



Effectivity of flavonoids on animal model psoriasis – thermographic evaluation

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Abstract

Background and purpose: Psoriasisform lesions are characterized by hyperproliferation and aberrant differentiation of epidermal keratinocytes, accompanied by inflammation, leading to a disrupted skin barrier with an abnormal stratum corneum. Psoriasis is a chronic inflammatory disease whose etiopathogenesis has not yet been fully resolved, and therefore there is no standardized therapeutical approach. This study examined the possible positive effects of propolis and its polyphenolic/flavonoid compounds on animal model psoriasis, induced by the Di-n-Propyl Disulfide irritant (PPD), and the possibility to assess usefulness of thermography in psoriatic lesion regression.

Material and methods: We monitored the inflammation process by monitoring the total number of inflammatory cells in peritoneal cavity, macrophage spreading index and thermographic scanning. Thermographic is scanning an effective and simple method which reproducibly records thermographic images of the examined area. The tested animals were divided into sixteen groups and locally processed during five days with PPD, water and ethanolic extract (WSDP or EEP) of propolis preparations and flavonoids (Epigallocatechin 3-gallate, Quercetin, Chrisin, Curcumin).

Results: The results of thermal imaging showed no statistically significant differences in temperature changes on skin locuses of psoriasis formed lesions among the examined groups. The total number of inflammatory cells in peritoneal cavity and the macrophage spreading index were reduced in psoriatic mice treated with test components.

Conclusions: These results demonstrate that topical application of propolis and the flavonoids present in propolis may improve psoriatic-like skin lesions by suppressing functional activity of macrophages and ROS production. Taken together, it is suggested that propolis and flavonoids offer some protection against psoriatic complications through their roles as inhibitors of inflammation and as free radical scavengers. Thermal imaging was realistic, and can be applicable in examining the inflammatory process in psoriasis and in evaluating the effectiveness of tested substances.

INTRODUCTION

Psoriasis is a chronic, recurrent, inflammatory skin disease which manifests by multiple erythematous squamous plaques, and it affects 1–3% of the general population (1, 2). It is understood today that the disease incurs as a result of interplay between genetic and surrounding factors (3). However, the immunopathogenic mechanisms of psoriasis, as well as optimal therapeutical approach, are yet to be fully resolved (4,

5). That is why, despite the development in new drug groups, traditional therapy is still used although its toxicity and negative side effects are well known (5).

Scientific information supports the view that an insufficient antioxidant system contributes to the pathogenesis of psoriasis (6). In psoriatic skin context, reactive species are generated by keratinocytes and activated leukocytes, mostly neutrophils (7). Psoriatic skin is also characterized by an advanced state of lipid peroxidation (8). Thus, it has been suggested that the antioxidant treatment could be part of a more specific and effective therapy for the management of this skin disease (9). Recent literature data continue to support the fact that polyphenolic compounds, found in most plants and bee products, can have a positive effect on many chronic diseases (10-13). Natural polyphenols, recognized as potent antioxidants, are multifunctional molecules that can act as anti-inflammatory (14) and antiproliferative agents (15) through the modulation of multiple signaling pathways. This characteristic could be advantageous for the treatment of multi-causal diseases, such as psoriasis. Polyphenols are ubiquitous constituents of plants and possess a broad spectrum of biological activities such as immune system activities (11, 16-18), oxygen radical scavenging (19-21), antimicrobial (22), anti-inflammatory (10) and antitumor activities (10, 11, 23, 24).

We therefore tried to investigate the possible positive effects of natural antioxidants, propolis and its polyphenolic/flavonoid compounds on animal model psoriasis, induced by the Di-n-Propyl Disulfide irritant (PPD), during 5 days (25). Taking into account that the basic pathohistologic properties of psoriasis, such as hyperkeratosis, inflammatory infiltrates and cardiovascular changes, can induce significant changes in skin temperature, we used thermographic imaging to monitor inflammatory reactions and the effectivity of tested compounds (26-28). In fact, many studies have shown that blood flow in psoriatic lesions is up to 10 times greater than in clinically normal skin, which leads to skin temperature increase (29). Thermography is an efficient and simple method which successfully and reproducibly records thermal images of tested areas, and is widely used in diagnostics and treatment of diseases (e.g. malignant diseases like breast cancer and melanoma, and scleroderma, osteoarthritis, rheumatoid arthritis, psoriasis and psoriatic arthritis, Raynauds phenomenon etc...), as well as different experimental studies, including possible effects and application of new compounds (28, 30-35). The development of new technologies and state-of-the-art thermovision cameras enabled thermovision inspection in numerous branches of science and industry. A typical new generation camera is the ThermoTracer TH7102WL (NEC San-ei Instruments, 2004.), with its uncooled sensor elements, and with which all the measurements in this study were done. The remote control and measuring using the WEB (ThermoWEB) are based on an expansion of the measuring and operable usage possibilities of the thermovision camera NEC Thermo tracer TH7102WL, and are used for operating, controlling and assaying the

images obtained in the process of thermal imaging of people, animals, etc. Because of its specific quantitative display in the field of medical processing, ThermoMed is used exclusively for images done with the ThermoWEB system (36). The aim of this study was to employ thermography in assessing the inflammatory skin changes and effectivity of flavonoids on animal model psoriasis.

MATERIAL AND METHODS

Animals

The present study was approved by the ethical committee (Faculty of Science, University of Zagreb, Croatia). Male Swiss albino mice 2 to 3 months old, weighing 20 to 25 g, obtained from Department of Animal Physiology, Faculty of Science, University of Zagreb, were used in this study. The animals were kept in individual cages during the experiment and at 12 hours of light per day. They were fed a standard laboratory diet (4 RF 21, Mucedola, Settimo Milanese, Italy) and given tap water ad libitum. Maintenance and care of all experimental animals were carried out according to the guidelines in force in the Republic of Croatia (Law on the Welfare of Animals, N.N. #19, 1999) and carried out in compliance with the Guide for the Care and Use of Laboratory Animals, DHHS Publ. # (NIH) 86-123.

Di-n-Propyl Disulfide

We used the Di-n-Propyl Disulfide irritant manufactured by Sigma-Aldrich Corporation, USA.

Water-soluble derivative of propolis (WSDP)

Water-soluble derivative of propolis (WSDP) was prepared by the method described in our previous paper (16). Briefly, Croatian propolis from beehives kept at the outskirts of Zagreb was extracted with 96% ethanol, and then the preparation was filtered and evaporated to dryness in vacuum evaporator. The resultant resinous product was added to a stirred solution of 8% L-lysine (Sigma Chemie, Deisenhofen, Germany) and freeze-dried to yield the WSDP, a yellow-brown powder.

The chemical profile of propolis from the northern hemisphere, often named as »poplar-type« propolis can be characterized by three analytical parameters: total flavonol and flavone content, total flavanone and dihydroflavonol content, and total polyphenolic content. According to Popova *et al.* (37), spectrophotometric procedures for quantification of the three main groups of bioactive substances in propolis could be used for quality assessment of different propolis samples, and results of those analyses correlate with biological activity, especially in the »poplar-type« of propolis. The spectrophotometric assay based on the formation of aluminum chloride complex was applied for quantification of total flavones/flavonols and expressed as Quercetin equivalent. For the quantification of flavanones and dihydroflavonols in propolis, we used 2,4-dinitrophenylhydrazine method

(38). Total polyphenolic content was measured by the Folin-Ciocalteu procedure (19). WSDP contained: flavones and flavonols: 2.13%, flavanones and dihydroflavonols: 9.06%, total flavonoids: 11.19%, total polyphenols: 70.48%.

Ethanolic extract of propolis (EEP)

Raw Croatian propolis was collected by scraping it off from hive frames. The collected propolis samples were kept desiccated in the dark until analysis at room temperature. Ethanolic propolis extract (EEP) was prepared by the method described elsewhere (39). Briefly, propolis (10 g) was crushed into small pieces in a mortar and mixed vigorously with 34.85 mL 80% (V/V) ethanol during 48 h at $37 \pm 1^\circ\text{C}$. After extraction, the ethanolic extract of propolis was filtered through Whatman N₀.4 paper and then the extract was lyophilized. Spectrophotometric analysis showed that EEP contained: flavones and flavonols: 1.6%, flavanones and dihydroflavonols: 38.60%, total flavonoids: 40.20%, total polyphenols: 84.40%.

Flavonoids

Epigallocatechin 3-gallate (EGCG) manufactured by Shanghai Angoal Chemical Co., Ltd., China; Curcumin and Chrysin, manufactured by Sigma-Aldrich Corporation, USA; Quercetin, manufactured by Fluka Chemie GmbH, Switzerland.

Induction of psoriatic skin lesions and application of test components

Di-n-Propyl Disulfide was used as an irritant as previously reported (40). Topical 5% propolis preparation or flavonoid application are relevant for evaluating the efficacy of test components in *in vivo* animal model, as reported previously in a study by Noh *et al.* (41). Test components (100 mg) were dissolved in 2 mL acetone/olive oil (1:1). The day before the trials were about to start, all animals had had an area of ~3 cm shaved on their abdomen.

Briefly, 30 μL Di-n-Propyl Disulfide was painted onto the surface of the shaved abdomen of mice during 5 days. During the one-time topical application on the skin of the tested animals, we used 30 μL of solution, and the analysis lasted 5 days. The animals were divided into 16 groups: the first group consisted of mice that were topically treated only with Di-n-Propyl Disulfide; the second group consisted of mice that were simultaneously analysed with Di-n-Propyl Disulfide and the solution; the next 6 groups consisted of mice that were simultaneously treated with Di-n-Propyl Disulfide and the Water-soluble derivative of propolis, WSDP (group 3), Ethanolic extract of propolis, EEP (group 4), EGCG (group 5), Quercetin (group 6), Chrysin (group 7) and Curcumin (group 8); further 6 groups consisted of mice that were treated solely with compounds of WSDP (group 9), EEP (group 10), EGCG (group 11), Quercetin (group 12), Chrysin (group 13) and Curcumin (group 14); group 15 consisted of mice treated solely with the solution, and

group 16 of untouched mice (healthy control). All animals were numbered within the group, and every group was in its separate cage with the label of the group on it. During 5 days, every animal was topically treated with its specific compound on the abdomen. The thermographic imaging was run on the 3rd and 5th day of trials, specifically on animals' abdomens.

Thermoscan aquisition and data analysis

The room in which the thermographic imaging is done has to be air-conditioned, and the temperature of the measuring area must not oscillate more than $\pm 1^\circ\text{C}$. Cages with laboratory animals were brought into the room 2 hours before the measuring, so that the animals can acclimatize to the room temperature. The temperature difference between the rooms was not more than 3°C in favor of the room in which the animals were placed (barn).

All thermographic imaging was done by using the same television camera, in the same room, using the same computer programme, and by the same examiner Darko Kolarić, PhD, a research associate from the Center for Informatics and Computing, Ruđer Bošković Institute, who used the ThermoMED programme (36). Every image had a marked circle of the tested locus on the abdomen, and the lowest, the highest, and average sample temperatures were noted within the circle. The basic statistical analysis (the lowest temperature, highest temperature, mean, standard error, interquartile range) was run over those circled measurements, either single or in groups. A p value equal to 0.05 or less was considered statistically significant.

For the statistical analysis of the obtained data, the R Version 2.10.1 statistical programme was used (Development Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria, 2009.).

Count of the total number of cells present in the peritoneal cavity

After disinfection of the external abdominal region, each animal was inoculated with 3 mL of saline solution and after gentle agitation of the abdominal wall, the solution containing peritoneal cells was removed for cellular evaluation. The total number of cells present in the peritoneal cavity was determined by counting in a Bürker-Türk chamber.

Determination of the functional activity of macrophages

The functional activity of macrophages in the peritoneal cavity was determined by the spreading technique (18) Thus, 10^3 cells in 0.1 mL of the cellular suspension obtained from the peritoneal cavity were placed in duplicate over glass cover slips at room temperature for 15 min. The non-adherent cells were removed by washing with phosphate buffered saline, and the adherent cells

were incubated in culture medium 199 containing 10 nM HEPES at 37°C for 1 h. Following this, the culture medium was removed and the cells were fixed with 2.5% glutaraldehyde. Then the cells were stained with a 5% Giemsa solution and examined by optical microscopy where the percentage of spread cells were determined using a ×40 objective. The spread cells were those that exhibited cytoplasmic elongation, while the non-spread cells were rounded (18) Using an ocular grid, 200 macrophages were scored as either round or spread. An index of macrophage spreading (SI) was then calculated for each monolayer of each glass cover slips, as follows: SI = (number of spreading macrophages × 100) / 200 adherent cells, i.e. SI = % of spreading macrophages.

RESULTS

The thermographic imaging

Results of the thermographic imaging showed no significant difference between the tested groups in temperature changes on the skin with the psoriasiform lesions on the animal model. In comparing the first and second thermographic measurements, no statistically significant differences between the groups were noticed. Detailed data is presented in Figures 1 and 2. Examples of the first and second measuring of a single animal from the group tested with PPD combined with Curcumin are presented in Figures 3 and 4.

Effect of propolis preparations and their flavonoids on the total number of peritoneal cells and functional activity of macrophages in PPD-induced psoriatic mice

The results (Table 1) showed no significant difference between PPD and PPD + solvent (vehicle) while a statis-

tically significant reduction was observed in the total number of inflammatory cells in the peritoneal cavity of experimental psoriatic mice treated with WSDP ($P < 0.05$). Other psoriatic groups topically treated with test components showed a lower total number of inflammatory cells without statistical difference. In addition, a statistically significant difference exists between nonpsoriatic animals ($P < 0.05$) treated with test components and PPD group. Psoriatic mice treated with WSDP do not show any statistically significant differences as compared with nonpsoriatic mice.

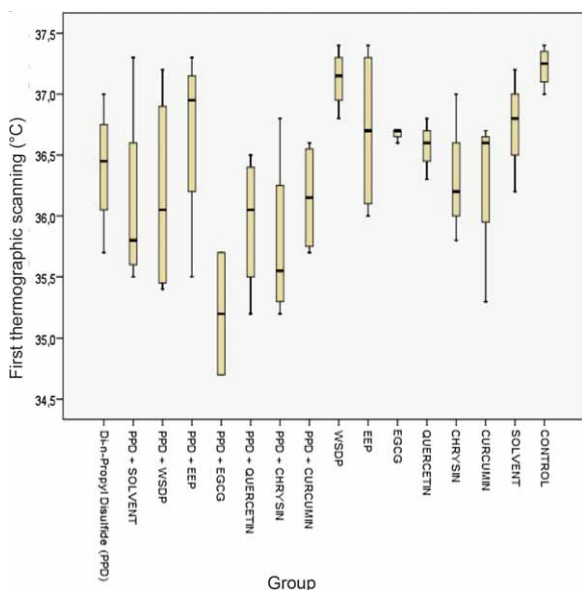


Figure 1. Results of the first thermographic measuring of tested animals topically processed with PPD and the compounds of propolis and/or flavonoids.

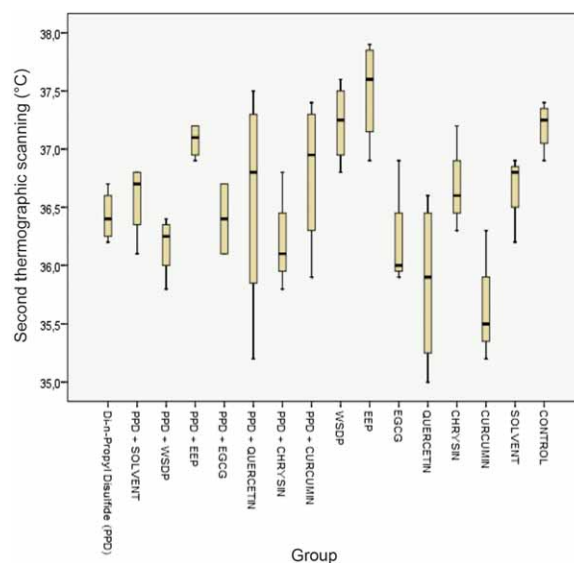


Figure 2. Results of the second thermographic measuring of tested animals topically processed with PPD and the compounds of propolis and/or flavonoids.

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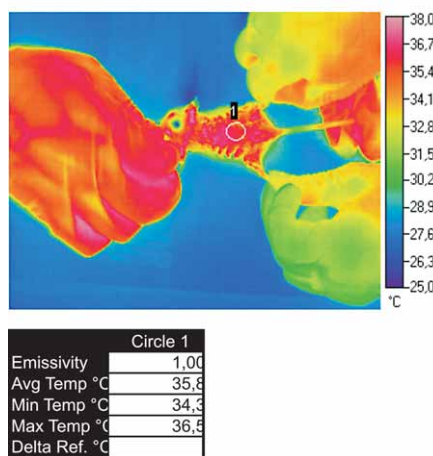


Figure 3. Thermographic display of the first measuring of abdominal skin, the first sample from the group processed with PPD combined with Curcumin.

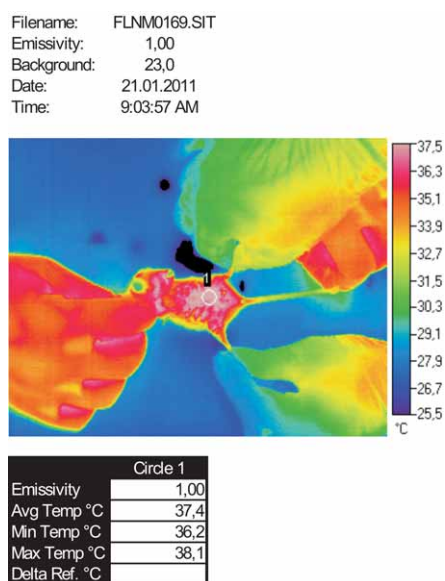


Figure 4. Thermographic display of the second measuring of the abdominal skin, the first sample from the group processed with PPD combined with Curcumin.

The results presented in Table 1 indicated statistically significant increase in the percent of activated macrophages in PPD mice ($P < 0.05$) compared with mice

treated with PPD in combination with test components. The percentages of functional activated macrophages in psoriatic mice treated with test components and non-psoriatic mice treated with test component were equal.

DISCUSSION

Today thermographic imaging has a great variety of uses in medicine, from research and diagnostics of many diseases to observing their path and assessing the effectivity of new compounds, especially in conditions characterized by an increased blood flow or inflammation development, such as psoriasis (29). However, although literature data on thermography use in psoriasis dates back to the 1970's, the thermographic systems of that era couldn't support quality research following today's standards (42). Ippolito *et al.* used thermography as a method of assessing the effectivity of cyclosporine treatment, and Zalewska *et al.* investigated the effectivity of thermography in assessing the activity of psoriatic lesions (30, 31).

Our thermographic imaging results showed no statistically significant difference between the tested groups in temperature changes on the skin with the psoriasiform lesions on the animal model. Furthermore, in comparing the first and second thermographic measurements, no statistically significant differences between the groups were observed. Considering the short irritation time, during

TABLE 1.

Total cells number and macrophage spreading in the peritoneal cavity of mice topically treated with PPD or propolis preparations and/or its flavonoids alone or in combination

Experimental group ^a	Total N ₀ of cells in peritoneal cavity × 10 ³ (mean ± SE)	Macrophage spreading index (%)
PROPIL DISULFID (PPD)	6796.87 ± 866.45 [#]	67.50 ± 2.63 [#]
PPD + solvent (vehicle)	5038.97 ± 1401.40	61.00 ± 1.30 [#]
PPD + WSDP	2871.09 ± 868.43*	45.50 ± 2.50*
PPD + EEP	4921.87 ± 1111.74	48.00 ± 2.45*
PPD + EGCG	4277.37 ± 1071.38	38.50 ± 4.99*
PPD + QUERCETIN	4335.94 ± 803.40	32.00 ± 6.88*
PPD + CHRYSIN	4628.90 ± 889.26 [#]	54.00 ± 4.55*
PPD + CURCUMIN	3808.59 ± 996.09	36.50 ± 6.24*
WSDP	6914.06 ± 564.77 [#]	48.50 ± 3.86*
EEP	2656.25 ± 563.37*	38.67 ± 5.81*
EGCG	4687.50 ± 993.34	54.67 ± 4.81
QUERCETIN	2890.62 ± 563.37*	46.00 ± 9.16
CHRYSIN	2109.37 ± 703.12*	36.67 ± 3.53* [▲]
CURCUMIN	1484.37 ± 434.98*	28.67 ± 5.81*
CONTROL +solvent (vehicle)	2031.25 ± 546.88*	42.67 ± 2.91*
CONTROL	1796.87 ± 340.54*	31.33±7.05*

^a Mice were treated topically with PPD or test components (WSDP, EEP, EGCG, quercetin, chrysin, curcumin) alone or in combination for 5 consecutive days.

* Statistically significantly different compared to PPD ($P < 0.05$)

[▲] Statistically significantly different compared to the corresponding combination with PPD ($P < 0.05$)

[#] Statistically significantly different compared to solvent control ($P < 0.05$)

which the inflammatory reaction probably could not developed, the obtained results can be considered as expected. Also, based on commonly known physiological properties of mice (their normal body temperature is between 36.5°C and 38°C), we conclude that significant changes in the inflammatory reaction could not be noticed. In their research, Maleszka *et al.* found that the hyperkeratonic lesions were hypothermic and therefore assumed that a serious skin infiltration, which leads to epidermal hypertrophy, could act as an isolation layer and cause a certain decrease in temperature (43). Zaleska *et al.* also divided psoriatic lesions into 4 groups, depending on their locality on the patients body, and noticed an increase in temperature in the chest and upper extremities in contrast to a decrease in the lower extremities (34). In our research we topically applied the tested compounds to the abdomen of the experimental animals, which is their lower body area, and therefore our results corresponded with the mentioned research. In addition, the total number of inflammatory cells in peritoneal cavity of psoriatic mice treated with test components were reduced without statistical significant difference, expect for the group treated with WSDP. The positive effect of test components was not only related to inflammatory cell infiltrates but also to significantly fewer macrophages spreading index in the abdominal cavity as a marker of inflammation induced by ROS generated by keratinocytes and activated leukocytes, mostly neutrophils (7). Dietary antioxidant compounds, including propolis and its flavonoids, offer some protection against psoriatic complications through their roles as inhibitors of inflammation and as free radical scavengers (10–12, 20, 21). There has been a growing interest in flavonoids which are widely distributed in plants and ingested by humans, due to their antioxidative, mild estrogenic, hypolipidemic, antibacterial, antitumor, anti-inflammatory, anti-diarrhoeal, antiulcer, antimutagenic, myocardial protecting, vasodilator, immunomodulator and hepatoprotective activities (10–12). Such remarkable spectrum of biochemical and cellular functions holds promise for the prevention and treatment of a variety of human disorders caused by oxidative stress, including psoriasis.

From the obtained data, we can conclude that the measured values were expected, which is to say that the thermal camera realistically registered thermal images and is an applicable method in assessing the inflammatory process in psoriasis. However, further investigation is necessary to confirm the obtained results.

CONCLUSIONS

The study implies that propolis and its flavonoids could be used as natural therapeutic drugs to prevent or treat psoriatic complications. Thermography seems to be a sensitive and realistic method, and can be applicable in examining of the inflammatory process in psoriasis and in evaluating the effectiveness of tested substances.

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