 – 2018

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Document information

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4. Revision history

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PU	Public	Yes
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Executive Summary

<table>
<tr><td>Background</td><td>In earlier tasks of the MILKQUA project, several EOs were obtained from distinct species (Table 1) and their chemical characterization achieved.
Table 1 – Sample identity.
<table><tr><th>EO</th><th>Species</th></tr><tr><td>1</td><td>Salvia officinalis</td></tr><tr><td>2</td><td>Laurus nobilis</td></tr><tr><td>3</td><td>Coriandrum sativum</td></tr><tr><td>4</td><td>Rosmarinus officinalis</td></tr><tr><td>5</td><td>Thymus capitatus</td></tr><tr><td>6</td><td>Nigella sativa</td></tr><tr><td>7</td><td>Juniperus oxycedrus</td></tr><tr><td>8</td><td>Pelargonium graveolens</td></tr><tr><td>9</td><td>Origanum vulgare</td></tr><tr><td>10</td><td>Artemesia herba alba</td></tr></table></td></tr>
<tr><td>Objectives</td><td>The main objective of the present deliverable is to assess the anti-inflammatory, immunomodulatory and antimicrobial properties of the EOs in use by the project.</td></tr>
<tr><td>Methods</td><td>Anti-inflamamtory and immunomodulatory properties
Cell model: human monocyte cells (THP-1) were used. At the time the experiment began, cells are incubated with PMA in order to induce differenciation into M0 macrophages. Each EO was then added in the concentrations presented below and cells pre-incubated for 2 hours, after which LPS was added in order to induce a pro-inflammatory phenotype.
Study of the NF-kB activation status: THP-1 cells containing a luciferase-based NF-kB reporter were used. After incubation with the EOs, culture media was collected and mixed with luciferase substrate, after which luminescense was read.
PCR: RNA was collected and converted to cDNA. RT-qPCR was conducted towards the genes presented below and relative expression levels calculated, using GAPDH as reference gene.

Antimicrobial properties.
Antimicrobial activity testing was done according to Vuddhakul et al. (2007) with slight modifications. The inocula of each of the three microorganisms were streaked, using a sterile swab, onto Mueller-Hinton agar for E. coli and Staphylococcus and a blood agar plates for Streptococcus strain. Then, sterile filter discs (diameter 6 mm, Whatman paper N°5) were impregnated with 10 µl of each EO and placed on the inoculated agar. After incubation (37°C for 18–24 h), the diameter of the inhibition zones around each disc was take. The same protocol was applied to the following antibiotics: Colistin, Amoxycillin, Ampicillin and Cephalexin usually used to treat mastisis. Each experiment was carried out 6 times and the mean diameter of the inhibition zone was recorded in mm. The diameter of disc (6 mm) was not took into consideration.</td></tr>
<tr><td>Results and implications</td><td>Among the 10 samples studied, four revealed significant anti-inflamamtory effect, their molecular targets having been identified.</td></tr>
</table>

1. Results

Anti-inflammatory and immunomodulatory

All samples being used in the project were first assessed for their toxicity towards the cells in use. This is an important step, as it allows removal of toxic concentration which could have a negative impact in subsequent assays. **Figure 1** presents the concentration range used and their impact in cell viability, as well as the statistical significance, when applicable. These results highlighted the importance of using distinct working concentrations depending on the sample.

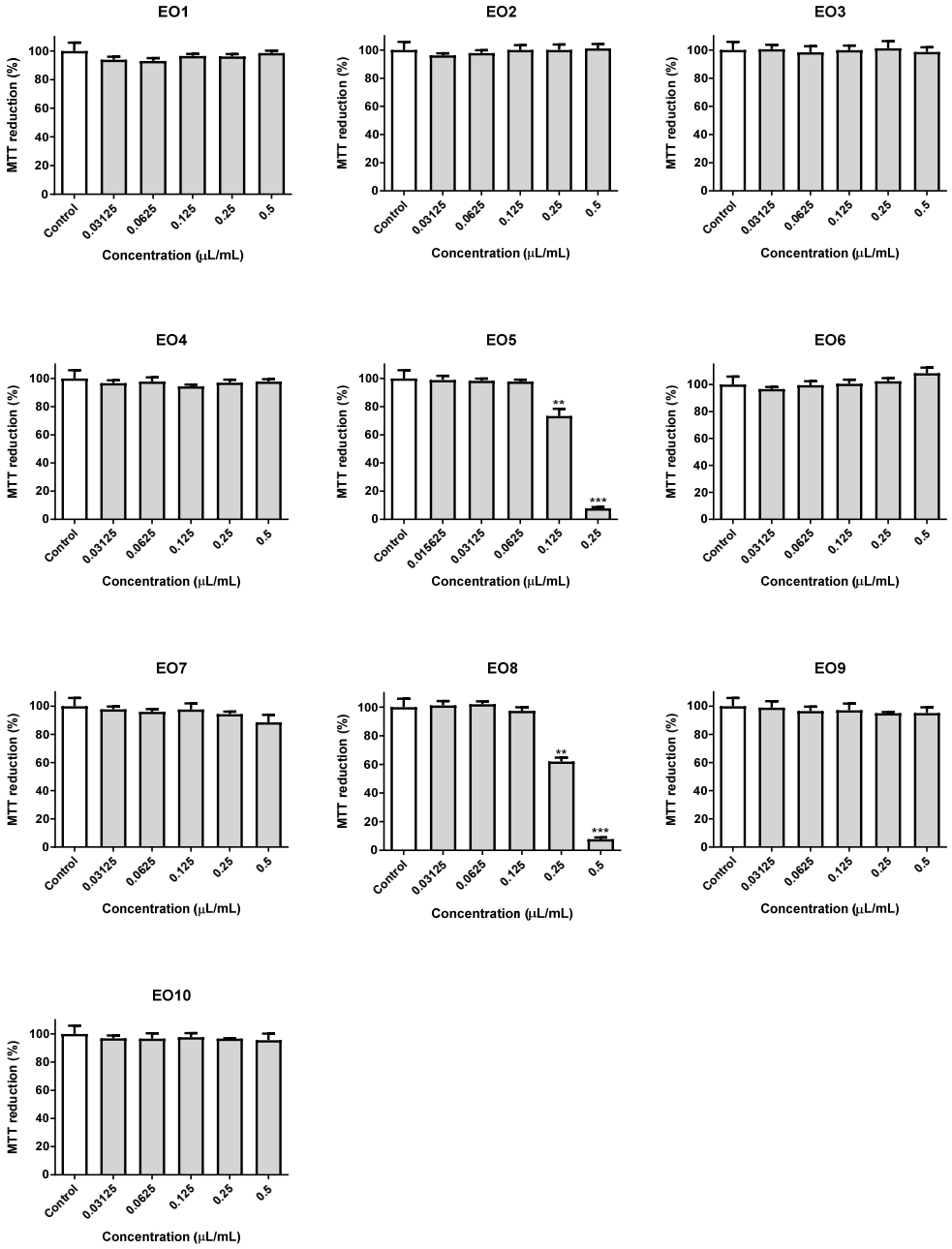

Figure 1: Impact of EO1-10 in the viability of THP-1 cells, in the concentrations indicated, as assessed by the MTT assay. ** p<0.01, *** p<0.001.

After selecting the working concentrations, all samples were assessed for their ability to prevent or ameliorate the activation of the NF-κB pathway in LPS-challenged cells. The NF-κB pathway comprises a set of key events for the inflammatory process, being frequently upstream of other genetic and phenotypic changes. To this end, a luciferase reporter assay was used comprising THP-1 Lucia cells.

Figure 2 presents the results found. Among the 10 samples tested, 4 of them (EO2, EO3, EO8 and EO10) were able to significantly prevent the activation of this pathway, in some cases by over 50%. These are promising results, as they suggest the EO under study may have a relevant role in the modulation of the inflammatory process, a key finding to the ongoing work in the framework of MILKQUA.

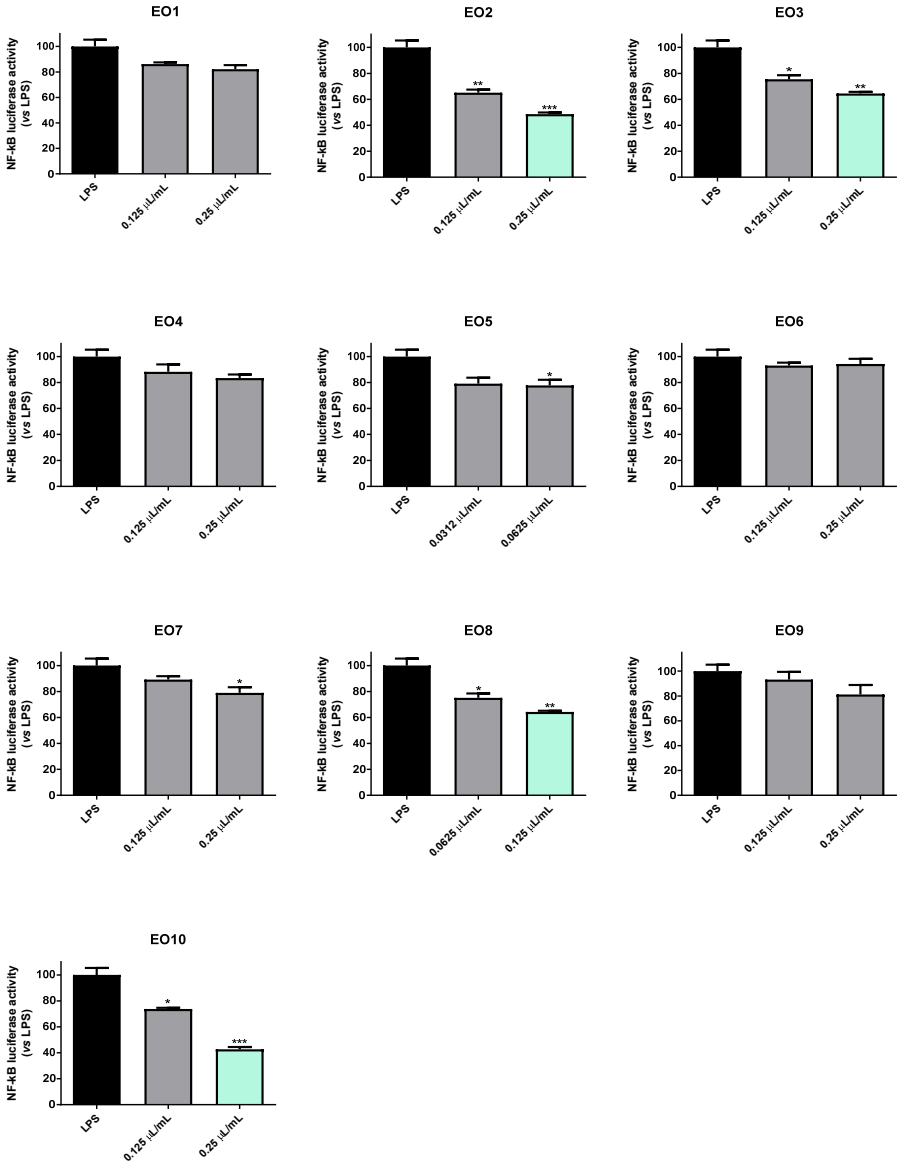

Figure 2: NF-kB activation status of THP-1 luciferase reporter-transfected cells for the different samples. *p<0.05, ** p<0.01, *** p<0.001.

After the capacity of EO 2, 3, 8 and 10 to negatively modulate the activation of the NF-κB pathway was identified, we were interested in assessing the putative targets that could be involved in this effect. To this end, the 4 samples were selected for subsequent studies. Specifically, we collected RNA from EO-treated cells in the presence of the pro-inflammatory molecule LPS. This RNA was then converted into cDNA and the effect of the samples in the expression of key genes involved in the inflammatory response evaluated. The selected genes were those corresponding to IL-6, IL-1β, COX-2 and TNF-α. As shown in Figure 3, all samples were capable of significantly lowering the expression of these genes, thus demonstrating their capacity to interefere in the inflammatory process.

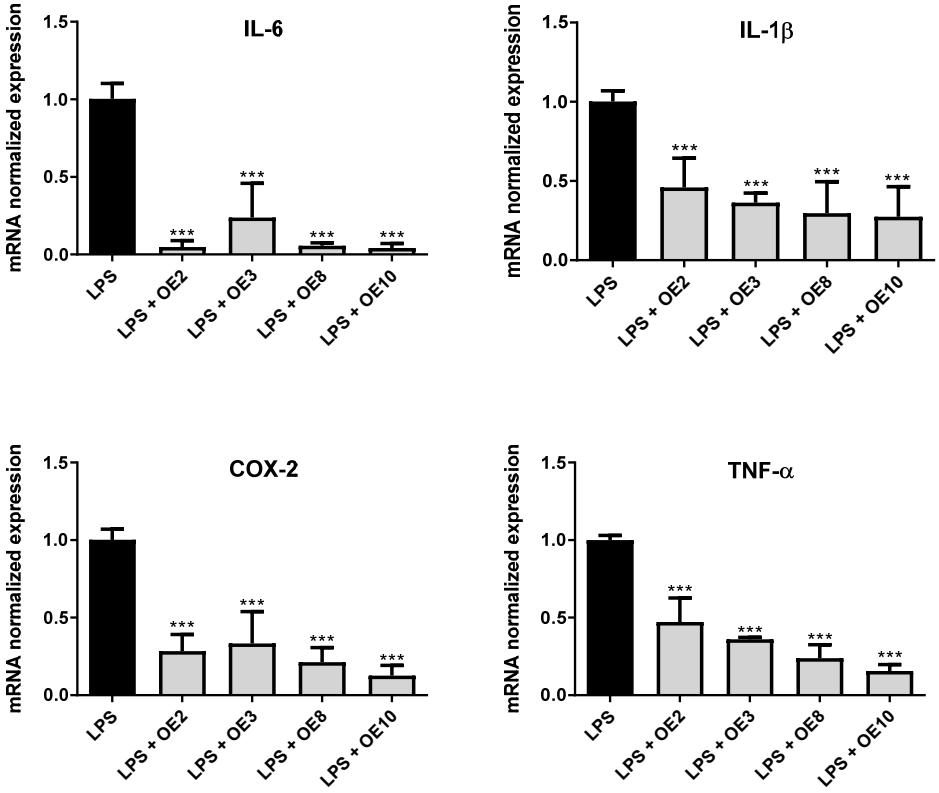

Figure 3: Expression levels of the indicated genes when compared with control conditions in cells incubated with the samples disclosed. Normalization was conducted using GAPDH as reference gene. *** p<0.001.

2. Antimicrobial activity

The essential oils of Artemisia herba alba, Coriandrum sativum, Juniperus oxycedrus, Laureus nobilis, Nigella sativa, Origanum majorana, Pelargonium graveolens, Rosmarinus officinalis, Salvia officinalis and Thymus capitatus were firstly studied for their bacterial growth inhibition potency. Figure 1, details the inhibition zones of E. coli growth recorded for each essential oil and for four standards antiobiotics. It can be seen that T. capitatus EO is clearly the most efficient sample exhibiting the largest inhibition diameter (24.6 mm). This activity is even significantly higher than that of the authentic antibiotic Cephamexin (13.8 mm). Thyme EO was followed by coriander, laurel and marjoram that process, statistically, the same anti-E. coli capacities with inhibition zones diameters around 11 mm. The third group of EO was formed by A. herba alba, R. officinalis, S. officinalis and P. graveolens with moderate inhibition zone ranged from 8 to 2.4 mm, while no activity was recorded for J. oxycedrus and N. sativa against this particular E. coli strain. Moreover, it is important to underline that two antiobiotics (Colisin and Ampicilin) were also unable of inhibiting E. coli growth (Inhibition zone = 0 mm).

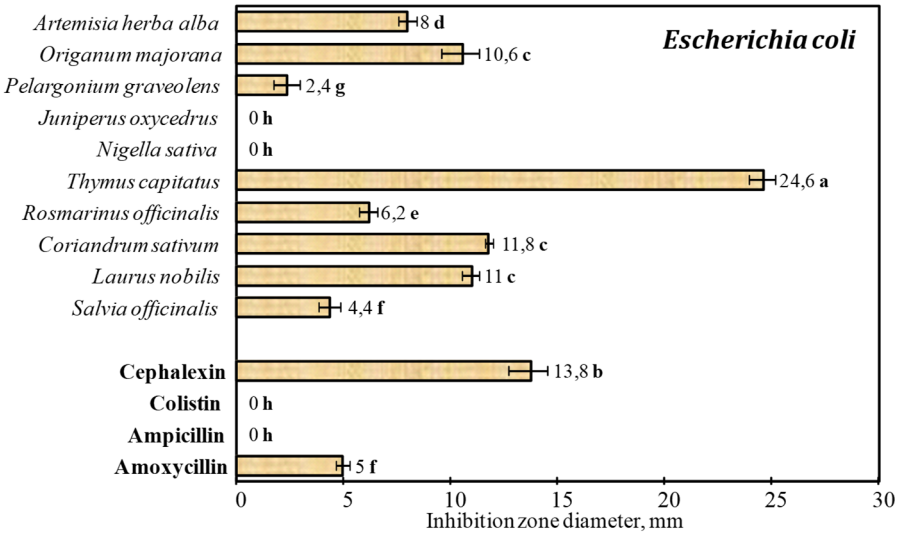

Figure 1. Comparison between the growth inhibitions zones (mm) obtained with ten essential oils against E. coli isolated from infected cow udder. Values followed by the same letter are not significantly different at p < 0.05. Inhibition zone <1 mm: Weak inhibition zone, inhibition zone 1 mm: Slight antimicrobial activity, inhibition zone 2–3 mm:Moderate antimicrobial activity, inhibition zone 4–5 mm: High antimicrobial activity, inhibition zone 6–9 mm: Strong antimicrobial activity, inhibition zone > 9 mm: Excellent antimicrobial activity.