Comparison of Antimicrobial Activity of Essential Oils, Plant Extracts and Methylparaben in Cosmetic Emulsions: 2 Months Study

Anna Herman

Received: 9 January 2014 / Accepted: 1 February 2014 / Published online: 12 February 2014
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Abstract The aim of the study was to compare the preservative effectiveness of plant extracts (*Matricaria chamomilla*, *Aloe vera*, *Calendula officinalis*) and essential oils (*Lavandulla officinalis*, *Melaleuca alternifolia*, *Cinnamomum zeylanicum*) with methylparaben in cosmetic emulsions against skin microflora during 2 months of application by volunteers. Cosmetic emulsions with extracts (2.5 %), essential oils (2.5 %), methylparaben (0.4 %) or placebo were tested by 40 volunteers during 2 months of treatment. In order to determine microbial purity of the emulsions, the samples were taken after 0, 2, 4, 6 and 8 weeks of application. Throughout the trial period it was revealed that only cinnamon oil completely inhibited the growth of bacteria, yeast and mould, as compared to all other essential oils, plant extracts and methylparaben in the tested emulsions. This result shows that cinnamon oil could successfully replace the use of methylparaben in cosmetics, at the same time ensuring microbiological purity of a cosmetic product under its in-use and storage conditions.

Keywords Antimicrobial activity · Essential oils · Plant extracts · Methylparaben

A challenge test is a controlled study of microbial contamination conducted in laboratory conditions, which may not fully reflect the realities of the actual exposure of a cosmetic to a broad spectrum of microorganisms, which

inhabit users' skin during cosmetics application. Therefore, the aim of the study was to compare the preservative effectiveness of plant extracts (*M. chamomilla*, *A. vera*, *C. officinalis*) and essential oils (*L. officinalis*, *M. alternifolia*, *C. zeylanicum*) with methylparaben in cosmetic emulsions against skin microflora during 2 months of application by volunteers.

The emulsions were prepared according to the procedure described by Herman et al. [1]. The composition of cosmetic formulations is shown in Supplementary Table 1. *M. chamomilla* flower, *A. vera* leaf, *C. officinalis* flower extracts (Greentech, France), *L. officinalis*, *M. alternifolia*, *C. zeylanicum* essential oils (Avicenna-Oil, Poland) and methylparaben (Cognis Polska Sp. z o.o.) were added at 2.5 and 0.4 % concentration, respectively. The emulsion without methylparaben, essential oils and extracts was a reference sample.

The microorganisms were activated through double passaging: bacteria (*Pseudomonas aeruginosa* ATCC 9027, *Escherichia coli* ATCC 8739, *Staphylococcus aureus* ATCC 6538) on TSA medium (Trypticase Soy Agar; BioMerieux, France) (37 °C, 48 h), yeast (*Candida albicans* ATCC 10231) and mould (*Aspergillus brasiliensis* ATCC 16404) on SDA medium (Sabouraud Dextrose Agar; BioMerieux, France) (25 °C, 72 h and 7 days, respectively). Several colonies of overnight cultures of individual microorganisms were suspended in saline to obtain density equal to 0.5 McFarland turbidity standard (approximate cell density of 1.5×10^8 CFU/mL). The antibacterial and antifungal activity was evaluated by using the disc diffusion method. The suspensions of microorganisms were spread over the agar plates by using sterile cotton swabs. The sterile paper discs of 6 mm in diameter (BTL, Poland) were impregnated with 15 μ L essential oils/extracts/methylparaben and placed on the agar surface.

Electronic supplementary material The online version of this article (doi:10.1007/s12088-014-0454-z) contains supplementary material, which is available to authorized users.

A. Herman (✉)
Academy of Cosmetics and Health Care, Podwale 13 street,
00-252 Warsaw, Poland
e-mail: anna.herman@wszkipz.pl

Kanamycin (BioMerieux, France) and amphotericin B (BioMerieux, France) were used as controls. All bacterial plates were incubated at 37 °C for 48 h and yeast and mould plates at 25 °C for 72 h and 7 days, respectively. The diameter of the zone of inhibition was measured in mm. Results were calculated as mean $\pm$ SD for 3 independent experiments.

Forty female volunteers, in the age range 25–50, in good general health, were assigned to one of the eight treatment groups: essential oils, plant extracts and methylparaben-free cosmetic emulsion ($n = 5$), cosmetic emulsion with methylparaben ($n = 5$), cosmetic emulsion with *C. zeylanicum*/L. *officinalis*/M. *alternifolia* oils (each $n = 5$), cosmetic emulsion with *M. chamomilla*/A. *vera*/C. *officinalis* extracts (each $n = 5$). The cosmetics were applied by volunteers on the forearm skin using fingers two times a day (morning and evening). The procedure on volunteers was performed with the consent of the Ethical Committee of the Academy of Cosmetics and Health Care. None of the volunteers enrolled in the trial experienced adverse effects during the course of the study and the cosmetic emulsions were well tolerated. The number of viable microorganism bacteria, yeast and mould in cosmetic formulations was determined by the plate count method at the proper times 2, 4, 6 and 8 weeks after starting experiments (cosmetics provided to volunteers). Time 0 was a control, namely, samples of cosmetic emulsions were taken from a container before transferring the cosmetics to the volunteers. A sample of 1 mL cosmetic emulsions was transferred to 9 mL saline and tenfold dilutions method was applied. Triplicate plating of each dilution was performed with TSA agar for bacteria (37 °C, 48 h) and SDA agar for fungi (25 °C, 72 h and 7 days). Counts the CFU per plate (30–300 colonies) determined the number of surviving microorganisms per gram of tested cosmetic product. The results were expressed as CFU in 0.1 mL of cosmetics. All determinations were performed in fivefold, and the results represent an average of two different experiments. Standard deviation for the test of microorganism population viability did not exceed 10 %.

Essential oils showed higher inhibitory activity against bacteria, yeast and mould than extracts and methylparaben in in vitro research (Supplementary Table 2). Among them, cinnamon oil was the most potent inhibitor of tested microorganisms growth. Similar results were obtained by Inouye et al. [2], which confirmed that cinnamon oil exhibited higher antimicrobial activity against *S. aureus* than lavender oil. Also Filoche et al. [3] showed that among all tested essential oils (cinnamon, tea-tree, manuka, arnica, eucalyptus, grapefruit) alone and in combination with chlorhexidine digluconate against *Streptococcus mutans* and *Lactobacillus plantarum* cinnamon oil exhibited the greatest antimicrobial potency. At the concentration of 0.125 % (v/v), cinnamon oil

showed a strong antibacterial activity against *S. aureus* and after 60 min reached 42.86 % cell viability reduction [4]. Cinnamon oil in concentration 2.5, 0.25 and 0.025 % (v/v) inhibited *S. aureus* growth in the time dependent manner [5]. Cinnamon oil at the concentration 2.5 % (v/v) completely killed microorganisms after 1 h of incubation. Cinnamon oils applied at the concentration of 0.25 and 0.025 % (v/v) inhibited microorganism growth starting from 1 h of incubation. However, the effect of 0.025 % (v/v) dose appeared to be rather bacteriostatic than bactericidal.

In the second part of our study, cinnamon oil at the concentration of 2.5 % completely inhibited bacteria, yeast and mould growth in cosmetic emulsion more firmly than methylparaben and plant extracts during 2 months of application by volunteers (Table 1). In addition to the mentioned studies, also the results of our previous experiment suggested a potential preservative effectiveness of cinnamon oil [1]. We showed that cinnamon oil added to cosmetic emulsion at concentration 2.5 % completely inhibited *S. aureus*, *E. coli* and *C. albicans* growth after 4 weeks of incubation during a challenge test. Since the results of these both in vitro and in vivo experiments, confirmed the strong antimicrobial activity of cinnamon oil, it can be considered as a promising natural preservative. Similarly to cinnamon oil, also methylparaben totally inhibited the growth of mould in cosmetic and inhibited the growth of new cells of bacteria and yeast in cosmetic emulsion during 2 months use by volunteers (Table 1). The total viable count for aerobic mesophylic microorganisms in emulsion with methylparaben complied with microbiological standards set out for the cosmetics. Other tested essential oils and all plant extracts showed the weakest protection of emulsion against microorganisms from volunteers' skin. After 4 weeks, total viable count for aerobic mesophylic microorganisms exceeded acceptable microbiological standards set out for cosmetics. These results are partially different from the results obtained in our previous study [1] because those results of challenge tests suggested the strong antimicrobial properties of essential oils and plant extracts. The differences in results obtained in both experiments may be due to the amount of microorganisms which had been in contact with cosmetic emulsion. The challenge test recommended five strains, which include gram-positive and gram-negative bacteria, yeasts and fungi, while in the experiment with volunteers' the cosmetics had contact with larger and more diverse microflora of human skin and its environment. A similar conclusion was reached by Zani et al. [6], who showed that a challenge test should include, in addition to recommended microorganisms from ATCC collection, wild strains which are generally believed to be more aggressive than laboratory strains and are more representative of potential contaminants that a product might encounter

Table 1 Growth of bacteria, yeast and mould in 0.1 mL cream formulation with essential oils, extracts and methylparaben in cosmetic emulsions during 2 months using by volunteers

Time [week]	Control	Methylparaben	<i>Cinnamomum zeylanicum</i> oil	<i>Lavandulla officinalis</i> oil	<i>Melaleuca alternifolia</i> oil	<i>Matricaria chamomilla</i> extract	<i>Calendula officinalis</i> extract	<i>Aloe vera</i> extract
Bacteria [CFU in 0.1 mL]								
0	1.1×10^2	1×10^2	0	9.8×10^1	9.3×10^1	1.2×10^2	1.1×10^2	1.15×10^2
2	1.2×10^5	9.7×10^1	0	1.34×10^2	1×10^2	1.3×10^4	1.32×10^2	1.27×10^5
4	1.33×10^6	1.1×10^2	0	1.17×10^5	9.9×10^4	1.17×10^5	1.1×10^5	1.6×10^6
6	– ^a	1×10^2	0	–	–	–	–	–
8	–	1.17×10^2	0	–	–	–	–	–
Yeast [CFU in 0.1 mL]								
0	10^2	9×10^1	0	0	0	0	0	0
2	1.1×10^3	0	0	0	1×10^2	1.2×10^3	1.13×10^4	1.1×10^2
4	1.2×10^5	9.8×10^1	0	1.01×10^2	1.1×10^4	1.14×10^4	1.21×10^5	1.2×10^2
6	–	1×10^2	0	–	–	–	–	–
8	–	1.1×10^2	0	–	–	–	–	–
Mould [CFU in 0.1 mL]								
0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0
6	–	0	0	–	–	–	–	–
8	–	0	0	–	–	–	–	–

^a Experiment was terminated, when the total viable count for aerobic mesophilic microorganisms exceed 10^3 CFU/g or 10^3 CFU/mL when tested in 0.1 g or 0.1 mL of cosmetic product (products category 2, cosmetics not including product specifically intended for children under 3 years, to be used in the eye area and on mucous membranes) [Guidelines on Microbiological Quality of the Finished Cosmetic Product, SCCNFP/0004/98 Final]

during its use and storage. Authors found that synthetic preservatives such as sodium benzoate-sorbic acid and p-hydroxybenzoate added to pharmaceuticals did not inhibit all microorganism strain use in the challenge test. Sodium benzoate-sorbic acid was proved effective against the wild strain of *Pseudomonas cepacia* isolated from the pharmaceutical environment and ineffective against the same strain from the ATCC collection. By contrast, p-hydroxybenzoate proved to be efficient in protecting products against the wild strain of bacteria as well as that from the ATCC collection. Therefore, what seems to be important is to incorporate a wider range of tested microorganisms in the challenge test than those recommended by European Pharmacopeia and the ISO standards for cosmetics. Moreover, conducting parallel in vitro and in vivo studies guarantees that the preservative system is properly matched to the product and ensures microbiological purity of cosmetics during their use and storage. Papageorgiou et al. [7] proved that *Lonicera caprifolium* and *L. japonica* extracts added to different aqueous and O/W formulations at the concentration of 0.2 % have excellent activity against *S. aureus*, *P. aeruginosa*, *E. coli*, *C. albicans*, *A. niger* in the acidic pH 5.5 during 28 days incubation and following 3 weeks of volunteers' use. Also Bernatoniene

et al. [8] found that 0.9 % *C. officinalis* extract added to hydrophilic cream inhibited the growth of *P. aeruginosa* and *S. aureus*. They also showed that no more than 10^4 microorganisms (aerobic bacteria plus fungi) per 1 g cream were found in samples collected directly after preparation and after 12 months of storage, what complied with microbiological standards provided for cosmetics.

The findings of the present study suggest that cinnamon oil inhibits the growth of microorganisms in cosmetic emulsions more effectively than methylparaben and plant extracts. Therefore, cinnamon oil can be recommended as an effective candidate for natural cosmetic preservatives and can replace the use of synthetic preservatives as methylparaben in cosmetics, simultaneously ensuring microbiological purity of a cosmetic product under its in-use and storage conditions.

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