International Journal of Phytomedicine 9 (2017) 279-288
http://www.arjournals.org/index.php/ijpm/index

Original Research Article

ISSN: 0975-0185

Oxidative stress and inflammation caused by n-hexyl salicylate in mouse skin: the effectiveness of flavonoids

Nada Oršoli [1]*, Vedran Balta[1], Dyana Odeh[1], Maja Matakovi [1], Jadranka Skuri [2]

*Corresponding author:

Nada Oršoli

[1]Department of Animal Physiology, Faculty of Science, University of Zagreb, Zagreb, Croatia.
[2]Clinic of Anaesthesia and Intensive Care Unit, Sveti Duh General Hospital, Zagreb, Croatia

Abstract

Context and purpose: Reactive oxygen species (ROS) play a role in a numbered of degenerative conditions including psoriasis. Psoriasis is a chronic inflammatory disease who's the etiopathogenesis is not yet completely understood, and therefore there is no standardized therapeutical approach. Flavonoids, recognized as potent antioxidants, are multifunctional molecules that can act as anti-inflammatory and antiproliferative agents through the modulation of multiple signaling pathways. The present study was designed to investigate the protective role of flavonoids [quercetin, chrysin, curcumin or Epigallocatechin 3-gallate (EGCG)] against n-Hexyl salicylate (HXS)-induced oxidative stress and inflammation in skin. Anti-oxidative and anti-inflammatory effect of flavonoids is quantified by histopathological assessment of skin, measuring the levels of lipid peroxidation and glutathione (GSH) in the skin, total number of inflammatory cells in peritoneal cavity, macrophage spreading index, and hematological and biochemical parameters.

Main findings: Topically applied of n-Hexyl salicylate caused significant increase in lipid peroxidation and decrease in GSH, which is accompanied by an increase total number of inflammatory cells in skin and peritoneal cavity, functional activity of macrophages, and enzymatic activity of ALP and AST. In contrast, topically applied 5 % preparation of flavonoids (quercetin, chrysin, curcumin or EGCG) with HXS effectively prevented these alterations and maintained the antioxidant status.

Summary: The results suggest that flavonoid preparations can serve as a potent antioxidant and anti-inflammatory agents in psoriatic-like skin lesions, without toxic effects.

Keywords: Flavonoids; Mice; n-Hexyl salicylate; oxidative stress; Skin inflammation.

Introduction

The skin is an external organ that covers the entire body surface. It is responsible for the communication between an organism and the environment and is constantly subjected to exogenous stimuli. The main function of the skin is to protect the organism from environmental insults [1]. More exact, the skin is able to activate a defense mechanism aimed at pathogen elimination and tissue repair [2]. The skin is a biological interface with the environment, and is frequently and directly exposed to prooxidative stimuli including chemical oxidants, ultraviolet and visible irradiation, which are known to promote the generation of ROSs and lipid peroxides [2]. Initiation of the defense response is characterized by the infiltration of neutrophils and the release of several pro-inflammatory mediators, which starts the inflammatory process. If this inflammatory response is not appropriately regulated, an inflammatory skin disease can be triggered [3]. The most common inflammatory skin disorders include atopic dermatitis and psoriasis.

Psoriasis is a chronic inflammatory, proliferative skin disease characterized by pathological skin lesions due to various exogenous and endogenous factors. It is associated with a number of biochemical and immunological disturbances. The disease is believed to be primarily mediated by T-cells dendritic cells (DCs), natural killer T cells, and macrophages, and affects approximately 0.2% to 2% of the world population [4], These cells release cytokines that stimulate keratinocyte (KC) hyperproliferation and altered differentiation leading to widespread development of erythematous plaques with adherent silvery scales [5]. In psoriatic skin context, reactive species are generated by keratinocytes and activated leukocytes, mostly neutrophils [6, 7] and macrophages [8] which play a key role in inducing psoriasis-like skin disease. This suggests that cellular redox status plays a pivotal role in a healthy skin environment and that an imbalance between pro-oxidant and antioxidant mechanisms could result in skin diseases, including psoriasis [7,9].

DOI:10.5138/09750185.2064

This article is distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use and redistribution provided that the original author and source are credited.

Since, that currently available therapeutics to treat chronic inflammatory skin diseases are mostly ineffective and produce a plethora of side effects [3, 4], the search for more effective and safer treatment alternatives is necessary. Natural products derived from plants have long been used in folk medicine, and topically applied antioxidants can protect the skin from oxidative damage [7, 10–13]. Natural polyphenols, recognized as potent antioxidants, are multifunctional molecules that can act as anti-inflammatory [7, 10-13] and antiproliferative agents [14-17] through the modulation of multiple signaling pathways.
The present study was designed to investigate the role of flavonoids in reducing oxidative stress and inflammation in HXS-induced psoriasis like lesions in skin.

Material and Methods

Animals

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 tap water ad libitum. Maintenance and care of all experimental animals were carried out according to the guidelines in force in 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.

Irritant

Psoriasiform lesions was induced by topical application of irritant n-Hexyl salicylate ($C_{13}H_{18}O_3$) in an area of ~3 cm shaved abdomen of mice. N-Hexyl salicylate (99.00 %) were purchased from Sigma-Aldrich, St. Louis, MO, USA. Figure 1 shows the chemical structure of selected irritant.

Flavonoids

Epigallocatechin 3-gallate (EGCG) manufactured by Shanghai Angola Chemical Co., Ltd., China; Curcumin (CURC) and Chrysin (CHRYS), manufactured by Sigma-Aldrich Corporation, USA; Quercetin (QU), manufactured by Fluka Chemie GmbH, Switzerland. The chemical structure of flavonoids used in the experiment is shown in Figure 1.

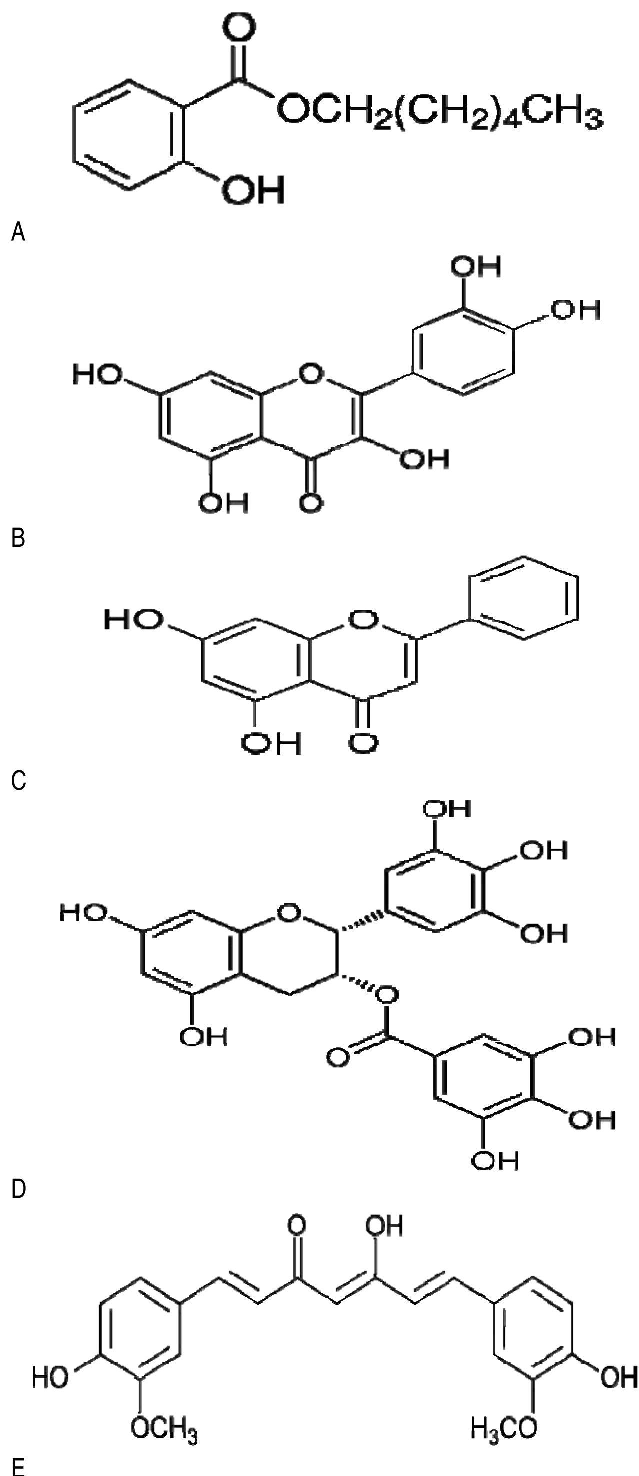

Figure 1. Chemical structures of selected materials used in experiment. (A) irritant n-Hexyl salycilate (HXS, $C_{13}H_{18}O_3$); (B) Quercetin ($C_{15}H_{10}O_7$); (C) Chrysin ($C_{15}H_{10}O_4$); (D) Epigallocatechin gallate (EGCG, $C_{22}H_{18}O_{11}$); (E) Curcumin ($C_{21}H_{20}O_6$)

Induction of psoriasis-like skin lesions and test components application

Hexyl salicylate were used as an irritant as previously reported [18]. Topical 5 % flavonoids application has relevance for evaluating the efficacy of test components *in vivo* in animal model, as was reported previously in the study by Noh et al. [19]. Test components (100 mg) was 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.

In the experiment we used seventy mice. The animals were divided into 10 groups (each group consisted of 7 mice: the first group consisted of mice that were topically treated only with n-Hexyl salicylate) and the acetone/olive oil (1:1) solution; the next groups (2 - 5) consisted of mice that were topically treated with irritant Hexyl salicylate and/or Epigallocatechin 3-gallate, quercetin, chrysin, curcumin), respectively; groups 6 -9 consisted of mice that were topically treated only with EGCG, QU, CRYS, or CURC, and control group treated with acetone/olive oil (1:1) solution (groups 11). 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 test compound on the abdomen.

Briefly, 30 µL Hexyl salicylate were painted onto their surface of 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.

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 well, 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 functional activity of macrophages

The functional activity of macrophages in the peritoneal cavity was determined by the spreading technique [15, 20]. Spread cells were those that exhibited cytoplasmic elongation, while the non-spread cells were rounded [15, 20] 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.

Pathology assessment

Mice were euthanized 5 days after induction of psoriasisform skin inflammation. Skin was removed and immediately fixed in a solution containing 10% formalin. Paraffin-embedded sections were cut at 0.5 µm and stained with H&E. The sections of the skin were examined histologically as described in paper Oršoli et al. [7].

Tissue preparations

Portions of skin samples of 100 mg were homogenized in 1 mL of 50 mM phosphate buffer (pH 7.0) by ultrasonic homogenizer SONOPLUS Bandelin HD2070 (Bandelin, Germany) using a probe MS73 (Bandelin, Germany) with a power of 10 %. Homogenates were centrifuged by Micro 200R centrifuge (Hettich, Germany) for 15 minutes at a speed of 10 000 rpm supernatant was used for measurements of glutathione and level of lipid peroxidation.

Lipid peroxidation

The presence of lipid peroxidation was determined by measuring the concentration of malondialdehyde (MDA) [7], which is one of the major products of lipid peroxidation. Malondialdehyde reacts with thiobarbituric acid and produces chromogen which was measured at 532 nm and 600 nm Libro S22 spectrophotometer (Biochrom, UK). The total absorbance was determined using the formula $A_{total} = A_{532} - A_{600}$. An array of known concentrations of tetrametoksipropan were used for creation of the calibration curve using the same protocol as for the homogenized samples. The concentration of lipid peroxides has expressed as a mg MDA/mL/mg protein.

Glutathione assay

The skin homogenate was used to measure the level of Glutathione [7] at 412 nm following the reaction between 5-5'-dithiobis[2-nitrobenzoic acid] (DTNB, Elman's Reagent) and GSH of sample supernatant. The results were calculated from the standard curve of array of dilutions of reduced glutathione measured by the same protocol.

Hematological analysis

The hematological analysis was performed on blood obtained from the tail vein of experimental and control mice on day 5 after topical treatment with test components. Blood was collected into EDTA tubes. The measurement of the leukocyte, erythrocytes, hemoglobin, hematocrit, MCV, MCH, MCHC and platelets was made in an automatic cell counter (Cell-Dyn® 3200, Abbott, USA).

Serum samples and biochemical determinations

Animals were treated with test components, blood samples were collected and centrifuged at 2200 rpm for 10 minutes. Serum was used for the determination of total protein, glucose, urea,

creatinine, bilirubin, alkaline phosphatase (ALP), aspartate and alanine aminotransferases (AST and ALT) and lactic dehydrogenase (LDH). Biochemical parameters were made using serum samples from both control and experimental groups in an automatic cell counter.

Peripheral blood micronucleus (MN) assay

Peripheral blood smear was prepared as described by Oršoli et al. [7, 15]. Blood was collected from tail tip, and smear was prepared on pre-cleaned slides. The smears were allowed to dry at room temperature and fixed in absolute methanol for 5 minutes. After fixation, slides were stained with acridine orange (AO) and washed twice with phosphate buffer (pH 6.8).

Statistical analysis

The data was presented as mean ± standard deviation (SD) of the representative experiment from two independent experiments. All data were analyzed by Kruscal-Wallis ANOVA. Further analysis of the differences between the groups was made with Multiple comparisons of mean ranks for all groups. Statistical analyses were performed using STATISTICA 12 software (StatSoft, Tulsa, OK, USA). The data were considered significant at $P < 0.05$.

Results

Effect of flavonoids on total number of peritoneal cells and functional activity of macrophages in HXS-induced psoriatic mice

Result (Figure 2) showed significant difference between HXS and combination HXS with tested flavonoids (EGCG, QU, CHRYS, CURC); a statistically significant reduction in the total number of inflammatory cells in the peritoneal cavity of experimental psoriatic mice treated with flavonoids were noticed (P <0.05). Psoriatic mice treated with test flavonoids do not show any statistically significant differences as compared with nonpsoriatic mice in the total number of inflammatory cells in the peritoneal cavity.

In addition, a statistically significant difference exists between nonpsoriatic animals (P <0.05) treated with test components and HXS group.

The results obtained in Figure 3 indicated statistically significant increase in the percent of activated macrophages in HXS-treated mice (P <0.05) compared with nonpsoriatic control mice and nonpsoriatic mice treated with CHRYS and CURC. In other groups, the percent of functional activated macrophages in psoriatic mice treated with test components and nonpsoriatic mice treated with test component were equal but lesser than HXS-induced psoriatic group.

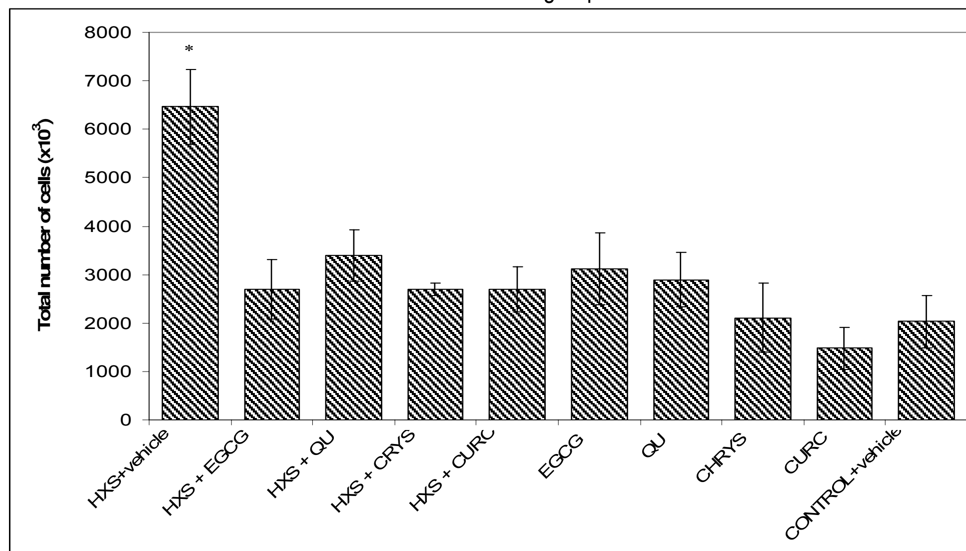

Figure 2. Total cells number in the peritoneal cavity of mice topically treated with HXS and flavonoids alone or in combination. Mice (n=7) were treated topically with HXS or test components (EEP, EGCG, quercetin, chrysin, curcumin) alone or in combination for 5 consecutive days. Data expressed as mean ± SE.*Statistically significantly different compared to solvent control (*P <0.05) Legends: HXS, n-Hexyl salycilate; EGCG, Epigallocatechin 3-gallate; QU, Quercetin; CHRYS, Chrysin; CURC, Curcumin

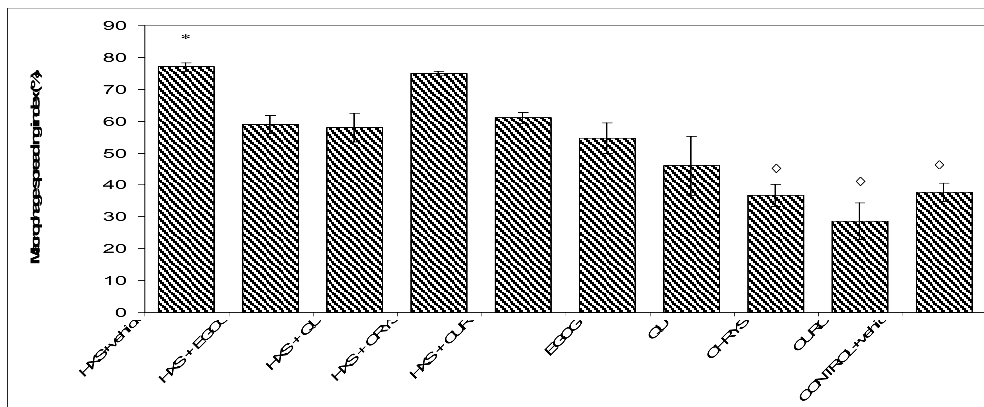

Figure 3. Macrophage spreading index in the peritoneal cavity of mice topically treated with HXS and flavonoids alone or in combination. Mice (n=7) were treated topically with HXS or test components (EGCG, quercetin, chrysin, curcumin) alone or in combination for 5 consecutive days. Data expressed as mean ± SE. Statistically significantly different compared to HXS (*P*<0.05)*Statistically significantly different compared to solvent control (**P* <0.05) Legends: HXS, n-Hexyl salycilate; EGCG, Epigallocatechin 3-gallate; QU, Quercetin; CHRYS, Chrysin; CURC, Curcumin.

Topical application of flavonoids reduces pathological markers of psoriasiform lesions

To evaluate the therapeutic effect of tested flavonoid on HXS-induce psoriasisform skin lesions we scored the clinical signs and symptoms such as intensity of inflammatory reaction, localization, number of mitosis and apoptosis and epidermal histology. HXS-treated skin exhibited extensive psoriasiform lesions, characterized by marked hyperkeratosis, mild parakeratosis, modest acanthosis, elongated rete ridges, and modest dermal inflammation, consistent with modest psoriatic-like dermatitis [7]. Topical application of HXS and EGCG, QU, CHRYS, or CURC significantly lowered intensity of inflammatory reactions and exhibited only slight hyperkeratosis, no parakeratosis, mild acanthosis, no elongated rete ridges, and mild dermal inflammation (Figure 4). Nonpsoriatic groups treated with tested flavonoids didn't show any changes in skin (data not shown).

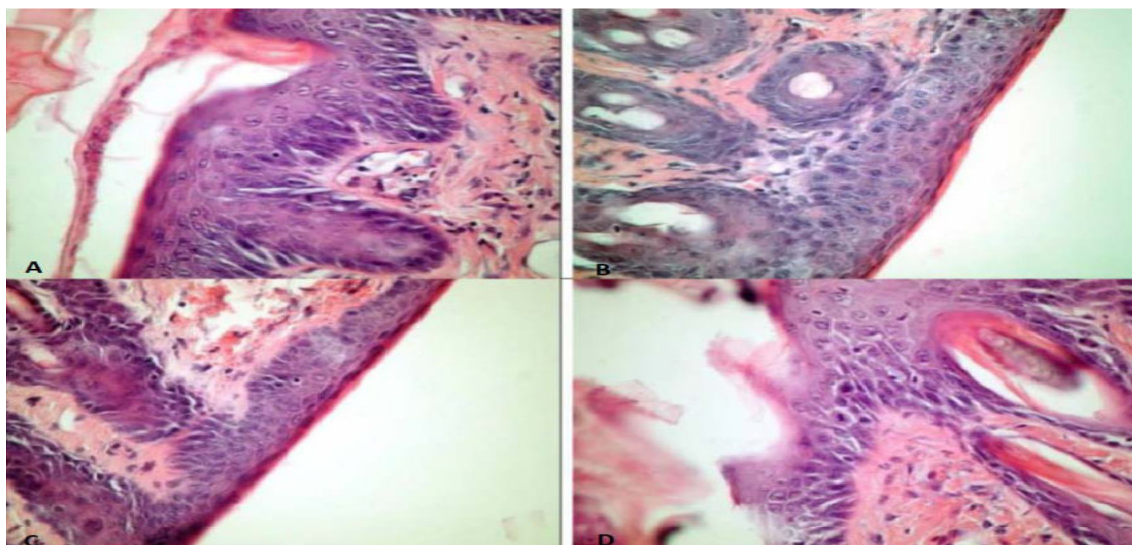

Figure 4. Changes of the total clinical severity score by topical of inflammatory reaction in skin of mice topically treated with HXS alone or in combination with flavonoids. Skin samples of mice topically treated with HXS (A), HXS+EGCG (B), HXS+ QU (C); HXS+CURC (D). Mice (n=7) were treated topically with HXS or test components (EGCG, quercetin, chrysin, curcumin) alone or in combination for 5 consecutive days.

Inflammation was scored on the following modified scale of 0–3: 0 - the absence of inflammatory reactions; 1 - weak inflammatory reaction (minimal inflammatory leukocyte infiltrates); 2 - moderately severe inflammatory reaction (mild inflammatory leukocytic and granulocytic infiltrates); 3 - severe inflammatory reaction and abundant inflammatory infiltrates (marked to severe inflammatory

infiltrates of primarily viable and degenerate neutrophils, eosinophils, lymphocytes, plasma cells admixed with cellular debris with altered or complete loss of normal histological structures). Description of the results is provided in the section "Results". The epidermal portion of the samples was photographed at a 400x magnification.
Legends: HXS, n-Hexyl salycilate; EGCG, Epigallocatechin 3-gallate; QU, Quercetin; CHRYS, Chrysin; CURC, Curcumin

Topical application of flavonoids markedly diminishes oxidative stress in HXS-induced psoriasis-like skin

ROS produced during the inflammatory process, in psoriasis, affects primarily the polyunsaturated fatty acid in a biological system, forming a lipid peroxidation product MDA, which serves as an important biological marker. MDA levels were significantly increased (P <0.01) in HXS-induced psoriasis-like skin compared to healthy control (Table 1).

Table 1. Lipid peroxidation levels in the skin tissues of mice topically treated with HXS and flavonoids alone or in combination

Experimental group[a]	MDA (nmol/mg skin protein)	Min value	Max value
HXS+vehicle	42.36 ± 3.70**	37.99	49.73
HXS + EGCG	13.54± 0.44	12.37	14.55
HXS + QU	16.83 ± 0.83*	16	18.49
HXS + CRYS	15.07 ± 0.40*	14.66	15.88
HXS + CURC	21.41 ± 1.45*	19.96	24.32
EGCG	5.61 ± 0.33	5.02	6.29
QU	12.11 ± 0.57	11.09	13.57
CHRYS	10.41 ± 1.80	6.87	12.80
CURC	7.95 ± 0.51	7.23	8.94
CONTROL+vehicle	9.80 ± 1.58	6.96	12.31

[a]Mice (n=7) were treated topically with HXS or test components (EGCG, quercetin, chrysin, curcumin) alone or in combination for 5 consecutive days. Data expressed as mean ± SE.Statistically significantly different compared to HXS (P<0.05; P<0.01) Statistically significantly different compared to the corresponding combination with HXS (P<0.05; P<0.01)*Statistically significantly different compared to solvent control ($^{*}P$ <0.05; ** P<0.01)Legends: HXS, n-Hexyl salycilate; EGCG, Epigallocatechin 3-gallate; QU, Quercetin; CHRYS, Chrysin; CURC, Curcumin; MDA, malondialdehyde

Topical application of HXS and EGCG, QU, CHRYS, or CURC reduced skin MDA activity significantly (P <0.05). Skin MDA levels in nonpsoriatic mice either treated or non-treated groups were decreased due to psoriatic mice, but MDA level between control and flavonoids did not reach any statistical significance (Table 1). GSH, which participates in the cellular system of defense against oxidative tissue damage, is determined as a key to antioxidant capacity. On the 5th day of HXS-induced psoriasis-like inflammatory skin, skin GSH levels was found to be decreased while treatment psoriatic mice with HXS+EGCG or HXS+QU increased GSH level (P<0.05). There were no significant changes in the levels of GSH between control and flavonoids nonpsoriatic treated mice (Table 2)

Table 2. Glutathione levels in the skin tissues of mice topically treated with HXS and flavonoids alone or in combination

Experimental group[a]	GSH(nmol/mg skin protein)	Min. value	Max. value
HXS+vehicle	0.37 ± 0.040*	0.32	0,45
HXS + EGCG	0,82± 0.037**	0.75	0.87
HXS + QU	0.64 ± 0.066**	0.50	0.81
HXS + CRYS	0.30 ± 0.018*	0.19	0.36
HXS + CURC	0.43 ± 0.064*	0.30	0.51
EGCG	0.22 ± 0.014	0.20	0.27
QU	0.23 ± 0.022	0.17	0.26
CHRYS	0.14 ± 0.047	0.14	0.17
CURC	0.13 ± 0.012*	0.090	0.15
CONTROL+vehicle	0.19 ± 0.014	0.15	0.22

[a]Mice (n=7) were treated topically with HXS or test components (EGCG, quercetin, chrysin, curcumin) alone or in combination for 5 consecutive days. Data expressed as mean ± SE. Statistically significantly different compared to HXS (P<0.05) Statistically significantly different compared to the corresponding combination with HXS (P <0.05; P <0.01)*Statistically significantly different compared to solvent control ($^{*}P$ <0.05; ** P <0.01) Legends: HXS, n-Hexyl salycilate; EGCG, Epigallocatechin 3-gallate; QU, Quercetin; CHRYS, Chrysin; CURC, Curcumin; GSH, glutathione

Effect of flavonoids on hematological and biochemical parameters in HXS-induced psoriatic mice

Tables 3 and 4 show hematological and biochemical parameters in control and experimental animals treated with irritant HXS and/or flavonoids. A statistically significant difference was observed in the number of leukocytes and MCH value in HXS + QU treated group and in MCH and MCHC values in HXS + CHRYS treated group compared to the healthy control group. MCHC value was also increased in the group HXS + EGCG compared to healthy controls. As shown in Table 4 irritant HXS elevated values of ALP and AST; a statistically significant difference exists between psoriatic mice (ALP, P <0.01 and AST, P <0.05) and healthy control animals. In psoriatic mice treated with CURC there was a reduction in level of AST (P <0.05) in relation to psoriatic animals without treatment. Animals treated with flavonoids (EGCG, QU, CHRYS, or CURC) do not show any difference compared to healthy controls, but there is a significant difference in ALP and AST between treatment with flavonoids and to appropriate combination of flavonoids and HXS (P<0.05)

Table 3 and 4 (separete file Landscape)

Flavonoids reduce the genotoxic effect of HXS

As shown in Figure 5, simultaneous application of HXS with flavonoids reduce the number of micronucleus in reticulocytes of peripheral blood as compared with HXS alone but without statistical significance. However, number of micronucleated cells in control mice was significantly lower (P <0.05) than that in psoriatic mice (HXS).

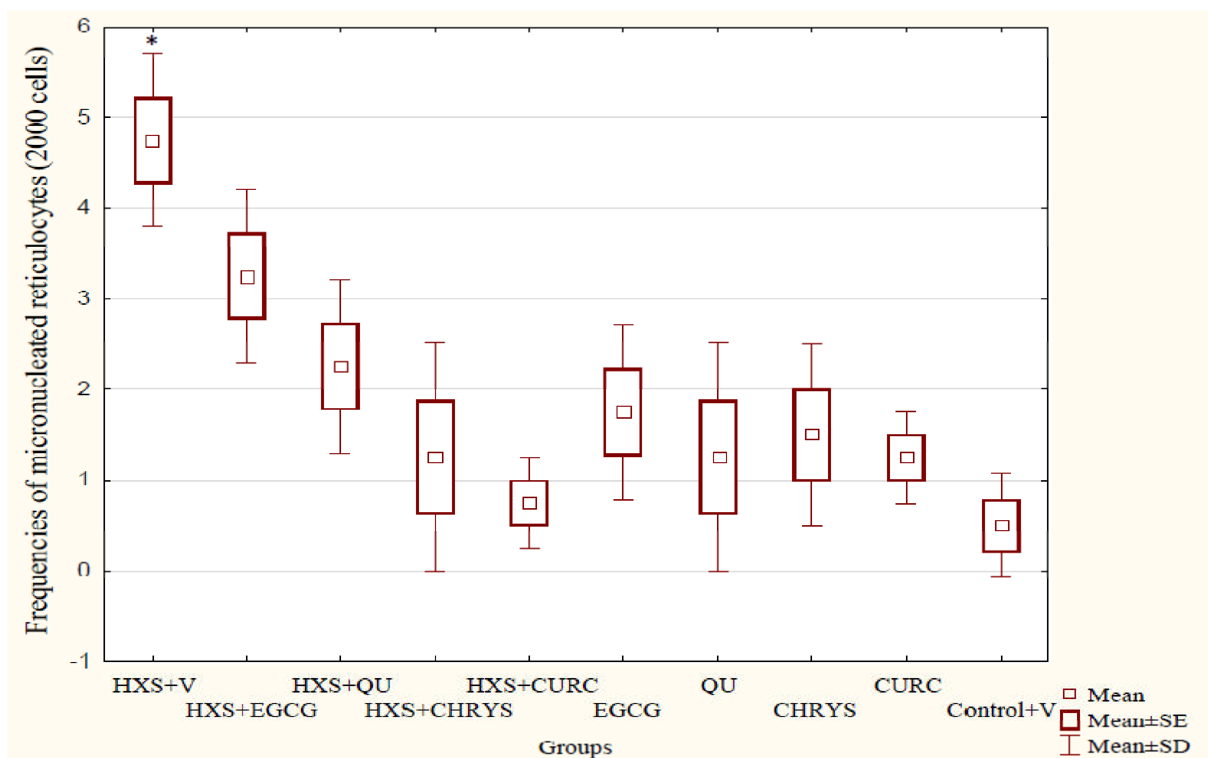

Figure 5 Frequencies of micronucleated reticulocytes (MN-RETs) in peripheral blood of mice topically treated with HXS and flavonoids alone or in combination. Mice (n=7) were treated topically with HXS or test components (EGCG, quercetin, chrysin, curcumin) alone or in combination for 5 consecutive days. Data expressed as mean ± SE.*Statistically significantly different compared to solvent control (*P <0.05) Legends: HXS, n-Hexyl salycilate; EGCG, Epigallocatechin 3-gallate; QU, Quercetin; CHRYS, Chrysin; CURC, Curcumin, V, vehicle

Discussion

In general, repeated exposure to HXS induces inflammation of the skin that results in the accumulation of inflammatory cells and the production of free radicals which cause lipid peroxidation (Table 1). Acute inflammation is characterized by classical symptoms, such as heat, redness, swelling and pain. Cellular infiltration represents an important feature in skin inflammation, and neutrophils are the predominant type of cells that infiltrate the area. These cells play a crucial role in cutaneous inflammation. In skin samples treated with HXS inflammatory infiltrate composed of lymphocytes, mononuclears, and some granulocytes and were localized predominantly in the dermis and epidermis were they showed mild hyperkeratosis and epidermal slightly elongated rete

ridges [7]. In contrast, flavonoids-treated skin samples exhibited slight hyperkeratosis, no parakeratosis, mild acanthosis, no elongated rete ridges, and lower dermal inflammation (Figure 4).

ROS produced during the inflammatory process, in psoriasis, affects primarily the polyunsaturated fatty acid in a biological system, forming a lipid peroxidation product MDA, which serves as an important biological marker of lipid peroxidation.

This study shows that the level of MDA increased significantly in HXS-induced psoriatic lesions in the skin of a mouse (Table 1). Plasma membranes of the skin cells in the psoriatic lesion have a significant increase in arachidonic acid, which is the natural substrate for synthesis of malondialdehyde (MDA), an end product of lipid peroxidation [7, 21, 22]. It seems that ROS that are produced by lipid peroxidation may activate phospholipase A2 and thus cause peroxidation of many mediators by arachidonic acid which finally metabolized to MDA [21, 22]. The increased levels of other reactive species, such as nitric oxide (NO•), superoxide anion ($O_2^{\bullet}$) and hydrogen peroxide (H_2O_2) have been determined in skin of psoriatic patients [22]. Hydrogen peroxide (H_2O_2) and superoxide anion ($O_2^{\bullet}$) can be generated by the action of the enzyme xanthine oxidase, which displays a higher activity in psoriatic epidermis [21,22]. Additionally, cytokines such as tumor necrosis factor alpha can contribute to H_2O_2 production [7, 11, 12, 23, 24].

Numbered authors demonstrated that in psoriatic skin context, reactive species are generated by keratinocytes and activated leukocytes, mostly neutrophils [6]. Our data obtained by histological analysis confirmed the above facts. Lactoferrin released by specific neutrophil granules can promote neutrophil-endothelial cell adhesion and, as a source of iron, may promote the Fenton reaction with the generation of the hydroxyl radical (OH•) [25]. Psoriatic skin is characterized by an advanced state of lipid peroxidation [21] and the depletion of intracellular GSH [7, 21, 26]. Previously, we have reported that high skin MDA levels and compromised levels of the antioxidant defense enzymes are observed at the time of diagnosis [7]. In present study we confirmed this hypothesis. Glutathione levels is decreased in HXS-induced psoriaticform skin while flavonoids can directly collect reactive radicals, prevent lipid peroxidation and induce the synthesis of cellular antioxidants such as GSH (Table 2) and the reactive radicals scavenging enzymes such as catalase and superoxide dismutase. It is known that GSH is an important constituent of intracellular protective mechanisms against various noxious stimuli, including oxidative stress.

It seems that the antioxidant treatment could be part of a more specific and effective therapy for the management of this skin disease; flavonoids can inhibit HXS-induced oxidative stress and inflammation in psoriatic skin. It appears that flavonoids through reduced level MDA and increase glutathione accelerate the healing process of damaged skin as indicated by histological analysis of skin. Antioxidants prevent oxidative injury of structural lipids and proteins contributing to barrier integrity, which is essential for healthy skin condition. This suggests that cellular redox environment plays a pivotal role in skin homeostasis and that skin disease could result from an imbalance between pro-oxidant and antioxidant stimuli.

The positive effect of test components was not only related to inflammatory cells in skin but also significantly reduced total number of inflammatory cells and macrophage spreading index in peritoneal cavity (Figure 2 and Figure 3) and inhibited the cellular migration of polymorphonuclear leukocytes in skin, an important step in the inflammatory process. Macrophage spreading index in the abdominal cavity may be important marker of inflammation induced by ROS generated by keratinocytes and activated leukocytes, mostly neutrophils [6]. In addition, flavonoids successfully reduce the values of ALP and AST and number of micronuclei in blood reticulocytes which was elevated in HXS-induced psoriasisform mice (Tables 3 and 4, Figure 5). Antioxidant compounds, including flavonoids, offer some protection against psoriatic complications through their roles as inhibitors of inflammation and as free radical scavengers [10-17, 20]. Mechanism involved in skin anti-inflammatory activity of the flavonoids have been proposed: i) antioxidant and/or antiradical properties; ii) the effect of nitric oxide (NO) on the metabolism; iii) the inhibition of lipoxygenase and the reduction of leukotriene B4 (LTB4) production; iv) inhibition of proinflammatory cytokine production v) preservation of the skin of microbes; vi) some flavonoid compounds have even been demonstrated to have the capacity to increase glutathione content, catalase and superoxidase activity. In addition, flavonoids have inhibitory effects on myeloperoxidase activity, NADPH-oxidase, ornithine decarboxylase, tyrosine-protein-kinase, and hyaluronidase [10-12]. It seems that flavonoids effectively reduce inflammation, possibly through inhibition of NF-κB, a redox-sensitive transcription factor that controls a number of genes involved in inflammation, proliferation, cell survival and apoptosis [11, 12, 27, 28]. Furthermore, flavonoids through increasing levels of glutathione (Table 2) accelerate the healing process of damaged skin. Their advantage compared to other drugs is as follows: 1. reduction of damage to healthy tissue; 2. faster healing of damaged tissue; 3. strong antioxidant, anti-inflammatory and antimicrobial activity; 4. low cost; and 5. easy availability.

Our data also suggest that development of a single suitable animal model could greatly facilitate research on the mechanism(s) of action that drive inflammatory and autoimmune processes associated with psoriasis [29, 30]. Such an animal model, for example, permit experiments using genetically uniform subjects within a controlled environment, as well as high-throughput screening of potential therapeutic agents. Given the many disparities between human and mouse skin, it cannot be expected that psoriasiform phenotypes in mice will mirror the human disease in every respect. For instance, relative to human skin, mouse skin has a denser distribution of hair follicles, thinner epidermis, and an underlying cutaneous muscle layer that is generally absent in humans [29, 30]. Additionally, mice possess subsets of inflammatory cells that are absent in humans [31, 32]. Despite

these challenges, psoriasis mouse models have already provided mechanistic insights into inflammatory skin diseases [7, 8, 33]. Inflammatory changes in the skin on a mouse model clearly can be monitored and using thermographic cameras that confirm our results presented in this paper [8, 33].

Conclusions

Taken together, these preliminary results support the use of flavonoids as an anti-inflammatory and antioxidative agent and open up new possibilities for its use in skin disorders. The protective effect of flavonoids against HXS-induced psoriatic lesions is probably based on the reduction of reactive oxygen species, increasing the levels of glutathione and reduced infiltration of macrophages and neutrophils. The protective role of the flavonoids against the inflammation induced by HXS in mice gives hope that flavonoids can control the production of ROS to reduce inflammation of the skin and achieve a similar protective effect in humans

Conflict of interest

There are no conflicts of interest to declare.

Acknowledgments

This work was supported by the Ministry of Sciences, Education and Sports of the Republic of Croatia project No. 119-0000000-1255.

Authors' contributions

NO - conceived and designed the study, participated in the data acquisition, analysed and interpreted the data, obtained the funding, supervised research personnel, performed statistical analysis and wrote the manuscript and made critical revision of the manuscript for important intellectual content. VB, DO, MM and JS - participated in the all data acquisition. JS - analysed and interpreted the histopathological activity of the test components. All authors reviewed the results and approved the final version of the manuscript.

References

[1]. Proksch E, Brandner JM, Jensen J-M. The skin: an indispensable barrier. Exp Dermatol. 2008; 17: 1063–1072.

[2]. De Benedictis C, Joubeh S, Zhang G, Barria M, Ghohestani RF. Immune functions of the skin. Clin Dermatol 2001; 19: 573–585.

[3]. Pietrovski EF, Magina MD, Gomig F et al. Topical antiinflammatory activity of Eugenia brasiliensis Lam. (Myrtaceae) leaves. JPP 2008; 60: 479–487.

[4]. Schön WP, Boencke WH. Psoriasis. N Engl J Med 2005; 352: 1899-912.

[5]. Conrad C, Boyman O, Tonel G, Tun-Kyi A, Laggner U, et al. Alpha1beta1 integrin is crucial for accumulation of epidermal T cells and the development of psoriasis. Nat Med. 2007; 13: 836–842.

[6]. Pelle E, Mammone T, Maes D, Frenkel K. Keratinocytes act as a source of reactive oxygen species by transferring hydrogen peroxide to melanocytes. J Invest Dermatol. 2005; 124: 793-797.

[7]. Oršoli N, Skuri J, Diki D, Stani G. Inhibitory effect of a propolis on di-n-propyl disulfide or n-hexyl salycilate-induced skin irritation, oxidative stress and inflammatory responses in mice. Fitoterapia. 2014; 93: 18-30.

[8]. Skuri J, Oršoli N, Kolari D, iki D, Benkovi V, Horvat-Kne evi A, Lisi i D, et al. Effectivity of flavonoids on animal model psoriasis – thermographic evaluation. Period boil. 2011; 113: 457-463.

[9]. Zhou Q, Mrowietz U, Rostami-Yazdi M. Oxidative stress in the pathogenesis of psoriasis. Free Radic Biol Med. 2009; 47: 891–905

[10]. Middleton E JR, kandaswami C, theoharides TC. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease and cancer. Pharmacol Rev. 2000; 52(4): 673-751.

[11]. Oršoli N, Baši I. Polyphenols from propolis and plants in control of allergy and inflammation. In: Oršoli N and Baši I, editors. Scientific evidence of the use of propolis in ethnomedicine. Ethnopharmacology - Review Book, Transworld Research Network, India. 2008. P. 311-336.

[12]. Oršoli N, Baši I. Propolis and plant flavonoids in human allergies and inflammations. In: Oršoli N and Baši I, editors. Scientific evidence of the use of propolis in ethnomedicine. Ethnopharmacology- Review Book, Transworld Research Network, India. 2008;337-376.

[13]. Oršoli N, Benkovi V, Horvat-Kne evi A, Kopjar N, Kosalec I, Bakmaz M, Mihaljevi , Bendelja K, Baši I. Assessment by survival analysis of the radioprotective properties of propolis and its polyphenolic compounds. Biol Pharm Bull. 2007; 30(5): 946-51.

[14]. Benkovi V, Oršoli N, Horvat-Kne evi A, Rami S, Diki D, Basi I, Kopjar N. Evaluation of the radioprotective effects of propolis and flavonoids in gamma-irradiated mice: the alkaline comet assay study. Biol Pharm Bull.2008; 31: 167-172.

[15]. Oršoli N, Benkovi V, Lisi i D, Diki D, Erhardt J, Horvat-Kne evi A. Protective effects of propolis and related polyphenolic/flavonoid compounds

against toxicity induced by irinotecan. Med Oncol. 2010; 27: 1346-1358.

[16]. Oršoli N, Baši I. Cancer chemoprevention by propolis and its polyphenolic compounds in experimental animals. In: Singh VK, Govil JN,.Arunahalam C. editors. Recent Progress in Medicinal Plants. Studium Press. Houston. 2007; 55-113.

[17]. García-Pérez ME, royer M, Duque-Fernandez A, Diouf PN, Stevanovic T, Pouliot R. Antioxidant, toxicological and antiproliferative properties of Canadian polyphenolic extracts on normal and psoriatic keratinocytes. J Ethnopharmacol. 2010; 132(1): 251-8.

[18]. Tornier C, Amsellem C, Fraissinette Ade B, Alépée N. Assessment of the optimized SkinEthic Reconstructed Human Epidermis (RHE) 42 bis skin irritation protocol over 39 test substances. Toxicol In Vitro.2010; 24(1):245-56.

[19]. Noh SU, Cho EA, Kim HO, Park YM. Epigallocatechin-3-gallate improves Dermatophagoides pteronissinus extract-induced atopic dermatitis-like skin lesions in NC/Nga mice by supressing macrophage migration inhibitory factor. Int Immunopharmacol. 2008; 8: 1172-1182.

[20]. Oršoli N, Kunšti M, Kukolj M, Gra an R, Nemrava J. Oxidative stress, polarization of macrophages and tumour angiogenesis: Efficacy of caffeic acid. Chem Biol Interact. 2016; 256:111-24

[21]. Yildirim M, Inaloz HS, Baysal V, Delibas N. The role of oxidants and antioxidants in psoriasis. J Eur Acad Dermatol Venereol. 2003; 17(1): 34-6.

[22]. Lin X, Huang T. Oxidative stress in psoriasis and potential therapeutic use of antioxidants. Free Radic Res. 2016; 50(6): 585-95.

[23]. Varga G, Ludwig S, Wixler V.Leite Dantas R, Masemann D, Schied T, Bergmeier V, Vogl T, Loser K. Brachvogel Macrophage-mediated psoriasis can be suppressed by regulatory T lymphocytes B. J Pathol. 2016; 240(3): 366-377.

[24]. Young, CN, Koepke, JI, Terlecky LJ, Borkin MS, Savoy LB, Terlecky SR. Reactive oxygen species in tumor necrosis fractor alpha activated primary human keratinocytes: implications for psoriasis and inflammatory skin disease. J Invest Dermatol. 2008; 128: 2606–2614.

[25]. Rocha-Pereira P, Santos-Silva A, Rebelo I, Figueiredo A, Quintanilha A, Teixeira F. The inflammatory response in mild and in severe psoriasis. Brit J Derm. 2004; 150: 917–928.

[26]. Kadam DP, Suryakar AN, Ankush RD, Kadam CY, Deshpande KH. Role of oxidative stress in various stages of psoriasis. Indian J Clin Biochem. 2010; 25(4):388-92.

[27]. Oršoli N, Saranovi A, Baši I. Direct and indirect mechanism(s) of antitumour activity of propolis and its polyphenolic compounds. Planta Med. 2006; 72(1): 20-27.

[28]. Oršoli N. A review of propolis antitumor action in vivo and in vitro. JAAS. 2010; 2(1): 1-20.

[29]. Gudjonsson JE, Johnston A, Dyson M, Valdimarsson H, Elder JT. Mouse Models of Psoriasis. J Invest Dermatol. 2007; 127: 1292-1308.

[30]. Boehncke WH, Schön MP. Animal models of psoriasis. Clin Dermatol. 2007; 25: 596–605.

[31]. Khavari PA. Modelling cancer in human skin tissue. Nat Rev Cancer. 2006; 6: 270–280.

[32]. Godfrey DI, Hammond KJ, Poulton LD, Smyth MJ, Baxter AG. NKT cells: facts, functions and fallacies. Immunol Today. 2000; 21: 573–583.

[33]. Skuri J, Oršoli N, Kolari D, iki D, Benkovi V, Lisi i D, Horvat Kne evi A., Maldini B. Thermography in Assessing the Inflammatory Skin Changes and Effectivity of Flavonoids on Animal Model Psoriasis. In: Jelena Bo ek, Mislav Grgi , editors. ELMAR-2011. Proceedings ELMAR-2011, Zadar; International Symposium Electronics in Marine –ELMAR. 2011; 313-316.