TREATMENT OF PRESSURE ULCERS WITH BIOAPIFIT® WOUND HEALING HERBAL OINTMENT - A PRELIMINARY STUDY

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Abstract

Objectives: The purpose of this work was development, formulation and testing of new herbal ointment for the treatment of pressure ulcers.

Patients and methods: 50 patients (27 males and 23 females) with total 84 ulcers of stage II and III were treated 28 days (twice a day) with the ointment containing the following ingredients: *Symphytum officinale*, *Plantago major*, *Calendula officinalis*, *Matricaria chamomilla*, *Bellis perennis*, *Achillea millefolium*, *Salvia officinalis*, *Hypericum perforatum*, *Olea europaea*, *Lavandula officinalis*, *Melaleuca alternifolia*, *Cympobogon martini*, *Origanum vulgare*, *Eugenia caryophyllata*, *Thymus vulgaris* ct. thymol, *Cera alba*, honey, and glycerol. The healing process was assessed by Pressure Ulcer Scale for Healing (PUSH) tool ver. 3.0.

Results: Prior to the therapy mean value and standard deviation of the PUSH score for ulcer surface area, quantity of exudate, type of tissue and the total score were 8.39±0.79, 1.35±0.84, 2.81±0.40 and 12.5±1.94, respectively. All the mentioned values decreased significantly after only seven days of the treatment ($p<0.00001$). Further treatment resulted in linear decrease of PUSH parameters reaching zero values after 28 days of the therapy. Slough disappeared after 14 days of the therapy and epithelial tissue was obtained on the edge of 67.86% of the ulcers. Following the 21 day of the treatment 17.86% of the ulcers were completely closed while after 28 days all the ulcers healed completely.

Conclusion: Four weeks of the topical treatment with Bioapifit® herbal wound healing ointment resulted in complete closure of all ulcers with mean healing time of 26.4 days. Such excellent results could be attributed to the ointment's formulation containing the ingredients with strong wound healing, anti-inflammatory and antimicrobial potential.

Key words- *pressure ulcers, wound healing, Bioapifit® herbal ointment, PUSH tool*

1. INTRODUCTION

According to the National Pressure Ulcer Advisory Panel a pressure ulcer (PU) is defined as localized injury to the skin and/or underlying tissue usually over a bony prominence, as a result of pressure, or pressure in combination with shear. According to the type of injury the ulcers could be categorized from stage 1 to stage 4 (Non-blanchable erythema of intact skin-stage 1; Partial-thickness skin loss with exposed dermis-stage 2; Full-thickness skin loss-stage 3; Full-thickness skin and tissue loss-stage 4) and Unstageable Pressure Injury (Obscured full-thickness skin and tissue loss). The PU present chronic, long lasting, hard to treat wounds associated with pain, suffering, and decreased quality of life (Stotts et al., 2001). Numerous predisposing factors are associated with development of pressure ulcers (Allam, 1997; Neloska et al., 2016) that could be roughly divided into two categories: (1) intrinsic (activity or mobility limitation, lack of alertness, poor nutritional status, dehydration, comorbidities, age associated skin sensitivity) and (2) extrinsic (pressure, friction, shear, incontinence). The most common places for development of PU are those

where the skin covering bony areas like heels, hips, elbows, shoulders, back of the head, knees, thighs and toes (Buzzi et al., 2016).

International guidelines for the treatment of pressure ulcers recommended necessary procedures to alleviate the symptoms of PU and among them are: pressure relief of the injury by repositioning of the patient every 2 hours; nutritional intervention by including protein rich diet and food supplements to support formation of granulation tissue; pain relief therapy, infection control using local antiseptics and antibiotic therapy; wound debridement of necrotic tissue and slough; specialized dressings for the preservation of the wound bed; surgical intervention in the case of necrotic tissue; and topical treatment in order to stimulate tissue repair and wound closure (Buzzi et al., 2016).

The purpose of this work was development, formulation and testing of new multi-herbal Bioapifit® wound-healing ointment for the treatment of pressure ulcers of stage II and III in elderly population.

2. PATIENTS AND METHODS

2.1. Study Design

50 patients at home care (27 females and 23 males) in the age range from 64 to 94 years with total 84 pressure ulcers were included. Only the patients with stage II and III pressure ulcers were included. The exclusion criteria were the patients with diabetes mellitus, necrotic wounds or severely infected wounds and immuno-compromised patients. Family member of each patient signed informed consent and completed the questioner. The patients were treated 28 days with the herbal ointment. The ointment was applied on the wound twice a day and covered with bandage during the whole course of the study. The healing progress was assessed by Pressure Ulcer Scale for Healing (PUSH) tool ver. 3.0 Stotts et al., 2001). This tool evaluates three basic parameters: (1) the ulcer surface area (length x width) scored from 0 (no ulcer present) to 10 when the surface area of the ulcer exceeded 24 cm^2; (2) the quantity of exudates scored from 0 to 3 (0-no exudates present, 1-light, 2-moderate, 3-heavy); (3) type of tissue scored from 0 to 4 (0-closed wound, 1-epithelial tissue, 2-granulation tissue, 3-slough, 4-necrotic tissue). The PUSH score could ranges from 0 (completely closed wound with no exudates) to maximum 17 with ulcer surface higher than 24 cm^2 with heavy exudates and the presence of necrotic tissue. The family members responsible for the patient care were advised to switch position of the patient every two hours in order to reduce pressure, use pressure-relieving cushions and pads, keep the skin clean and free of body fluids, wash with gentle soap and warm water and protect the skin with appropriate cream, include protein rich food in the patient's diet, include food supplements, and provide enough fluid to the patient in order to ensure good hydration. Four follow-ups (after every 7 days) were carried out in order to assess the efficacy of the therapy.

2.2 Preparation of the Ointment

The basic criteria for the inclusion of each component in the formulation was possession of one or more of the following properties: induction of wound healing and re-epithelization, anti-inflammatory effect, antibacterial activity, antifungal activity that was proven on *in vitro* and/or *in vivo* experiments on animal models and human clinical trials (Orescanin et al., 2015a; Orescanin et al., 2015a; Orescanin, 2016). The process of the production of the macerate and ointment was described in details in our previous papers (Orescanin et al., 2015 a,b; Orescanin and Findri, 2016). The final product consists of: 12% macerate of plantain leaves (*Plantago major*), 12% macerate of comfrey root (*Symphytum officinale*); 10% macerate of marigold flowers (*Calendula officinalis*), 10% macerate of chamomile flowers (*Matricaria chamomilla*), 10% macerate of aerial part of yarrow (*Achillea millefolium*), 4% macerate of daisy flowers (*Bellis perennis*), 4% macerate of aerial part of St. John's wort (*Hypericum perforatum*), 4% macerate of aerial part of sage (*Salvia officinalis*), 4% macerate of olive leaves, 2.5% macerate of lavender flowers (*Lavandula officinalis*); essential oils: 0.3% of tea tree (*Melaleuca alternifolia*), 0.3% of palmarosa (*Cympobogon martini*), 0.3% of thyme (*Thymus vulgaris* ct. thymol), 0.3% of clove (*Eugenia caryophyllata*), 0.3% of oregano (*Origanum vulgare*); 10% of bee wax (*Cera alba)*; 10% of honey; 6% of glycerol.

2.3. Statistical Analysis

For statistical evaluation Statistica 11.0 software package was employed. The description of the treated population was done by basic statistics and frequency tables. Statistical significance was set to $p<0.05$ in all the tests performed. The differences in the mean values of each parameter prior and after the therapy as well as different treatment periods were assessed by Newman-Keuls test. The influence of the predictor variables on the dependent variable was tested by Multiple regression method (Oreĉčanin et al., 2015a).

3. RESULTS AND DISCUSSION

3.1 Description of the Population

Basic variables of the tested population were presented in Table 1. The study included 27 males ranging from 64 to 91 years with 43 ulcers (10 of stage II and 33 of stage III) and 23 females in the age range from 65 to 94 years with 41 ulcers (6 of stage II and 55 of stage III). The study included long-term completely immobile patients who have spent in bed or wheelchair between 3 and 21 years. Most of them were malnourished or on the border of malnourishment. Majority of them (77.8% males and 78.3% females) have lack of bladder control while 14.8% males and 14.3% females have lack of alertness. According to the multiple regression analysis there was statistically significant correlation between total number of ulcers and predictor variables ($R=91.4$; $p<0.00000$). The variables *time spent in bed and / or wheelchair* ($p<0.00000$), *age* ($p<0.00023$), *BMI* ($p<0.00371$) and *lack of bladder control* ($p<0.04021$) had the highest, statistically significant contribution to the total number of ulcers.

Table 1. Description of the tested population

Variable	Males (N=27)	Females (N=23)
Age	64-91 (72.3±12.7)	65-94 (73.7±11.3)
Weight	61-84 (67.2±6.6)	47-69 (53.1±7.4)
BMI	16-22 (18.4±3.2)	16-23 (18.1±3.4)
Enable to change positions by themselves	27	23
Time spent in bed and / or wheelchair (yr)	3-18 (8.1±7.2)	4-21 (10.2±8.9)
Lack of bladder control	21	18
Lack of alertness	4	3
Total No. of ulcers	43	41

3.2 The Response to the Therapy

The results of the assessment of pressure ulcer healing according to the Pressure Ulcer Scale for Healing (PUSH) tool following the treatment with Bioapifit® wound healing herbal ointment were presented in Table 2. Prior to the therapy the majority of the ulcers had score 9 (44.05% of them) with surface area ranging from 12.1–24 cm^2 followed by score 8 (36.90% of ulcers) ranging from 8.1–12 cm^2 while only 4 ulcers had the area higher than 24 cm^2. When quantity of exudates was considered the majority of the ulcers (44.05% of them) were classified by PUSH tool as score 2 with moderate quantity of exudates followed by score 1 (32.14% of ulcers) with small amount of exudates. In 16 ulcers (19.05%) the wound was completely dry (score 0) while in 4 ulcers of grade III heavy exudates was obtained. According to the type of ulcer tissue in the majority of grade III ulcers white or yellowish slough was obtained (80.95% of ulcers) which was graded by score 3 by PUSH tool. None of the ulcers had necrotic tissue present while in 16 ulcers red pink granulation tissue was obtained (score 2).

Mean values and standard deviations for total score and three basic parameters of PUSH tool before the treatment and after each treatment period with Bioapifit® wound healing herbal ointment were presented in Fig. 1. Prior to the therapy mean value and standard deviation of the score for ulcer surface area, quantity of exudates, type of tissue and the sum of these three parameters (total score) were 8.39±0.79, 1.35±0.84, 2.81±0.40 and 12.5±1.94, respectively.

All three mentioned parameters, and consequently, the total score decreased after only seven days of the treatment. The following mean values and standard deviations for the surface

area, quantity of exudates, type of tissue and total score were determined respectively: 7.39±0.78, 0.87±0.64, 2.44±0.50 and 10.70±1.75. Further treatment resulted in linear decrease of the scores of all the parameters (Fig. 1) reaching zero values after 28 days of the therapy with Bioapifit® wound healing herbal ointment.

After 14 days of the therapy the surface area of the ulcers ranged between 0.7 and 4.0 cm^2 with the 50% of them in the range of 2.1-3 cm^2 (Table 2). The wounds in 57.14% of the ulcers were dry while in another 42.86% of them only small amount of exudate was found. None of the ulcers showed white to yellowish slough while epithelial tissue was obtained on the edge of 67.86% of the ulcers.

Following the 21 day of the treatment 15 ulcers were completely closed, 22 of them had surface area less than 0.3 cm^2 and 47 of them ranged from 0.3-0.6 cm^2. 80 ulcers were completely dry and in only 4 of them small amount of exudate still existed. Closed wounds were obtained in 17.86% of the ulcers while in 82.14% of them epithelial tissue was found (Table 2). Such results were reflected on the PUSH scores reaching the values of 1.94±1.25 for the surface area, 0.05±0.21 for the quantity of exudate, 1.32±0.47 for the type of tissue and 2.81±1.60 for the total score (Fig.1).

Complete closure of all ulcers was achieved after 28 days of the treatment reaching zero values of all PUSH parameters (Table 2; Fig. 1).

Table 2. Assessment of the pressure ulcer healing according to the Pressure Ulcer Scale for Healing tool following the treatment with Bioapifit® wound healing herbal ointment.

Parameter	Score/category	Before the treatment		After the treatment							
				7 days		14 days		21 days		28 days	
		N	%	N	%	N	%	N	%	N	%
Length × Width	(0) 0 cm^2	0	0	0	0	0	0	15	17.86	84	0
	(1) < 0.3 cm^2	0	0	0	0	0	0	22	26.19	0	0
	(2) 0.3-0.6 cm^2	0	0	0	0	0	0	47	55.95	0	0
	(3) 0.7-1.0 cm^2	0	0	0	0	6	7.14	0	0	0	0
	(4) 1.1-2.0 cm^2	0	0	0	0	24	28.57	0	0	0	0
	(5) 2.1-3 cm^2	0	0	0	0	42	50.00	0	0	0	0
	(6) 3.1-4.0 cm^2	0	0	8	9.52	12	14.29	0	0	0	0
	(7) 4.1-8.0 cm^2	12	14.29	42	50.00	0	0	0	0	0	0
	(8) 8.1-12 cm^2	31	36.90	27	32.14	0	0	0	0	0	0
	(9) 12.1-24 cm^2	37	44.05	7	8.34	0	0	0	0	0	0
	(10) > 24 cm^2	4	4.76	0	0	0	0	0	0	0	0
Quantity of exudate		N	%	N	%	N	%	N	%	N	%
	(0) None	16	19.05	23	27.38	48	57.14	80	95.24	84	100
	(1) Light	27	32.14	49	58.33	36	42.86	4	4.76	0	0
	(2) Moderate	37	44.05	12	14.29	0	0	0	0	0	0
	(3) Heavy	4	4.76	0	0	0	0	0	0	0	0
Type of tissue		N	%	N	%	N	%	N	%	N	%
	(0) Closed wound	0	0	0	0	0	0	15	17.86	84	100
	(1) Epithelial tissue	0	0	0	0	57	67.86	69	82.14	0	0
	(2) Granulation tissue	16	19.05	47	55.95	27	32.14	0	0	0	0
	(3) Slough	68	80.95	37	44.05	0	0	0	0	0	0
	(4) Necrotic tissue	0	0	0	0	0	0	0	0	0	0

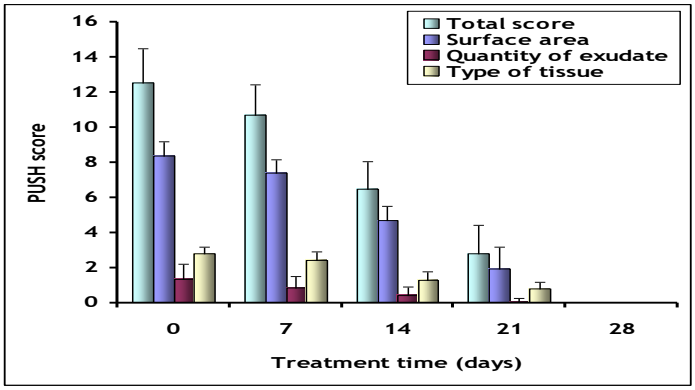

Figure 1. Mean values and standard deviations for thee basic parameters and the total score of Pressure Ulcer Scale for Healing Tool before the treatment and after each treatment period with Bioapifit® wound healing herbal ointment.

The results of Newman-Keuls test (Table 3) showed statistically significant difference in the mean values of the scores for the ulcer's surface area, quantity of exudate, type of tissue as well as total PUSH score before the therapy and after each follow-up period. Moreover, a significant difference was obtained among each treatment period for all PUSH parameters with the exception of quantity of exudate between 21st and 28th days of the treatment.

Table 3. The results of Newman-Keuls test testing for difference for thee basic parameters of Pressure Ulcer Scale for Healing Tool and total PUSH score among different treatment periods expressed in days. *Marked effects are significant at p < 0.05

Surface area (Length × Width)					
	Before the therapy	7th day	14th day	21st day	28th day
Before the therapy		0.000009*	0.000022*	0.000008*	0.000017*
7th day	0.000009*		0.000009*	0.000022*	0.000008*
14th day	0.000022*	0.000009*		0.000009*	0.000022*
21st day	0.000008*	0.000022*	0.000009*		0.000009*
28th day	0.000017*	0.000008*	0.000022*	0.000009*	
Quantity of Exudate					
	Before the therapy	7th day	14th day	21st day	28th day
Before the treatment		0.000009*	0.000022*	0.000008*	0.000017*
7th day	0.000009*		0.000009*	0.000022*	0.000008*
14th day	0.000022*	0.000009*		0.000012*	0.000022*
21st day	0.000008*	0.000022*	0.000012*		0.560981
28th day	0.000017*	0.000008*	0.000022*	0.560981	
Type of tissue					
	Before the therapy	7th day	14th day	21st day	28th day
Before the treatment		0.000009*	0.000022*	0.000008*	0.000017*
7th day	0.000009*		0.000009*	0.000022*	0.000008*
14th day	0.000022*	0.000009*		0.000009*	0.000022*
21st day	0.000008*	0.000022*	0.000009*		0.000009*
28th day	0.000017*	0.000008*	0.000022*	0.000009*	
Total score					
	Before the therapy	7th day	14th day	21st day	28th day
Before the therapy		0.000009*	0.000022*	0.000008*	0.000017*
7th day	0.000009*		0.000009*	0.000022*	0.000008*
14th day	0.000022*	0.000009*		0.000009*	0.000022*
21st day	0.000008*	0.000022*	0.000009*		0.000009*
28th day	0.000017*	0.000008*	0.000022*	0.000009*	

Such excellent results could be attributed to the composition of the herbal ointment containing the components with strong wound healing, anti-inflammatory and antimicrobial potential that act synergistically to speed up the healing process. Either mono-herbal or multi-herbal preparations as well as honey have long tradition both in folk and modern medicine for the treatment of fresh and chronic wounds as chipper alternative to the standard therapeutic approaches. Pressure ulcers represent a special challenge due to chronic wounds that are very difficult to heal.

Although inflammation is normal step in the wound healing process, the prolonged wound inflammation could decrease collagen synthesis, delay angiogenesis and slower re-epithelialization and consequently delay wound closure (Alam et al., 2014). Consequently, the preparations with high anti-inflammatory potential are highly beneficial in the wound healing process. Keeping that in mind, ten medicinal plants (*Symphytum officinale*, *Plantago major*, *Calendula officinalis*,

Matricaria chamomilla, Bellis perennis, Achillea millefolium, *Salvia officinalis, Hypericum perforatum*, *Olea europaea*, *Lavandula officinalis*) with well proven anti-inflammatory potential were selected.

The comprehensive literature review reviled that *Plantago major* leaves extract possess numerous beneficial biological effects and among them are anti-inflammatory, wound healing, analgesic, antioxidant, antimicrobial, immuno-modulating and anti-ulcerogenic activity (Samuelsen, 2000). Leaves have been traditionally used for the treatment of skin lesions and combat bacterial infections (Holetz et al., 2002). It was used in folk medicine as a wound healing remedy in almost all parts of the world. Either whole or crushed leaves are used for the treatments of various skin injuries in order to stop bleeding and enhance wound healing process. The leaves of *P. major* have thus been prescribed for the treatment of wounds caused by dog bites (Roca-Garcia, 1972). Normally, it is sufficient to apply only the juice from leaves to heal superficial wounds (Brondegaard, 1987). Recent studies have justified its use in folk medicine as wound healing remedy. Zubair et al., 2012 reported significant increase of epithelial cell proliferation *in vitro* following the treatment with various extracts of *P. major*. Thomé et al., 2012 confirmed excellent wound healing potential of *P. major* on *in vivo* animal model. Complete wound closure occurred on 15th day of the treatment. Moreover, the plant extract showed no mutagenic effect with or without S9 metabolic activations. *P. major* leaf extracts stimulated wound healing in *ex-vivo* porcine wound-healing model (Zubair et al., 2016). Wound-healing effect of *P. major* could be attributed to the presence of proteins with physicochemical properties and biological activity similar to those of membrano-tropic homeostatic tissue-specific bio-regulators recently isolated from *P. major* leaves that showed significant wound healing effect on the skin of vertebrates both *in vitro* and *in vivo* models (Krasnov et al., 2011).

Symphytum officinale has 2000 years long tradition and is still been used for external treatment of wounds, inflammatory joint disorders, gout, bone fractures, distortions, haematomas and thrombophlebitis (Staiger, 2007). Leaves extracts of *Symphytum officinale* were found highly effective in wound healing tested by open wound rat model (Araújo et al., 2012). Collagen deposition increased from 40% to 240% and reduction on cellular inflammatory infiltrate from 3% to 46% from day 3 to 28 of the treatment.

Human study also showed beneficial effect of comfrey on wound healing. Following the treatment of 161 patients with decubitus ulcers with *Symphytum officinale* 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). 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).

Calendula officinalis is used in the traditional medicine world wide, especially for wound healing, jaundice, blood purification, and as an antispasmodic. Its beneficial health effects could be directly linked with its phytochemical composition containing triterpenoids, flavonoids, coumarines, quinones, volatile oil, carotenoids and amino acids, charbohydrates, lipids and other phytochemicals like calendin, calendulin and n-paraffins (Muley et al., 2009).

C. officinalis extract showed beneficial effect on the thermal induced wound healing on animal model expressed trough significant increase of the collagen-hydroxyproline and hexosamine contents as well as haptoglobin and orosomucoid proteins in the treated group (Chandran and Kuton, 2008). Significantly higher percentage of wound closure and significant decrease of re-epithelization time was observed following the topical application of the *C. officinalis* extract compared to non-treated group of animals (Preethi and Kutton, 2009). A significant anti-inflammatory effect of the extract against carrageenan and dextran-induced acute paw edema in mice was reported by Preethi et al., 2009. They also observed a significant inhibition of pro-inflammatory mediators induced by lipopolysaccharides like TNF-α, IL-1beta, IL-6, IFN-γ, C- reactive protein (CRP) and cyclooxygenase-2 (Cox-2) and consequently inhibition of prostaglandin synthesis.

Human study also showed beneficial effect of calendula preparations in wound healing process. Following the treatment of venous ulcers twice a day for three weeks with *C. officinalis* based ointment complete epithelialization was observed in 21% of the patients while the total surface of all ulcers were reduced for 41.71% compared to only 14.52% in the control group treated with saline solution (Đuran et al., 2005). Application of calendula cream three times a day during 4 weeks resulted in complete healing of pressure ulcers in 56.6% of the patients (Esmaili et al., 2008). After the treatment of pressure ulcers of grade II and III with Plenusdermax® *Calendula officinalis*

spray for 30 weeks the complete closure of the wounds was observed in 88% of the patients (Buzzi et al., 2016a). After using the same preparation for the treatment of diabetic foot ulcers in females the complete wound closure was 54%, 68%, and 78% after 11, 20, and 30 weeks, respectively (Buzzi et al., 2016b). The application of topical cream containing 3% of calendula extract decreased skin erythema significantly which confirmed its anti-inflammatory effect. Besides, the cream showed skin moisturizing effects and decreased trans-epidermal water loss (Akhtar et al., 2011).

The application of *C. officinalis* extract preparation either prevented the development of radiation-induced oropharyngeal mucositis or significantly reduced its symptoms (Banaee et al., 2013). The topical application of calendula preparations significantly reduced the occurrence of acute dermatitis of grade 2 or higher in the breast cancer patient undergoing postoperative radiotherapy (Pommier et al., 2004). Significantly better results were observed with calendula compared to standard topical treatment with trolamine.

Matricaria chamomilla was included in the formulation due to its excellent wound healing potential obtained on both incision and excision animal models that was exhibited trough significantly higher percentage of wound closure, faster re-epithelization, higher wound-breaking strength, higher hydroxyproline content and higher percentage of collagen fibers in chamomile treated groups compared to the control (Nayak et al., 2007; Jarrahi, 2008; Martins et al., 2009; Jarrahi et al., 2010; Duarte et al., 2011). Another reason for the inclusion was its proven anti-inflammatory potential (Reis et al., 2011; Curra et al., 2013).

The results of human clinical trials also justified its usage in the wound healing formulation. The topical application of chamomile extract (Glowania et al., 1987) resulted in significantly faster wound healing and epithelialization compared to the control in the patients who underwent dermabrasion of tattoos. Maiche et al., 1991 reported less frequent radiation-induced skin reactions that appeared later in chamomile-treated areas compared to the control. Merfort et al., 1994 confirmed that chamomile bioactive components apigenin, luteolin and apigenin 7-O-beta-glucoside could penetrate into deeper skin layers and could be successfully used as topical antiphlogistic agents. Oral application of Kamillosan Liquidum® chamomile preparation (Carl and Emrich, 1991) by cancer patients during head and neck irradiation and/or systemic chemotherapy either completely prevented or reduced the intensity of oral mucositis.

Yarrow *(Achillea millefolium L.)* is one of the most widely used medicinal plants in the world, primarily for wound healing, hemorrhage, digestive problems, respiratory infections, and skin conditions. Preclinical studies indicate that it may have anti-inflammatory, anti-ulcer, hepatoprotective, anxiolytic, and perhaps antipathogenic activities (Applequist and Moerman, 2011). *A. millifolium* extract (5%) in Eucerin cream base significantly increased healing rate compared to phenytoin in excision based animal model. Bioactive components from the extract may stimulate the myofibroblasts contraction which resulted in faster wound closure. Moreover, the presence of hydolysable tannins in yarrow extract may cause coagulation of surface proteins and prevention of wound infection as well as faster wound closure (Hemmati et al., 2002). Those findings were in agreement with the results obtained by Nirmala and Karthiyayini, 2011 confirming significant increase in the rate of wound contraction, skin breaking strength, the weight of dry and wet granulation tissue as well as breaking strength of granulation tissue in *Achillea millefolium* treated animals both in incision and excision models. Besides, increased level of collagen was also observed in yarrow treated groups. *A. millifolium* extract significantly accelerated wound healing process and increased epithelialization in excision animal model (Rezaei et al., 2013). Excellent wound healing potential could be attributed with its anti-inflammatory potential (Benedek et al., 2007). Temamogullari et al., 2009 also reported beneficial effects of *A. millifolium* extract on wound healing in rabbits that were expressed as decreased bleeding, higher contraction and thickness of the scar, decreased level of inflammatory cell infiltrate and faster epithelization compared to the control group. Jalali et al., 2012 reported accelerated burn wounds healing and reduced the microbial wound count in rabbits during the course of treatment with yarrow extract.

Beneficial effect of yarrow on wound healing was also reported in human clinical trial. The treatment of the patients with venous leg ulcers by herbal preparation containing 7.5% of yarrow extract for three weeks resulted in 39.64% reduction of total surface area of all ulcers compared to only 15.1% reduction in the control group treated only with saline solution dressings (Matić et al., 2009). The parameters like granulation, epithelization and dermatitis also showed better results in the group treated with yarrow ointment compared to the control.

Hypericum perforatum was included in the formulation due to its strong wound healing, anti-inflammatory and antimicrobial potential obtained both *in vitro* and *in vivo* on animal study and human clinical trials. Olive oil extract of *Hypericum perforatum* showed excellent wound healing and anti-inflammatory effect in dose-dependent manner (Süntar et al, 2010). A dose dependent anti-inflammatory activity *in vitro* and *in vivo* was also reported by other researchers (Sosa et al., 2007; Kumar et al., 2001; Hammer et al., 2007; Abdel-Salam, 2005; Schempp et al., 2000; Menegazzi et al., 2006; Zdunić et al., 2009; Tedeschi et al., 2003; Öztürk et al., 2007). Besides, numerous authors reported antimicrobial activity of various extract of *H. perforatum* (Reichling et al., 2001). Excellent antibacterial activity of *H. perforatum* and its constituents was observed against gram-positive bacteria including penicillin-resistant (PRSA) and methicillin-resistant (MRSA) *S. aureus* (Schempp et al., 2003; Schempp et al., 1999). Antimicrobial activity against Gram-positive bacteria, *B. subtilis* and *B. cereus* was also reported by Avato et al., 2004. Meral and Karabay, 2002 reported antimicrobial activity of *H. perforatum* against Gram-positive (*S. aureus*, *S. epidermidis*, *E. faecalis*) and Gram-negative (*P. aeruginosae*, *E. cloacae* and *E. coli*) species which was comparable or better compared to standard antibiotics.

Wound healing properties were also obtained in human studies. The study of *Hypericum perforatum* olive oil extracts for the treatment of the 15 patients with lower back bedsores reviled significantly higher ($p < 0.001$) reduction in the total wound area (37.6%) compared to the 15 control patients treated with pure olive oil (17% reduction of wound area) following the 15 days treatment. The deep area was reduced by 37% and 12% in experimental and control group, respectively (Lomagno and Lomagno, 1979). An ointment prepared from olive oil macerate of *H. perforatum* was used in the treatment of 1st, 2nd, and 3rd degree burns. First degree burns healed within 48 hours. Second and third degree burns healed at least 3 times as rapidly as burns treated with conventional methods (Saljic, 1975). A randomized, double-blind clinical trial on the efficacy of *H. perforatum* applied 3 times a day for 16 days for cesarean section wound healing and hypertrophic scar reduction reviled significantly better results in wound healing compared to placebo or control groups. Besides, significantly lower pain and pruritus were reported by the experimental compared to the placebo and control groups (Samadi et al., 2010).

Salvia officinalis was included in the ointment formulation due to its strong bacteriostatic and bactericidal activity against broad range of pathogens involved in wound infections like both methicillin resistant and methicillin susceptible *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Escherichia coli*, *Proteus mirabilis*, *Salmonella typhi* (Snowden et al., 2014; Balouiri et al., 2014; Stefanović et al., 2012). Its anti-inflammatory potential which was mostly based on the suppression of Prostaglandin E_2 synthesis by direct interference with microsomal PGE_2 synthase-1 (Bauer et al. 2012; Rodrigues et al., 2012; Oniga et al., 2007) was also the reason for the inclusion. Besides, its astringent activity is beneficial during the course of wound healing.

Olea europaea L. leaves have been widely used in traditional herbal preparations due to their proven anti-inflammatory, antioxidant, antihypertensive, antiatherogenic, hypoglycemic, and hypocholesterolemic properties (El and Karakaya, 2009) that are directly connected with the presence of bioactive constituents, especially oleuropein as well as other secoiridoids, flavonoids, and triterpenes. *Olea europaea* leaves extract enriched in oleuropein showed significantly higher percentage of wound contraction on circular excision model as well as significantly higher wound tensile strength on incision animal model compared to the control group treated with Madecassol® ointment (Koca et al., 2011) which justified its traditional application for the treatment of wounds and ulcers. Oleuropein accelerated wound healing in aged male Balb/c mice on incision model which was exhibited trough reduced cell infiltration in the wound site, a significant increase in type 1 collagen fiber deposition and faster re-epithelialization in the experimental compared to the control group (Mehraein et al., 2014a). Similar study conducted by Mehraein et al., 2014b on young male Balb/c mice also confirmed excellent wound healing potential of oleuropein which was expressed trough accelerating the re-epithelialization process, enhancing collagen fiber synthesis, and increasing the blood supply to the wounded area by upregulation of vascular endothelial growth factor protein expression.

Bellis perennis was included in the formulation due to its traditional use for the treatment of wounds and bruises. Karakaş et al., 2012 reported excellent wound healing potential of the extract of *B. perennis* flowers on circular excision wound model. Following 30 days of the treatment with *B. perennis* based ointment significantly higher wound closure was observed compared to the

control group. Furthermore, no scars were observed in *B. perennis* treated rats. It was reported that oleanane-type triterpene saponins isolated from *Bellis perennis* flowers has promoting collagen synthesis in normal human dermal fibroblasts (Morikawa et al., 2015).

Lavandula officinalis was used in both folk and official medicine for wound healing and disinfectant (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 lavender was reported by Hajhashemi et al., 2003. Analgesic effect of lavender was also confirmed on human study resulted in significantly lower degree of pain following episiotomy in lavender treated group (Sheikhan et al., 2012; Vakilian et al., 2011).

Multiherbal preparations containing above mentioned plants also showed a significant potential for wound healing. Kundaković et al., 2012 used the ointment prepared from the extracts of garlic, *Calendula officinalis* and *H. perforatum* for the treatment of 25 patients with venous ulcers. Following the seven weeks treatment period a significant improvement of the epithelialization was observed with a mean reduction of the ulcer area by 55%. In another clinical trial 12 patients undergone cesarean section were treated twice a day with the formulation prepared from the combination of *Calendula officinalis* and *H. perforatum* macerate in wheat germ oil. The formulation accelerated wound healing significantly compared to the control group treated with wheat germ oil only, with 38% vs. 16% reduction of the wound area (Lavagna et al., 2001). Similar study (Đuran et al., 1997) was conducted with the Plantoderm ointment containing the extracts of four different medicinal herbs (*Achillea millefolium*, *Calendula officinalis*, *Symphytum officinale*, *Salvia officinalis*). 40 patients with a total number of 66 venous ulcers were treated for three weeks which resulted in 58.55% decrease of total ulcer surface while complete epithelization was recorded in 22 ulcers.

Honey has numerous beneficial effects to the human health. The antimicrobial activity against the pathogens connected with invasive wound infections including methicillin-resistant *Staphylococcus aureus* (MRSA) was linked either to the production of hydrogen-peroxide by glucose oxidize enzyme or non-peroxide antimicrobial activity which could be connected to the presence of polyphenols and flavonoides, low pH value, osmotic effect of sugar, carbohydrate and its break-down Maillard products, aromatic acids, 10-HAD defensin-1 protein, 1,2-dicarbonyl compound methylglyoxal and bacillomycin F antibiotic like polypeptide (Lusby et al., 2005; Simon et al., 2009; Al-Waili et al., 2011; Orescanin et al., 2015a; Orescanin et al., 2015a).

Numerous authors reported beneficial effects of topical honey application on wound healing. The treatment of pressure ulcers with honey alginate (Vandamme et al., 2003) resulted in a rapid and complete wounds healing, reduced inflammation and deodorizing effect on the wounds due to its antimicrobial and prebiotic activity. It was confirmed that natural honey suppresses inflammation, reduces scarring and stimulate angiogenesis (Molan, 2002). Subrahmanyam et al., 2001 reported significantly faster wound healing in the patients treated with honey dressing compared to those treated with silver sulphadiazine. Moreover, completely sterile wounds were obtained in 90% of honey treated patients. It was reported that pH of the wound has critical influence on its healing potential since the wounds with pH higher than 8 showed no reduction in size (Gethin et al., 2008). The authors concluded that reducing the wound pH value could decrease protease activity, increase fibroblast synthesis, increase oxygen release that all together promote wound healing. Alam et al., 2014 summarized beneficial effects of honey for the treatment of diabetic associated wounds that ware mostly connected to its strong antioxidant and antimicrobial activity, low pH value, hydrogen peroxide activity which stimulates both fibroblast proliferation and angiogenesis, debridiment of slough and necrotic tissue through autolytic debridement induced by honey proteases, minimizing wound odor either trough its antimicrobial activity against anaerobic bacteria causing malodor or by converting glucose into lactic acid by lactic bacteria which replaced malodor of the by-products of amino acids metabolism, minimizing scar formation and by inflammation control.

A significant improvement of venous ulcer wound healing was observed following the treatment with the honey-based dressing (Alcaraz and Kelly, 2002). Following four weeks treatment of diabetic patient amputation wound with natural honey on a daily basis resulted in complete wound closure (Mohamed et al., 2014). The treatment of foot ulcers with natural honey once a day resulted in complete wound closure within three weeks with no contractures or scars (Mohamed et al., 2015). Natural honey was also found efficient in preventing and decreasing the oral mucositis in radiotherapy treated patients. The treatment of the patients with neuropathic diabetic foot ulcers

with manuka honey impregnated dressings (Kamaratos et al., 2014) resulted in complete healing after 31±4 days while in app. 78% of the patients wound became sterile following one week of the treatment. Significantly later onset and lower grade of mucositis was developed in honey treated group compared to those treated with 0.15% benzydamine hydrochloride or 0.9% normal saline (Jayachandran).

In addition to the above mentioned medicinal plants macerates and honey, five essential oils (*Melaleuca alternifolia, Thymus vulgaris, Cymbopogon martinii, Origanum vulgare, Cinnamomum camphora* and *Eugenia caryophyllata*) were added in the formulation due to their strong antibacterial and antifungal activity against wide range of pathogens including those responsible for invasive wound infections (Orescanin et al., 2015a; Orescanin et al., 2015b). So, they served in the ointment to prevent wound infection and to sterilize already infected wounds. Besides, their proven bacteriostatic and fungistatic activity against broad range of food-borne pathogens made them excellent natural preservatives in the ointment (Orescanin and Findri Gustek, 2015c).

Bee wax was used in the formulation not only as emulsifying and thickening agent but also for wound isolation and protection from microbial infection. Moreover, its bioactive components showed anti-inflammatory effect on animal model (Carbajal et al., 1995; Noa and Mass, 1998).

Glycerol was used in order to provide enough moisture content of the wound necessary for the healing process.

4. CONCLUSION

Bioapifit® wound healing ointment containing medicinal plants in the form of olive oil macerate and essential oils in the combination with honey presents excellent choice for the treatment of chronic wounds like pressure ulcers and could be used as cheep alternative to the standard treatment approaches. Four weeks of the treatment of PU of stage II and III resulted in complete closure of all ulcers with mean healing time of 26.4 days. Considering the fact that pressure ulcers presents, long lasting, hard to treat chronic wounds that may never heal completely such excellent results could be ascribed to the synergistic and additive effect of bioactive constituents from the medicinal plants and honey with well known wound healing, anti-inflammatory and antimicrobial potential.

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