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Ex Vivo and In Vivo Antiinflammatory Evaluations of Modulated Flavanones Solutions †

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Abstract: Interest has developed in natural molecules due to their clinically proven effects on skin diseases. Flavanones display several biological activities, and recently have been the focus of studies due to their anti-inflammatory effect. To improve their pharmacological profile four flavanones (**A**, **B**, **C** and **D**) were synthesized by structural modification of one natural flavanone **1** (semi-systematic name: (2S)-5,7-dihydroxy-6-prenylflavanone) extracted from *Eysenhardtia platycarpa*. The hydroalcoholic flavanone solutions (FS) were assayed to investigated their anti-inflammatory effect on two in vivo cutaneous inflammation models. *Materials and methods*: the topical anti-inflammatory effect of FS were evaluated against models of 12-*O*-tetradecanoylphorbol acetate (TPA) induced mouse ear edema and arachidonic acid (AA) in rat ear edema. *Results*: The vinylogous cyclized derivative (flavanone **D**) caused edema inhibition in the TPA- induced models with an inhibition of 96.27 ± 1.93 %; equally effective and potent in inhibiting the mouse ear edema as Indometacine had been. In addition, the AA-induced increase in ear thickness was reduced the most by the topical application of modulated ether (flavanone **B**). *Conclusions*: The in vivo and histology results suggest that flavanones **B** and **D** are effective as a topical anti-inflammatory agents in inflammatory processes. Thus, this new compounds represents a promising agent for the management of skin diseases with an inflammatory component.

Keywords: flavanones; *Eysenhardtia platycarpa*; anti-inflammatory activity

1. Introduction

Skin inflammation is one of the most common skin problems. There are widespread dermatological diseases that include inflammatory responses in the skin and can present different ranges in severity. It is manifested by swelling, redness, heat, and pain in the affected tissue [1]. The most effective route of drug administration where higher concentration of the drug can be accomplished is the topical administration. Non-steroidal anti-inflammatory drugs (NSAID) are

currently used to treat inflammation, but severe adverse effects make these drugs unsuitable for chronic therapies [2]. Natural products for human skin problems has been used since ancient times. Recently, they have gathered considerable attention as new anti-inflammatory compounds because their long stablished usage promises the development of safe and effective medicaments [3]. Flavanones have been the focus of much research and development due to their several biological activities, included anti-inflammatory effects [4]. They have been a potential source in the search for lead compounds and biologically active components [5]. In recent times, five flavanones were isolated from a methanolic extract of *Eysenhardtia platycarpa* and they showed an anti-inflammatory effect during in vivo study [6–8]. Molecular modification represents one method used by medicinal chemistry for the rational variation of lead compounds with the aim of improving the efficacy, potency and the reducing of undesirable side effects [9]. Based on the above-mentioned interesting facts, the aim of this research was the in vivo anti-inflammatory evaluation of four flavanones derivatives in solution using one flavanone extracted from *E. platycarpa* as the starting material. The therapeutic efficacy of flavanones was checked by TPA edema mouse and AA (arachidonic acid) edema rat model. Also the histopathology in rat ear was observed. We explored the relationship between chemical structure and the therapeutic efficiency of flavanones.

2. Experiments

2.1. Extraction and Isolation of Plant Material

E. platycarpa leaves were collected from the municipality of Tetipac, Guerrero State (Mexico), and they are kept in the Faculty Herbarium of Facultad de Ciencias de la Universidad Nacional Autónoma de México. The plant material was authenticated by Professor Ramiro Cruz (Register number 1325). Experimental procedures detailed for the extraction, isolation, purification and structure elucidation of flavanone **1** (Figure 1), isolated from the methanolic extract of leaves of *E. platycarpa* have been described previously [8]. Briefly, the dried leaves of *E. platycarpa* (100 g) were extracted with MeOH (1000 mL). Then, the extracts were merged and concentrated *in vacuo*, to obtain the crude extracts. Next, the flavanone **1** was isolated by silica gel column chromatography. Finally, it was purified by direct thin-layer chromatography (TLC). The yellow powder precipitate obtained was characterized by comparison with previously published melting point data and with ^{1}H-NMR results [10].

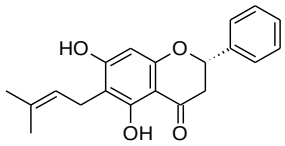

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Figure 1. Natural Flavanone **1** (2*S*)-5,7-dihydroxy-6-(3-methyl-2-buten-1-yl)-2-phenyl-2,3-dihydro-4*H*-1-Benzopyran-4-one) extracted from *E. platycarpa*.

2.2. Semi-Synthesis from Natural Prenylated Flavanone

The flavanones **A**–**D** were obtained following the method previously reported [10] to yield the derivatives flavanones (2*S*)-5,7-bis(acetyloxy)-6-(3-methyl-2-buten-1-yl)-2-phenyl-2,3-dihydro-4H-1-Benzopyran-4-one **A**; (2*S*)-5-hydroxy-7-methoxy-6-(3-methyl-2-buten-1-yl)-2-phenyl-2,3-dihydro-4H-1-Benzopyran-4-one **B**; (8*S*)-5-hydroxy-2,2-dimethyl-8-phenyl-3,4,7,8-tetrahydro-2H,6H-Benzo[1,2-b:5,4-b']dipyran-6-one **C**; and (8*S*)-5-hydroxy-2,2-dimethyl-8-phenyl-7,8-dihydro-2H,6H-Benzo[1,2-b:5,4-b']dipyran-6-one **D** (Figure 2).

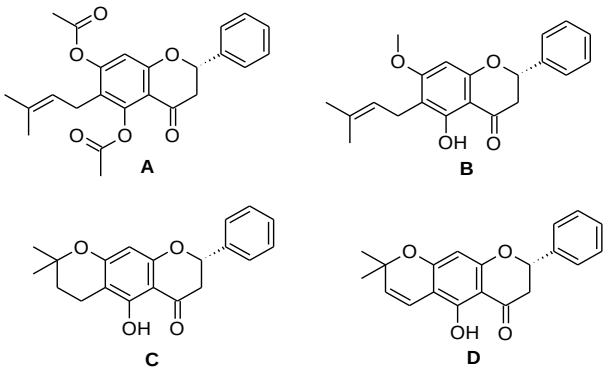

Figure 2. Derivative flavanones (2*S*)-5,7-bis(acetyloxy)-6-(3-methyl-2-buten-1-yl)-2-phenyl-2,3-dihydro-4*H*-1-Benzopyran-4-one (**A**); (2*S*)-5-hydroxy-7-methoxy-6-(3-methyl-2-buten-1-yl)-2-phenyl-2,3-dihydro-4*H*-1-Benzopyran-4-one (**B**); (8*S*)-5-hydroxy-2,2-dimethyl-8-phenyl-3,4,7,8-tetrahydro-2*H*,6*H*-Benzo[1,2-b:5,4-b'] dipyran-6-one (**C**); and (8*S*)-5-hydroxy-2,2-dimethyl-8-phenyl-7,8-dihydro-2*H*,6*H*-Benzo[1,2-b:5,4-b'] dipyran-6-one (**D**).

2.3. Anti-Inflammatory Testing

TPA-induced mouse ear edema was carried out using male Wistar CD-1 mice (n = 3 for each of the falvanone **A–D**, 20 to 25 g) based on the protocol previously described. Edema was induced by the topical application of 2.5 μg per ear of TPA (12-O-tetradecanoylphorbol-13-acetate) dissolved in 20 μL ethanol (10 μL each ear side). The standard drug indomethacin was used as reference. It was dissolved in acetone and applied to both sides of the right ear (1 mg/ ear) simultaneously with TPA. In the same way, 1 mg of each flavanone (**A–D**) was dissolved in acetone and applied on both sides of the right ear with TPA. Similarly, acetone were applied to both sides of the left ear. Four hours after the flavanone solutions were applied in one go, the animals were sacrificed by dislocating their neck. Subsequently, the left and right ears were perforated by punching bear (7 mm diameter), and the resulting tissues were accurately weight. The edema weight and inhibition percentage were assessed according to the following equation:

$$Inhibition\ (\%) = \frac{difference\ in\ weight\ of\ ear, control - difference\ in\ weight\ of\ ear, treated}{difference\ in\ weight\ of\ ear, control} \times 100 \quad (1)$$

The studies were conducted under a protocol in acordance with Mexican Official Norm for Animal Care and Handing (NOM-062-ZOO-1999) and, with the approval of the Academic Committee of Ethics of the Vivarium of the Autonomous University of the Morelos State of Mexico with number 0122013.

2.4. Histological Analysis

The anti-inflammatory histological effect of the flavanones (**A–D**) was assessed using arachidonic acid (AA) in rat ear edema model [11]. Adult male Sprague Dawley® rats were used (n = 5 for each flavanone solution, 200–240 g). Firstly, 5 mg of AA was dissolved in 1 mL of Phosphate buffered –saline solution. Then, 60 μL of AA solution was applied on both sides of the ears to induce the inflammatory process and left for 20 min of exposure. The animal in the positive control were treated only with AA solution. A solution diclofenac sodium (5 mg/mL) in EtOH/H_2O (7:3) was used as reference drug (ref). One animal group was treated only with the mixture EtOH/H_2O (7:3) without any flavanone (nFS). The animals, except the negative and positive control, were treated with 50 μL of the respective flavanone solution (FS **1**, FS **A**, FS **B**, FS **C** and FS **D**) 20 min after AA exposition and the treatment was effected for 20 min. To finish, the animals were sacrificed using carbon dioxide, following the recommendations for euthanasia of experimental animals from the European Commission [12]. Then, the ears were cut off and the tissues were rinsed with PBS pH 7.4, and left to stand overnight in 4 % buffered formaldehyde and embedded in paraffin wax at the end. Transversal

sections (5 μm) were stained with hematoxylin and eosin. The ear inflammation was observed under a light microscope (Olympus BX41 and camera Olympus XC50) on blind coded samples. Ears from the non-treated animals were used as the control condition.

Additionally to histology study, the stratum corneum hydration (SCH, arbitrary units AU) of rat ear was measured before and after the AA application and treatments with a corneometer CM825 (Courage & Khazaka electronics GmbH, Köln, Germany). Similarly, ears thicknesses were verified before and after the AA and the different treatments with a digital micrometer (Wisamic Digital Thickness Gauge 0–12.7 mm). The edema reduction was calculated by the following equation [13]:

$$\Delta Edema\ reduction = thickness\ after\ treatment - thickness\ before\ treatment \quad (2)$$

3. Results

3.1. Model of Mice Ear Inflammation Induced with TPA

The anti-inflammatoy study results of the flavanones are depicted in Table 1 as mean values ± the standard deviation (SD). The flavanone solutions showed good results of the anti-inflammatory efficacy studies. The flavanone natural **1** reveled a significant reduction of the dermal edema with inhibition percentage of 66.67 ± 1.55. However, only the flavanone modulated **D** showed an inhibition percentage of 96.27 ± 1.93 compared to the indomethacin of 91.35 ± 0.47.

In previous studies [14], the solutions of the flavanone natural **1** and the derivatives flavanones **A**-**D** were evaluated in ex vivo diffusional studies in Franz cells using human skin. This was to evaluate their intrinsic permeation and human skin retention (Table 1). The skin retention results of that study were correlated with the inhibition percentage of mouse edema induced by TPA. The function that best fitted to FS **1**,FS **C** and FS **D** was the first order with a correlation coefficient (R^2) equal to 1 (Figure 3).

Table 1. In vivo anti-inflammatory efficacy after TPA (12-*O*-tetradecanoylphorbol 13-acetate) induced mouse edema. Mean ± SD (n = 3).

Solutions	FS 1	FS A	FS B	FS C	FS D	Indometacin
% Inhibition	66.67 ± 1.55	10.27 ± 0.21	25.69 ± 0.52	40.61 ± 0.81	96.27 ± 1.93	91.35 ± 0.47
Human Skin Retention * (μg/g.cm²)	50.22 ± 7.51	321.52 ± 45.23	381.75 ± 57.26	23.78 ± 5.46	116.14 ± 17.24	

* Results of the permeation studies expressed by mean and SD (n = 3) reported previously [14].

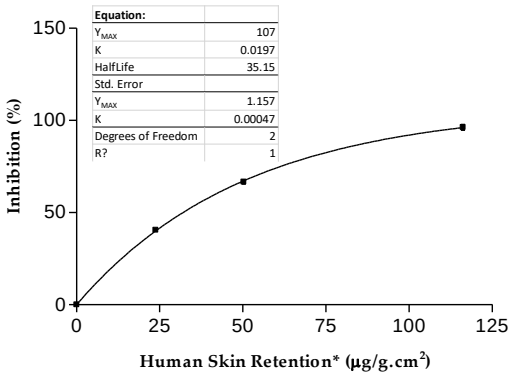

Figure 3. Correlated Function Inhibition vs Human Skin Retention.

3.2. In Vivo Rat Model and Anti-Inflammatory Response after Flavanones Solutions Treatment

The edema reduction, associated with the flavanones solutions **A**–**D** treatment in an in vivo ear rat model of inflammation induced by arachidonic acid, was evaluated by the difference of thickness compared to initial ear measures. In the same way, the nFS and a solution of diclofenac sodic were

evaluated. The results are despicted in Figure 4. The reference solution of diclofenac sodic reduced the ear thickness compared with the FS **D**. This treatment was also used for the flavanone solution **1** and nFS, producing the same effect in the edema ear. Thereupon, the flavanone **1** had not contributed with any additional anti-inflammatory effect compared with the excipients. Similarly, the FS **A** with the FS **C** presented almost the same edema reduction. On the other hand, it was interesting to note that the FS **B** had a higher efficacy since it reduced the thickness of the rat ears after 20 min of it application. Is important to point out that the ethanol per se can produces an effect of constriction and dehydration that it could translate in an anti-inflammatory action.

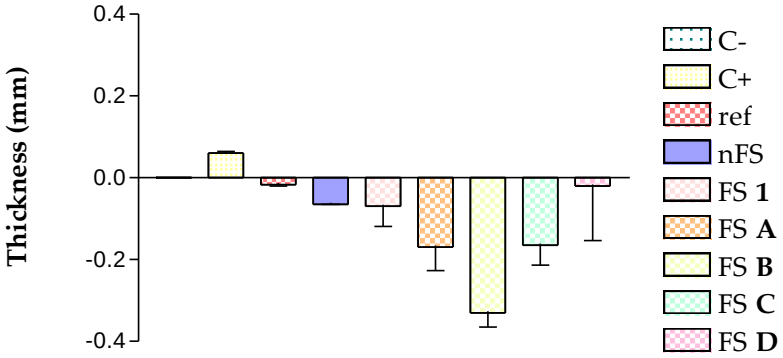

Figure 4. In vivo rat model anti-inflammatory response after FS (**A**–**D**) treatment in AA-induced edema model as the increment or decrement of thickness respect to initial conditions. Results are expressed as Mean ± SD (n = 5). C− = negative control, C+ = positive control, ref = reference drug, nFS = ethanol:water, FS = flavanone solution (**A**–**D**).

The skin hydratation data may also reveal the importance of the treatment with FS. With regards to this, the skin hydration of rat ears was measured and the results are shown in Figure 5 as the difference in stratum corneum hydration (SCH) after the formulation treatment on swelled ears and the basal SCH conditions as arbitrary units (AU). When the ears' hydration were measured, it was found that the skin's hydration changed with the application of all flavanone solutions. All of them reduced the skin's hydration except in the case of FS **B**, which increased the hydratation initial value.

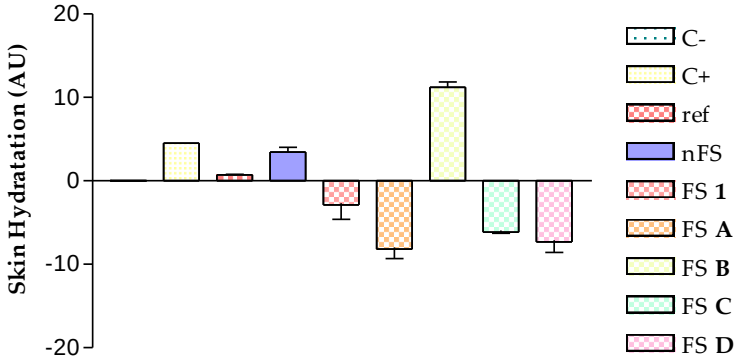

Figure 5. In vivo skin hydration after application of after FS (**A**–**D**) in AA-induced rat ear edema as the difference in hydration compared to initial conditions. Results are expressed as Mean ± SD (n = 5). C− = negative control, C+ = positive control, ref = reference drug, n FS = ethanol:water, FS = flavanone solution (**A**–**D**).

3.3. Histological Analysis

Histological analysis of ear sections was carried out for the assessment of the anti-inflammatory effect of the FS. Ears treated with AA (Figure 6) showed a mild inflammation characterized by edema, increased epidermal thickness, and infiltration of polymorphonuclear (PMN) leukocytes.

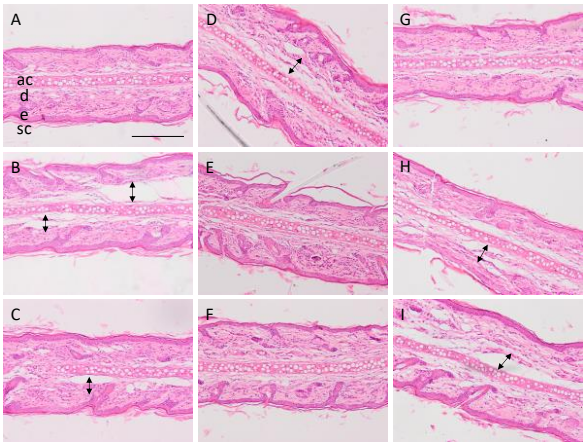

Figure 6. Representative micrographs of rat's ear (×100 magnification). (**A**): control −, (**B**): control +, (**C**): (nFS), (**D**): (ref), (**E**): (FS **1**), (**F**): (FS **A**), (**G**): (FS **B**), (**H**): (FS **C**), (**I**): (FS **D**). e-epidermis, d-dermis, ac-auricular cartilage, sc-stratum corneum. Arrows indicate presence of edema. Scale bar= 200μm.

4. Discussion

The results obtained in in vivo studies using different irritant agents (TPA and AA), in Table 1 and Figure 4, showed that the natural flavanone extracted **1** and the derivatives flavanones (**A**–**D**) have topical anti-inflammatory activity. All five FS were able to reduce the epidermal thickness present in the AA-treated ears. Furthermore, it is important to point out that the ethanol per se can produce constriction and dehydration effects that could translate into an anti-inflammatory action. Histological analysis of the ear of FS-treated animals confirmed the reduction of edema and stratum corneum swelling. Topical administration of reference drug slightly decreased these inflammatory indicators. The effect of the solutions diluent (EtOH:H_2O) was also assayed in order to observe its effect on inflammation and this showed some reduction of the edema. FS **B** (Figure 6G) was the best solution in reducing the inflammation induced by AA, showing better results than the reference. Another matter is that FS **1** and FS **A** also showed less edema although FS **1** showed greater presence of PMN. Furthermore, FS **C** and FS **D** were also able to reduce the edema but to a lower degree than the previous solutions. The before mentioned show us that the chemical modification of flavanone **1** played an important role in exercising an anti-inflammatory activity. Some SARs studies of flavonoids revealed that a planar ring system is vital in the flavonoid molecules so that they exhibit the anti-inflammatory action and that hydroxyl groups at 5- and 7- position of A-ring seem to be favorable to structural features to the inhibition of AA-induced mouse ear edema [15–17]. These facts could be the reasons why the molecules structure of flavanones **B** that posses hydroxyl group at 5-position and flavanone **D** with a more rigidid structure favored the anti-inflammatory effect in the models evaluated in this research. The natural flavanone **1** and derivatives flavanones **C** and **D** showed a correlation of accumulation into the human skin with their local anti-inflammation effect evaluated in TPA-induced model. This correlation could be owed to the fact that these three flavanones have similar physicochemical properties, such as area and molecular mass, as well as bond energy.

The working mechanisms of flavonoids as anti-inflammation agents are still not clearly defined. These kind of compounds may act on several molecular targets simultaneously. Different mechanisms may also be involved in the activity of each flavanone assayed. The anti-inflammatory effect of some plants used in skin illnesses could be explained by their obstruction effect in the

synthesis of inflammatory mediators such as leucotrienes and prostaglandins [18]. Although AA- and TPA-induced models are used to evaluate the anti-inflammatory effect, there are differences in the inflammation process that could help us to understand the inhibitory effects of the FS assayed on these models. It is know that AA produces only a modest increase in epidermal DNA synthesis, while TPA dramatically increases epidermal DNA synthesis and cell proliferation producing a long-lasting hyperplasia [19]. The edema caused by TPA can be reduced by cyclooxygenase (COX) and 5-lipooxigenase (5-LOX) enzyme inhibition, and the blockage of LTB4 receptors. Also, the protein kinase C (PCK) and groups of enzymes such as the mitogen activated protein kinases (MAPKs) and phospholipase A2 could be involved. It is reported that dexamethasone is a phospholipase A2 (PLA2) inhibitor more active against TPA-induced than AA-induced ear edema [20]. According to our obtained results, FS **D** anti-inflammatory activity, was greater against TPA-induced than AA-induced edema. Based on all these results, it can be suggested that FS **D** has a similar activity profile to PLA2 inhibitors. On the other hand, the ear edema caused by topical application of AA has been widely used to evaluate COX and 5-LOX inhibitors [21]. Considering that BW755C is a dual COX/LOX inhibitor and zileuton is a 5-LOX inhibitor, they showed a higher anti-inflammatory activity against AA-induced than TPA-induced edema in previous studies [20]. We hypothesize a dual COX/LOX inhibitory activity for FS **B**. However, this would need to be confirmed by additional studies.

5. Conclusions

Based on obtained results, it can be concluded that the derivatization of natural flavanone **1** to yield flavanones **A**, **B**, **C** and **D** allowed us to comprehend the importance of molecular structure to derive in an anti-inflammatory action on skin. The FS **B** and FS **D** showed better anti-inflammatory efficacy values. For these reasons, data obtained reflect that FS **B** and FS **D** could and should attract considerable attention for skin inflammatory treatment. Future studies can add to current findings leading to better understanding of these flavanones, with the potential to develop dermatological treatments and skin care products using these compounds. Moreover, the probable mechanism of action through which flavanones exert their effects could involve several targets, resulting in the reduction of important inflammatory mediators in the cutaneous tissue. Investigations into the mechanism of action of the anti-inflammatory activity and into the compounds responsible for the activity of flavanones must be completed.

Author Contributions: M.L.G.-R. and A.C.C. conceived and designed the experiments; P.B.-S., V.D., B.A.-C. M.J.R.-L. performed the experiments; P.B.-S., A.C.C. and M.J.R.-L. analyzed the data; M.L.G.-R. contributed reagents/materials/analysis tools; P.B.-S. wrote the paper. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

Abbreviations

The following abbreviations are used in this manuscript:

AA	Arachidonic acid
TPA	12-*O*-tetradecanoylphorbol-13-acetate
FS	Flavanone solution
nFS	Solution without any flavanone
TLC	Thin-layer chromatography
NSAID	Non-steroidal anti-inflammatory drugs

MeOH	Methanol
^{1}H-NMR	Proton nuclear magnetic resonance
ref	Reference drug
EtOH	Ethanol
H_2O	Water
PBS	Phosphate buffered saline
SCH	Stratum corneum hydration
AU	Arbitrary units
PMN	Polymorphonuclear
COX	Cyclooxygenase
LOX	Lipooxigenase
SAR	Structure activity relationship
DNA	Deoxyribonucleic Acid
PCK	protein kinase C
MAPKs	mitogen activated protein kinases
PLA2	phospholipase A2

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