Development and Aplication of New Herbal Pessaries for the Treatment of Squamous Endocervical Metaplasia

INTRODUCTION
Squamous metaplasia refers to the cellular non-cancerous changes in the epithelial linings of the cervix when normal epithelial cells are converted or replaced with squamous epithelium. Such process is triggered with constant stress or irritation that causes a reversible maturation process which converts one differentiated epithelial cell type into another. Among young women, the junction between two types of epithelium is typically found on the ectocervix, while in the generative age process of squamous metaplasia is activated resulting with the transformation of the columnar to squamous cells. This is the area of intense cellular activity and as such, is assumed to be a predilection point of vulnerability to HPV infection. Papilloma infection depends on hosts epithelial differentiation and replication for completion of its own life cycle events and survival (Hwang et al., 2012; Doolbar et al, 2005). Therefore, the squamous-columnar junction is a “locus minoris” for developing HGSIL through low-grade squamous intraepithelial lesion (LGSIL) where the basal cells of mature metaplastic epithelium are infected with oncogenic HPV types. These lesions are usually presented with cytological low-grade findings and some of them might progress to HGSIL. Other pathways links oncogenic HPV infection with the reserve cells of the glandular epithelium or with an early metaplastic cell described as atypical immature metaplasia (AIM). This entity embraces a varied group of lesion which includes reactive conditions, LSIL or can be instantaneously transformed into the HGSIL. For distinction between AIM and HSIL, beside morphological signs usage of immunoreactivity of p16INK4A and CK17 was suggested. For the cytokeratin 17 is been shown to be expressed in immature squamous metaplasia of the transformational zone of cervix (Skapa et al., 2013), and on the other hand p16INK4a is well known and used marker for cervical dysplasia and can be used as an indirect marker of HPV infection (Von Knebel Doeberitz, 2002).

Although, squamous metaplasia represents reversible changes of epithelial cells, on the other hand, if not treated properly it might progress to HGSIL, hrHPV or even cancer. So, the purpose of this work was development and testing of new herbal based pessaries consisted of oil based extracts and essential oils of medicinal plants with well known wound healing, anti-inflammatory, antimicrobial and antiviral properties for eradication of the squamous metaplasia compared with the results obtained by conventional treatment with albothyl pessaries. Total clearance was observed in 91.15% and 66.67% of the patients in the experimental and control group, respectively. Among the predictor variables, health status of the patients had the highest, statistically significant influence on the outcome of the therapy. The synergistic effect of the medicinal plants with well known wound healing, anti-inflammatory, antimicrobial and antiviral properties could be responsible for high treatment potential of the pessaries. This new product could present good alternative to the conventional treatment of squamous endocervical metaplasia due to high treatment efficiency and no observed side effects during the therapy.

ABSTRACT
The purpose of this work was to test the efficiency of newly developed herbal pessaries for the treatment of the squamous endocervical metaplasia compared to the conventional treatment with albothyl pessaries. The influence of the predictor variables on the outcome of the therapy was also assessed. 192 women in the age range from 20 to 59 years (38.2±8.2) represented experimental group and 30 women ranging from 24 to 59 years (39.4±7.9) represented control group, both diagnosed with squamous endocervical metaplasia were included in the study. The patients from the experimental group were treated locally for 10 days with herbal pessaries containing macerates (Calendula officinalis, Matricaria chamomilla, Lavandula officinalis, Hypericum perforatum L., Achilea millefolium, Thymus serpyllum, Salvia officinalis, Mentha piperita l., Symphytum officinale, Plantago major L., Alchemilla vulgaris) and essential oils (Melaleuca alternifolia, Tymus vulgaris, Pelargonium graveolens, Cympobogon martinii, and Origanum vulgare L.) The patients were treated 10 days with either albothyl (control group) or herbal pessaries (experimental group). New herbal pessaries showed significantly higher efficiency ($q^2=8.39; P=0.0038$) in eradication of metaplasia compared to albothyl. Total clearance was observed in 91.15% and 66.67% of the patients in the experimental and control group, respectively. Among the predictor variables, health status of the patients had the highest, statistically significant influence on the outcome of the therapy. The synergistic effect of the medicinal plants with well known wound healing, anti-inflammatory, antimicrobial and antiviral properties could be responsible for high treatment potential of the pessaries. This new product could present good alternative to the conventional treatment of squamous endocervical metaplasia due to high treatment efficiency and no observed side effects during the therapy.
effects could be directly linked to its phytochemical composition consisting mostly of triterpenoids, flavonoids, coumarines, quinones, volatile oil, carotenoids and amino acids while carbohydrates, lipids and other phytochemicals like calendin, calendulin and n-paraffins (Muley et al., 2009). Chandran and Kuton, 2008 reported the beneficial effect of C. officinalis extract on the thermal induced wound healing. Oral and topical application of the C. officinalis flower extract resulted in significantly higher percentage of wound closure compared to non-treated group of animals. Re-epithelization time was decreased in the treated group compared to control group (Preethi and Kutton, 2009). This extract also showed a significant anti-inflammatory effect (Preethi et al., 2009) after oral administration.

M. chamomilla is well known medicinal plant with multi-therapeutic properties. Among them anti-inflammatory, anti-septic, antiplogistic, and spasmyloptic properties (Singh et al., 2011) are most common. Significantly higher wound closure percentage compared to the control group was reported following the treatment with M. chamomilla oil extract (Jarrahi, 2008; Jarrahi et al., 2010). The treatment of the tongue wound with M. chamomilla extract resulted in significantly higher, time dependent re-epithelization and percentage of collagen fibers (Duarte et al., 2011). The M. chamomilla based treatment of the ulcer resulted in significantly faster wound healing compared to the control (Martsins et al., 2009). Significantly higher percentage of wound closure, faster re-epithelization, higher wound-breaking strength and higher hydroxyproline content compared to negative control (Nayak et al., 2007) was assessed following the treatment with M. chamomilla extract.

Lavandula officinalis was used in both folk and official medicine for wound healing as well as a disinfectant, antispasmodic, carminative, cholagogue, diuretic, stimulant and sudorific and flavouring agent (Bayoub et al, 2010). Sosa et al., 2005 reported a significant, dose dependent anti-inflammatory effect of lavender extract which was comparable to that of indomethacin. Both anti-inflammatory and analgesic effect of L. angustifolia was reported by Hajhashemi et al., 2003.

Olive oil extract of Hypericum perforatum showed excellent wound healing and anti-inflammatory effect in dose-dependent manner (Suntar et al, 2010). A dose dependent anti-inflamatory activity of H. perforatum was also reported by other researchers (Sosa et al., 2007; Hammer et al., 2007; Abdel-Salam, 2005; Menegazzi et al., 2006; Zdunić et al., 2009; Tedeschi et al., 2003; Öztürk et al., 2007).

Sadigc, 2003 reported, both bacteriostatic and bactericidal activity of Thymus serpyllum against various strain of E. coli, S. aureus and Y. enterocolitica. The antimicrobial activity comparable or better than standard antibiotics (Ur Rahman and Gul, 2003) was observed against both, gram positive (B. megaterium, B. subtilis, L. acidophilus, M. leu- teus, S. albus, S. aureus, V. cholera) and gram negative bacteria (E. coli, S. typhimurium, S. ferrarei).

A strong bacteriostatic and bactericidal activity of Salvia officinalis extract against S. aureus with MIC value of 60 μg/mL was reported by Snowden et al., 2014. S. officinalis showed strong antibacterial activity against B. subtilis and S. aureus (Balouri et al., 2014) and strong synergistic effect with amoxicillin against S. aureus ATCC 2923, E. coli ATCC 25922, P. aeruginosa ATCC 27853 and clinical isolates S. aureus, B. subtilis, E. cloacae, K. pneumoniae, E. coli and P. mirabilis. Anti-inflammatory effect S. officinalis was also demonstrated by Zubair et al., 2012 and Velasco-Lezama et al., 2006 with significant increase of epithelial cells proliferation compared to the negative control.

Alchemilla vulgaris is often used in folk medicine especially for the treatment inflammations, bleeding as well as various gynecological disorders. Wound-healing properties of A. vulgaris in vitro and in vivo was confirmed by Shrivasta-va et al., 2007 by increasing cell proliferation and decreasing the lesion diameter. The topical application A. vulgaris extract to the ulcers resulted in complete healing in 75% of the patients following the 3 day treatment (Shrivastava and John, 2006).

The beneficial effect of Symphytum officinale to wound healing could be directly linked with its phytochemical composition especially to its component allantoin (Staiger, 2012). Following the treatment of 161 patients with decubitus ulcers with Symphytum based cream during four weeks resulted in complete healing of the pressure sores in 85.9% of the patients and reduction of the total decubitus area for 89.2% (Stepán et al., 2014). S. officinale was found highly effective in wound healing in anime model with collagen deposition increase up to 240% and reduction of cellular inflammatory infiltrate up to 46% (Araújo et al., 2012). The wound healing mechanism induced by allantoin occurs via the regulation of inflammatory respon- se and stimulus to fibroblastic proliferation and extracellular matrix synthesis (Araújo et al., 2010). A significant wound healing potential of the topically applied preparation Traumaplant® containing 10% active ingredient from medicinal comfrey was confirmed on the patients with fresh abrasions (Barna et al., 2007; Barna et al., 2012).

Among the essential oils those with well known antimicrobial properties were selected in our study.

Melaleuca alternifolia essential oil showed excellent anti-fungal activity against filamentous fungi associated with invasive fungal wound infections (Homeyer et al., 2015) and
could be safely used for topical wound treatment. It was superior to fluconazole in growth inhibition of C. albicans (Dialwai et al., 2014) including fluconazole resistant strains (Vazquez et al., 2000; Ergin i Arik, 2002) with MIC ranging from 0.25-4% and inhibition zone diameter ranging from 14 to 42 mm. Its antifungal activity was also confirmed against yeasts (Candida spp., S. pombe, D. Hansenii) with the concentration ranged from 0.12% to 0.50% (v/v) (D’Auria et al., 2001). Tea tree oil at the concentration of 0.25% and higher inhibited significantly germ tubes formation by C. albicans (Hammer et al., 2000). It was found effective against 57 Candida isolates with MIC90 of 0.5% (v/v) (Hammer et al., 1998). Comparable MIC concentrations were also obtained for various intra-vaginal tea tree oil based products. The MIC and MBC for 60 isolates of meticillin-resistant S. aureus (MRSA) and mupirocin-resistant S. aureus were 0.25% and 0.50%, respectively (Carson et al., 1995a). Carson et al., 1995b reported MIC90 of tea tree oil of 0.25% for E. coli and 0.50% for S. aureus.

Thymus vulgaris essential oil has very strong antimicrobial potential and depending on the bacterial strain tested it showed 3-8 times higher inhibitory activity compared with all tested antibiotics. It exhibited the same strength of antifungal activity as amfotericin and miconazol against C. albicans (Herman and Mlynarczyk, 2014). The thyme essential oil showed excellent inhibitory effect against both Gram-positive and Gram-negative bacteria with inhibition zones ranging from 9 to 36 mm (Béjaoui et al., 2013). Thymus vulgaris essential oil were found active against 30 strains of clinical isolates of E. coli including multi drug resistant strains (Sienkiewicz et al., 2011). It also exhibited strong antibacterial activity against S. aureus, B. cereus and P. vulgaris (Al-Bayati, 2008).

Origanum vulgare showed a significant antimicrobial activity against various strain of E. coli, S. aureus and Y. enterocolitica (Sadigc, 2003). Strong antibacterial activity was also found against Clostridium genpus which was demonstrated in up to 93% growth inhibition after 30 minutes of the exposure (Kačániová et al., 2014). The essential oils of Origanum vulgare obtained from the plants at different phenological stages showed antibacterial activity against Gram positive bacteria (S. aureus, S. pyogenes and P. pneumoniae) with the growth inhibition zone ranging from 10-54 mm for 25% dilution (Kulenova et al., 2000). Thymus vulgaris essential oil were found active against 30 strains of clinical isolates of E. coli including multi drug resistant strains (Sienkiewicz et al., 2011). It also exhibited strong antibacterial activity against S. aureus, B. cereus and P. vulgaris (Al-Bayati, 2008).

Cymbopogon martini essential oil exhibited very good antimicrobial activity against broad range of the microorganisms (Fsai et al., 2010) and reduced pro-inflammatory cytokines secretion in vitro. A strong antibacterial activity was also obtained against various strains of E. coli (Duarte et al., 2007).

Statistical Analysis
For statistical evaluation Statistica 7.0 software package was employed. Statistical significance was set to p<0.05 in all the tests performed. The differences in the percentage of each parameter between control and experimental group were assessed by c2 test. The influence of the predictor variables (age, number of births, number of abortions, number of miscarriages, menopause, other health problems, other therapies) on the response to the therapy was tested by Multiple regression method, General regression model and Canonical correlation analysis (Findri-Guštek et al., 2012).

MATERIALS AND METHODS

Study Design
400 patients in the age range from 20 to 59 years were subjected to Pap test, cervical swabs for the presence of aerobic bacteria, yeasts, Ureaplasma urealyticum, Chlamydia trachomatis, Mycoplasma, and hrHPV DNA. Among them 286 were diagnosed with squamous endocervical metaplasia and represented our target group while the other patients were withdrawn from the study and subjected to conventional treatment depending on the diagnosis. Among those 289 patients 37 of them were also diagnosed with either bacterial, fungal or viral infections and they were excluded from the study. The rest of the patients were scheduled for the follow up after three months. On the second Pap test performed on those 252 patients 30 of them were free from metaplasia and excluded from the study. Finally, among the rest of the patients 30 of them was selected into control group and treated ten days with albuthol pessaries while the rest 192 patients represented experimental group were treated with newly developed pessaries also for ten days. Prior to the treatment all the patients signed the informed consent. To avoid possible intergroup differences the patients in both groups were of similar age and health status. Following the therapy all the patients were subjected to the Pap test and cervical swabs in order to determine the efficiency of the therapy. The sample preparation and analysis was described in details in our previous work (Findri-Gustek et al., 2012).

Preparation of the Pessaries
For the production of the macerate the following plants were used: Calendula officinalis (20%), Matricaria chamomilla (10%), Lavandula officinalis (5%), Hypericum perforatum L. (10%), Achillea millefolium (5%), Thymus serpyllum (5%), Salvia officinalis (5%), Mentha piperita L., (5%) Symphytum officinale (10%), Plantago major L. (20%), Alchemilla vulgaris (5%). The macerate was prepared from the dried plants obtained from herbal pharmacy and extra virgin olive oil with solid/liquid ratio = 1:5. The plants were macerated 30 days on 50°C and filtered. 40% of the macerate heated to 60°C was mixed with 4.5% of melted Cera alba and 54.6% of melted Witosphate E75 and mixed slowly for 10 minutes and allowed to cool to the room temperature. The essential oils of Melaleuca alternifolia (0.2%), Thymus vulgaris (0.2%), Pelargonium graveolens (0.1%), Cymbopogon martini (0.1%), and Origanum vulgare L (0.1%) were added into cooled mixture, mixed thoroughly and packed.

Pelargonium graveolens essential oil exhibited promising antimicrobial activity against broad range of microorganisms with inhibition zones ranging from 12 to 34 mm and MICs values from 0.039 to 10 mg/mL (Ben Hsouna and Hamdi, 2012). It exhibited strong antibacterial activity against S. aureus which was comparable or even better compared to chloramphenicol and amoxicillin (Ghannadi et al., 2012). Strong synergistic activity of Pelargonium graveolens essential oil and ciprofloxacin was observed against K. pneumoniae, P. mirabilis and S. aureus uropathogens (Mašlik et al., 2011).
RESULTS AND DISCUSSION
Description of the Population
The age range in the experimental group was from 20 to 59 years (38.2±8.2) while the control group ranged from 24 to 59 years (39.4±7.9). Women ranging from 31 to 40 years present the highest percentage of the studied population for both groups (43.33% and 41.67% for control and experimental group, respectively).

The number of births varied from 0 to 3 in the control and from 0 to 6 in the experimental group with the prevalence of two births in both groups (40% in control and 35.94% in experimental group). 96.67% and 97.92% of the control and experimental population respectively had no abortions, while 93.33% and 88.54% of control and experimental population, respectively had no miscarriages.

Menopausal women represent 10.00 % of the control and 5.73% of the experimental population.

Various health problems including allergies, heart condition, high blood pressure, diabetes, hypothyroidism, benign tumors of the breasts and ovaries, malign tumors, depression, kidney dysfunction, skin conditions, stress were obtained in 27.77% of the control and 21.87% of the experimental population. When distributed according to the age of the patients (Figure 1) the health problems increased with age in both groups. The lowest percentage in the experimental group was obtained among the women ranging from 31 to 40 years (17.5%) and the highest in the age group ranging from 51 to 59 years (28.57%). In the control group various health conditions increased from 24.11% in the youngest to 28.13% in the oldest age group. Malign and benign tumors of the breasts and ovaries, allergies, hypothyroidism, skin conditions, stress and depressions prevailed in the patients younger that 40 while the patients ranging from 41-59 years suffered mostly from high blood pressure, diabetes, heart and kidney conditions, equally in experimental and control group. 20% of the control and 11.94% of the experimental group were on constant therapy for above mentioned health conditions. According to the results of c² test there was no significant difference in the percentages between the control and experimental population for neither of the predictor variables.

The Outcome of the Therapy
Control group
Following the treatment with albothyl pessaries (Table 2) total clearance of metaplasia was observed in 20 of 30 (66.67%) of the patients. Three of them (10%) were diagnosed with milder metaplasia while in other seven patients there was no improvement following the therapy. The efficiency of the therapy decreased with age of the patients from 69.23% in the youngest group to 65.7% in the age group from 51-60 years.

The reason for those findings may lie in the fact that the oldest group was characterized with the highest percentage of various health issues that could weaken the body and extend the recovery time. Among the seven patients with no improvement following the therapy six of them suffered from other health problems. Although, the differences in the percentage of total clearance of metaplasia among the age groups were visible, according to the c² test those differences were not statistically significant.

All the swabs were negative to yeasts, bacteria and hrHPV.

Table 1. The response to the therapy (%) of the control patients treated with albothyl pessaries and control group treated with albothyl pessaries. TC-total clearens; MM-milder metaplasia; M-metaplasia

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In order to check whether there is a statistically significant correlation between the outcome of the therapy and predictor variables multiple correlation, general regression model and canonical analysis were used (Table 3, Fig. 3). According to the multiple regression only weak, but not statistically significant correlation was observed (R = 0.29; p<0.78063). Based on the beta coefficient the variables Other health problems and Age had the highest contribution to the correlation. However, none of the tested variables had statistically significant influence to the outcome of the therapy. Canonical analysis (Table 3) also confirmed weak, non-significant correlation between the outcome of the therapy and predictor variables (canonical R=0.29; c² = 3.99; p=0.77999). The variables Other health problems and Age showed higher values of canonical weights compared to other predictor variables confirming their somewhat higher influence on the outcome of the treatment. The highest (non-significant) influence of those two variables was also confirmed by general regression model expressed as pareto charts of t-values (Fig. 3).

### Table 3. Results of multiple regression and canonical analysis testing for the correlation between the response to the therapy and the predictor variables for the experimental and control group.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Multiple regression</th>
<th>Canonical analysis</th>
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<tbody>
<tr>
<td></td>
<td>Beta</td>
<td>p</td>
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<td>Age</td>
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<td>No. of births</td>
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<td>No. of abortions</td>
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<td>No. of miscarriages</td>
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<td>0.2319</td>
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<tr>
<td>Other therapies</td>
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<td>0.8650</td>
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According to the multiple regression analysis (Table 4) there was statistically significant correlation between the response to the therapy and selected predictor variables (R = 0.66; p<0.00000). Among the predictor variables only Other health problems showed statistically significant contribution (b=0.64; p=0.000000) to the correlation. Significant correlation was also confirmed by canonical correlation analysis (canonical R=0.66; c² = 90.08; p=0.00000). The variable Other health problems showed the highest value of canonical weight compared to other predictor variables. As presented by Pareto charts of t-values Other health problems was the only variable with high, statistically significant influence on the response to the therapy while all other variables had negligible effect (Fig. 3).

Our results showed almost 25% higher treatment efficiency of newly developed pessaries compared to albothyl used as the standard therapeutic approach. These results could be explained by the differences in their mechanisms of action. The Albothyl containing sulfonic acid acts in a way that peels squamous epithelium which promotes restoration of normal epithelial cells. This product has no anti-inflammatory, wound healing or antimicrobial properties.

On the other hand, herbal pessaries are composed of the extracts and essential oils of the plants with well-known an-

### Experimental group

All the swabs were negative to yeasts, bacteria and hrHPV. New herbal pessaries showed significantly higher efficiency (c²=8.39; P=0.0038) in the eradication of metaplasia (Table 2) compared to albothyl. Total clearance was observed in 91.15% of the patients, milder metaplasia in 2.60% of them while in 6.25% of the patients there was no changes following the therapy. The best results (Figure 2) were obtained in the youngest group (94.59%) while the lowest response was determined in the group ranging from 41 to 50 years (88.52%). Those differences were not statistically significant at p<0.05. Among the 12 patients with no improvement following the therapy 10 of them suffered from other health problems including allergies (4 cases), benign tumors (4 cases) and malign tumors (2 cases). Among them four of the patients belong to the group ranging from 41 to 50 years (two cases of benign tumors and two cases of malign tumors). High response to the treatment was found in the oldest age group (92.86%) which was explained by lower sexual activity among this group leading to less irritation of the gentle columnar cells.
CONCLUSIONS
Considering the obtained results it could be concluded that new herbal pessaries based on the combination of macerates and essential oils of the selected medicinal plants could be used successfully for the treatment of squamous endocervical metaplasia with treatment efficiency over 91%. The outcome of the therapy was highly dependent on the overall health status of the patients. The lowest efficiency was obtained on the patients with malignant diseases and severe allergies. When comparing with albithol, herbal pessaries showed significantly higher treatment efficiency (c²=8.39; P=0.0038). The synergistic effect of the medicinal plants with well known wound healing, anti-inflammatory, antimicrobial and antiviral properties could be responsible for high treatment potential of new herbal pessaries.