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Evaluation of Anti-Propionibacterium Acnes and Anti-inflammatory Effects of Polyphenolic Extracts of Medicinal herbs in Jordan

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Acne vulgaris is one of the most common health problem where medical treatment is sought in adults worldwide. It has been long described the integral role of *Propionibacterium acnes* in the pathogenesis of this disease. In this study, a group of local herbs known for their antimicrobial effects were selected for the evaluation of potential anti-acnes effects in vitro.Phenolics and flavonoid contents of methanolic extracts of *Eucalyptus globulus, Mentharotundifolia, Inulaviscosa, Utricadioica, Malvasylvestris,Quercuscalliprinos, Arum palaestinum*and*Achilleaodorata*collected from different regions in Jordan during 2016-2017 were screenedfor antimicrobial activity against *Propionibacterium acnes* by disc diffusion and by broth microdilution method.Measurement of release of interleukin 1 alpha from human skin explants by ELISA was used for the evaluation of anti-inflammatory effects of the herbal preparations and extracts. *M.rotundifolia* and *E.globulus*, showed the highest phenolic and flavonoid contents in contrast to *M.sylvestris* which showed the least phenolic contents. Moreover, polyphenolic fractions exhibited modest anti-acne activity of herbal extracts of *E.globulus* and *A.palaestinum* (MIC 0.125 mg/ml), *U.dioica* (0.25 mg/ml) and *I.viscosa* (0.5 mg/ml), compared to not significant antimicrobial activity for others (MIC >1mg/ml). Regarding anti-inflammatory effects of the tested fractions, *E. globulus* and *A.palaestinum* extracts showed inhibition of interleukin 1 alpha release by more than 60 % for concentrations of 0.5 mg/ml respectively. The presence of anti-inflammatory and anti-acne activities in the polyphenolic extracts of local medicinal plants would increase the potential of using these herbs in the control of Acne vulgaris.

Keywords: Acne vulgaris, polyphenolics, Antimicrobial and Anti-inflammatory effects.

Acne vulgaris is considered the most frequent skin disease of sebaceous glands with characteristic inflammatory lesions called comedones in the face, back, and trunk. A growing group of evidence stated that the presence of the obligated anaerobic Gram positive bacterium *Propionibacterium acnes*(*P. acnes*) in the follicular and sebum canals is implicated in the development of inflammatory acne[1]. This is generally explained by the significant capability of this microbe to interact with the components of the immune system. For instance *P. acnes* is remarkably capableof metabolising sebum fats into free fatty acids, leading to chemotactically attracting

neutrophilsand induction of monocytes to produce various pro-inflammatory mediators including tumor necrosis factor (TNF) and Interleukin -8 (IL-8)[2]. Routinely, major treatment options for acne vulgaris include isotretinoin,use of antibiotics liketetracyclines and hormonal drugs. However factors like the long term use of such drugs with their serious side effectsin addition to the emergence of antibiotic resistance among clinical strains of *P. acnes,* constituted considerable limitations of optimal eradication and treatment of this disease[3, 4].

Medicinal herbs have been extensively investigated as a remarkable approach in encountering infections especially those caused by multi-drug resistant pathogens[5, 6]. As part of the Arabian folk medicine, several preparations of local herbs and oils, like olive oil and water extracts of pomegranate, were used for the treatment of skin infections[7, 8]. Several studies showed that phenolic extracts exhibited potent antibacterial and anti-inflammatory effects[9, 10].These fractions contain high quantities of different phenolic acids includingcaffeic acid and ferulic acidswhich exhibited potent antimicrobial, antiseptic, preservative and anti-oxidant activities[11].

Our previous work on polyphenolic extracts of herbs collected from Algeria showed potent anti-microbial activity against *Helicobacter pylori,*the causative agent of peptic ulcer in humans[12]. However, their antimicrobial effects on *P. acnes* and anti-inflammatory effects were not well characterized. This study aims to determine the antimicrobial effects against *P. acnes* of polyphenolic extracts of methanolic extracts of *Eucalyptus globulus, Mentharotundifolia, Inulaviscosa, Utricadioica, Malvasylvestris, Quercuscalliprinos*, *Arum palaestinum*and*Achilleaodorata* collected from different regions in Jordan and their potential anti-inflammatory effects which, in our opinion, might provide new therapeutic options for the management of acne vulgaris.

MATERIALS AND METHODS

Plants and extraction of polyphenolic fractions

During 2016-2017, leaves of *M.rotundifolia, I. viscose, U.dioica, E. globules, A.odorata, Q.calliprinos, A.palaestinum*and *M.sylvestris* were collected from different regions of Jordan. The plant material was stored at room temperature in a dry place until subsequent use. Leaves were firstly air dried, ground by an electric grinder until becoming a fine powder[13]. Next, stitches 50ìm in diameter were used for sifting purposes. The plant powder was then kept in small bottles of tinted glass to avoid the oxidization of their contents. Polyphenolic extraction was performed by maceration in methanol-water 80/20 (v/v) at a solid-liquid ratio 1/10 (w/v) with continuous stirring at ambient temperature for 48h followed by filtration with filter papers. Defatting with hexane of the aqueous phase was carried out as described by Yu *et al*. 2005 [14]. Subsequently, the obtained methanol phase was concentrated using a rotary evaporator with a vacuum controller, at a temperature of 40°C.

Total phenolic and flavonoid content measurements

Determination of the total phenols content was performed according to Folin–Ciocalteu's method[15]. Briefly, a total of 0.3 mL of sample was dissolved in 1.7 mL of 1:10 Folin–Ciocalteu reagent. The solutions were then mixed and incubated at room temperature for 10 min. Two mL of 7.5% sodium carbonate (Na_2CO_3) solution were then added and incubation for 90 min at room temperature was performed. Finally, measurement of the optical density of the solutions at 725 nm was carried out in relation to gallic acid calibration curves where the results were expressed as Gallic acid equivalents (GAE). Total flavonoid content was measured spectrophotometrically at 430 nm. 1:5 diluted samples were equally mixed with 2% aluminium chloride solution and incubated at room temperature for 30 min[16]. Quercetin was used to construct the calibration curves and the flavonoids content was expressed in mg ofquercetin equivalent (QE) per gram of crude extract. All experiments were carried out in at least three replicates.

Screening for antibacterial effect against *P. acnes* by disc diffusion method

The extracts were tested for their potential anti-bacterial effect on *P. acnes* by the method of Hayes and Markovic[17] with some modifications. In brief, standard aliquots with 1.0×10^8 CFU/ mLof a control strain of *P. acnes* (NCTC 747) provided by the Jordan Company for Antibody Production (Monojo) was incubated in tryptic soy broth supplemented with 1% glucose for 48

h under anaerobic conditions using CampyGen atmosphere generating system (Oxoid, UK). The agar base was constituted of molten TSA with glucose. The prepared bacterium inoculum was then added to the molten agar, mixed and evenly poured over the surface of the agar base until it was completely solidified. A sterile paper disc was impregnated with 25 ìl of 50 mg/mL of each extract and fraction and then placed on the surface of the agar plates. Plates were then incubated at 37^0C for 48 h under anaerobic conditions in an anaerobic jar (Oxoid, UK) under aerobic conditions. Antibacterial activity was determined by the production of an inhibition zone around the impregnated disc with the extracts[18]. Positive control discs were made of Clindamycin (10 µg/ml) while discs impregnated with solvents served as negative controls. Growth of*P. acnes* was confirmed according to Gram staining, biochemical reactions and subsequently by standard PCR of 16S rDNA test. [19]*P. acnes*cultures were stored at -70°C in TSB containing 1% glucose and 15% glycerol.

Determination of minimal inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs)

Broth Microdilution method was used to measure MIC values of the extracts and fractions against *P. acnes*. Cultures of *P. acnes* containing 1 x 10^5 cells/mL were prepared in Nutrient yeast glucose broth (NYG). A total of 20 µl of the bacterial suspension was added to 200 µl of two fold serially diluted plant extracts dissolved in NYG broth with a concentration range of 0.06to 8mg/mL.subsequently, plates were then incubated for 48 h at 37^OC under anaerobic conditions. The growth of *P. acnes*was measured as a function of turbidity at 600 nm.The MIC was defined as the lowest concentration of the compound to inhibit the visible growth of microorganisms. Triplicates of each extract were performed and the average MIC values were taken.

Evaluation of Anti-inflammatory effects by Interleukin release assay

Measurement of the interleukin-1a secretion from UVB irradiated human skin cultures (epiCS® skin model, (CellSystems, Germany) was carried out using Human IL-1a immunoassay ELISA Kit (R&D Systems GmbH,Germany) as an indicator for inflammatory process[20]. First, human skin explants were exposed for UVB 2 J / cm^2 UVB irradiation maximum at 312 nm for 20 min. Then, immediately after irradiation, polyphenolic extracts of each tested herb (two different concentrations; 0.25 and 0.5 mg / mL) were applied to the irradiated skin explants for 5 min. After 24 h, IL-1a secretion was measured in pg / mL. The baseline level of IL-1a was set to the amountof IL-1a in the non-treated non-exposed skin while the maximal IL-1a production as considered as the level in the non-treated UVB-exposed skin. Dexamethazone in three different concentrations (1 uM, 10 uM and 50 uM) was used as anti-inflammatory positive controls and the inhibitory effects of the tested extracts were statistically compared with the non-treated UVB-exposed control according to a one-way anova followed by Dunnett's t-test ($P < 0,05$).

RESULTS

Total phenols and flavonoids content

The total phenolic and flavonoid contents in the methanloic extracts of each studied plant material, was calculated as gallic acid and quercetine equivalents, respectively. The reference standard curve of $y = 0.0024x + 0.0886$, $r^2 = 0.9642$ was used for this purpose. The phenolic contents of extracts from *M.rotundifolia*and *E.globulus* showed the highest concentrations of 720.56±1.27 and 670.66±1.55 mg GAE/g extractrespectively. In contrast, *A.palaestinum* showed the least total phenolic contents (48.67±0.65 mg GAE/g extract) (Table 1). The flavonoid contents were highest in *E.globulus* and *A. odorata* with concentrations of 110.56±0.82 and 101.56±1.27 mg QE/g extract respectively. The percentage of flavonoids in the phenolic fraction was highest in *M.sylvestris* (37.6%).

Growth inhibition of *P. acnes* by herbal extracts

In this study, the antimicrobial effect of eight herbal extracts was evaluated (table 2). Notable growth inhibitory effects against P. *acnes*of polyphenolic extracts of *E.globulus* ,*A. palaestinum*, *U.dioica* and *I.viscosa* were reported. Both extracts from *E.globulus,* and*A. palaestinum* presented the highest antimicrobial activity with MIC values of 0.125 mg / mL.Moderate antimicrobial activity of *I. visocosa* was observed with MIC values of 0.5 mg / mL.

Anti-inflammatory effects of the herbal extracts

The anti-inflammatory effects of the

Table 1. Concentrations of phenolic and flavonoid contents of methanolic extracts of plants materials used in this study

Plant	Total phenols content (mg GAE/g extract)	Total flavonoids content (mg QE/g extract)	% of flavonoids in the phenol fraction
E.globulus	670.66±1.55	110.56±0.82	16.4
A.odorata	420.88±1.04	101.56±1.27	24.0
M. sylvestris	165.32±0.87	62. 40±0.37	37.6
Q.calliprinos,	80.36±0.24	18.45±0.19	22.5
A.palaestinum	48.67±0.65	12.36±0.84	25.0
I.viscosa	360.45±0.75	51.34±0.56	14.1
U.dioica	410.99±1.66	41.25±1.51	10.0
M.rotundifolia	720.56±1.27	114.17±0.63	15.8

Table 2. Antimicrobial activity of methanolic extracts against *P. acnes*

Plant	Stock concentration (mg / ml)	Antimicrobial effect Zone of inhibition (mm)	MIC (mg / ml)
E. globulus	50	28	0.125
A. palaestinum	50	26	0.125
U. dioica	50	22	0.25
I. viscosa	50	19	0.5
M. rotundifolia	50	9	2
M. sylvestris	50	None	> 8
Q. calliprinos	50	None	> 8
A. odorata	50	None	> 8

herbal extracts were summarized in figure 1. The IL-1a release was remarkably increased by 10 folds in the non-treated UVB exposed skin explants, compared with the non-irradiated skin. The UVB exposed skin explants treated with 0.5 mg / ml extracts of *E. globulus* and *A. palaestinum*showed marked inhibition of release of IL-1a with figures reading 60% and 75% respectively.Less inhibition of IL-1a release with figures ranged from 5-30% for 0.25 and 0.5 mg / mL was observed for extracts of other herbs.

DISCUSSION

Recent trends toward natural products have increased as sources of innovative therapeutic

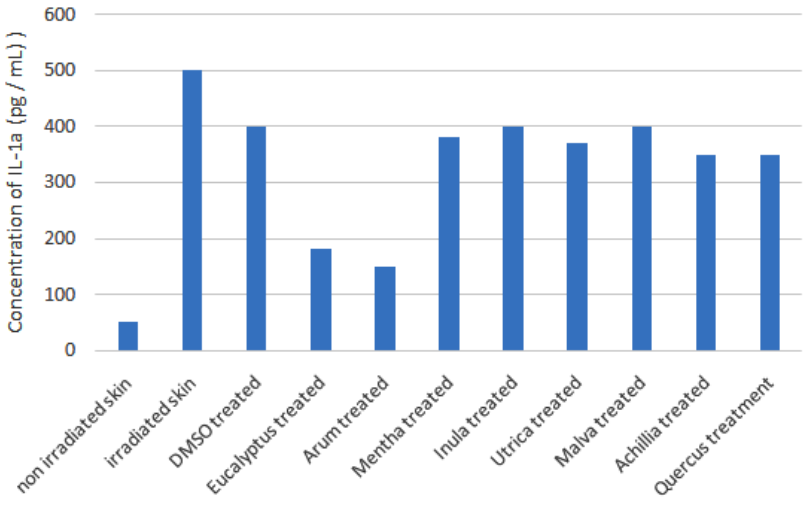

Fig. 1. Effects of herbal extracts on the production of the pro-inûammatory mediator such IL-1a in UVB irradiated skin explants. Data are expressed asthe mean ±SD. $p < 0.05$ compared to the DMSO solvent was set as significant. Control was made of non irradiated skin explants

agents which, would in principle constitute adjunctive and alternativesto the currently used antimicrobial agents[21]. In Jordan, there is a wide variety of plants that have been widely used in traditional medicine for different types of diseases including skin infections[22, 23]. However, little is known regarding the potential antimicrobial and anti-inflammatory effects of these plants as a model for the prevention and control of Acne vulgaris.

The present study, investigated the antimicrobial activity of polyphenolic extracts of eight local herbs including against *P. acnes*. In addition, and for the first time, their anti-inflammatory effects were evaluated in vitro using skin explants. Our preliminary screening revealed that the polyphenolic fractions of some of the selected herbs possess considerable anti-P. acnes activity with a marked anti-inflammatory effects. The results also showed significant variations in the total phenolic and flavonoid contents among the extracts of the tested plants. With particular concern, *E.globulus* showed both strong antimicrobial (MIC 0.125 mg / mL) and inhibition of IL-1a release (more than 60% at concentrations 0.5 mg/mL). Both *A. palaestinum* and *U. dioica* showed modest antimicrobial activities in comparison to other herbs which insignificantly affected the growth of *P. acnes*.Our results are in agreement with most of the studies conducted on the evaluation of biological activities of *Eucalyptus* extracts. Oils of *Eucalyptus* showed potent antimicrobial activity against macrolide resistant P. acnes with a notable decrease of sebum production[24]. In contrast to our results, a stronger effect of *Achillea* against *P. acnes* with MIC of 0.83 mg / mLwas reported[25]however, the activity was related to the petroleum ether extract which is known to contain Tannins and alkaloids rather than flavonoids as in our study.

In regards to the anti-inflammatory effects of the selected herbs, *Arum* exhibited a unique potent inhibitory effects on the release of IL-1a pro-inflammatory cytokine. The inhibition was measured to be more than 70% on the concentration of 0.5 mg/ mL of the polyphenolic extract of *Arum*. This is the first study to report the anti-inflammatory effect of *Arum* leaves as a raw model for the management of Acne. Our results are however in contrast with a study conducted by Khalaf*et al*., 2015 in which no significant anti-inflammatory effect of Arum was reported compared to extracts from leaves of *Rosmarinus officinalis*[26].

Based on the results of this study, the presence of anti- acne specific phenolic compound/ compounds among the examined plants is not the same. The total polyphenolic and the flavonoid contents have not been shown to be solely linked to the antimicrobial and anti-inflammatory effects of the tested herbs. Variation of the chemical compositions of numerous natural phenolic compounds like rosmarinic acid,ferruginol and kaempferolwould significantly affect the results of the antimicrobial activity of the fractions[27, 28]. Moreover,synergistic effects of the extracts with various phenolic compounds could not be excluded in the antimicrobial activity of the herbs. As previously shown, the presence of anti-oxidants including ascorbic acid and ferulic acid, alone or in combination with other phenols play a key role in the antimicrobial effects of many medicinal herbs [6, 12, 29]. This can also be postulated for the anti-inflammatory effects of the tested plants in this study, since the activity was shown to be independent and not linked to the antimicrobial activity.

In conclusion, the presented work demonstrated that polyphenolic fractions of extracts from local plants in Jordan have considerable in vitro activities against *P. acnes* and its associated inflammatory potential. further examination of these herbs as adjunct to or as an alternative treatment of acne vulgaris is required.

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