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1 # The Biological Evaluation of Fusidic Acid and Its Hydrogenation
2 # Derivative as Antimicrobial and Anti-inflammatory Agents

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15 **Running title**: Difunctional Bioactivity of FA and Its Derivative

16 **Key words**: fusidic acid; derivative; antimicrobial; anti-inflammatory

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18 **Abstract**

19 Fusidic acid (WU-FA-00) is the only commercially available antimicrobial from
20 the fusidane family that has a narrow spectrum of activity against Gram-positive
21 bacteria. Herein, the hydrogenation derivative (WU-FA-01) of fusidic acid was
22 prepared, and both compounds were examined against a panel of six bacterial strains.
23 In addition, their anti-inflammation properties were evaluated using a
24 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced mouse ear edema model. The
25 results of the antimicrobial assay revealed that both WU-FA-00 and WU-FA-01
26 displayed a high level of antimicrobial activity against Gram-positive strains.
27 Moreover, killing kinetic studies were performed, and the results were in accordance
28 with the MIC and MBC results. We also demonstrated that the topical application of
29 WU-FA-00 and WU-FA-01 effectively decreased TPA-induced ear edema in a
30 dose-dependent manner. This inhibitory effect was associated with the inhibition of
31 TPA-induced up-regulation of pro-inflammation cytokines IL-1β, TNF-α and COX-2.
32 WU-FA-01 significantly suppressed the expression levels of p65, IκB-α, and p-IκB-α
33 in the TPA-induced mouse ear model. Overall, our results showed that WU-FA-00 and
34 WU-FA-01 not only had effective antimicrobial activities *in vitro*, especially to the
35 Gram-positive bacteria, but also possessed strong anti-inflammatory effects *in vivo*.
36 These results provide a scientific basis for developing fusidic acid derivatives as
37 antimicrobial and anti-inflammatory agents.

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39 ## Introduction

40 Over the past few decades, the appreciation of the key role of inflammation in
41 disease diagnosis, prevention and treatment has burgeoned (1, 2). Inflammation has
42 been defined as a complex biological response of vascular tissues to different types of
43 harmful stimuli (3, 4), such as damaged cells, irritants or pathogens. Inflammation has
44 also been linked to the release of pro-inflammatory cytokines (5, 6), including tumour
45 necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6) and
46 cyclooxygenase-2 (COX-2), all of which could be a sign of many diseases (4, 7).
47 Therefore, inflammation is a biological response wherein the organism attempts to
48 remove the injurious stimuli and initiate the healing process for the tissue; thus, it
49 could be regarded as a protective effect (4).

50 Currently, steroids and non-steroidal anti-inflammatory drugs are proverbially
51 used in clinical application as effective therapeutic anti-inflammatory agents (4).
52 Despite the widespread use of anti-inflammatory drugs, there may be some residual
53 risks of inflammation and the side effects of their long-term oral administration (8),
54 especially in infectious diseases, in which patients suffer from not only the
55 inflammatory responses but also pathogenic microorganism infections (9-11).

56 Fusidic acid (FA, WU-FA-00) (Figure 1), which has a steroid-like scaffold
57 structurally and is derived from the fungus *Fusidium coccineum*, is the only marketed
58 antibiotic from the fusidane family. Sodium fusidate, the sodium salt of fusidic acid,
59 was primary introduced into practice as an anti-staphylococcal therapy in 1962
60 (12-14). However, FA has a narrow spectrum of biological activity against some

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61 anaerobic gram-negative organisms and most gram-positive bacteria, especially the
62 staphylococci, including the methicillin-resistant *Staphylococcus aureus* (MRSA) and
63 coagulase-negative staphylococci (15-17). Although some antimicrobial activity and
64 reasonable anti-inflammatory effects have been discovered (18, 19), there is no
65 in-depth study of FA and its derivatives as potential anti-inflammatory agents.
66 Therefore, the therapeutic efficacy of FA and its derivatives as antimicrobial and
67 anti-inflammatory agents should be explored.

68 **< Insert Fig.1 Here>**

69 In the present study, the *in vitro* antimicrobial activities of fusidic acid and its
70 hydrogenation derivative (WU-FA-01) were investigated against six bacterial strains,
71 including five Gram-positive bacteria strains and one Gram-negative bacteria strain.
72 In parallel, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was used as an inducer to
73 explore both compounds' inhibitory activity on skin inflammation in a mouse ear
74 edema model (20-22). Moreover, immunohistochemical analysis was introduced to
75 reveal their inhibitory effects on the expression of TPA-induced TNF-α, IL-1β and
76 COX-2 in mouse ears. Furthermore, the anti-inflammatory mechanisms of FA and its
77 hydrogenation derivative were also discussed to gain insight into their effects.
78 Accordingly, fusidic acid or its derivatives, especially the hydrogenation compound,
79 may be developed as promising di-functional parent drugs, which could be underlying
80 anti-inflammatory and antimicrobial agents.

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82 # Results

83 **Chemistry**. To obtain the hydrogenation derivative of FA, structural modifications
84 (according to a previous study) were made at the double bond position of C-24 and
85 C-25 (23). The synthetic route is shown in Scheme 1. The 24, 25-dihydrofusidic acid
86 (WU-FA-01) was prepared by Palladium catalysed hydrogenation in quantitative
87 yielding. Its structure was confirmed by high-resolution mass spectrometry (HRMS),
88 CHNS-O elemental analyser, ^{1}H NMR and ^{13}C NMR, and it was in accordance with
89 the previous research (23).

90 **< Insert Scheme 1 Here>**

91 **Antibacterial activity**. The antibacterial activity of WU-FA-00 and WU-FA-01 were
92 tested against six microorganisms, including reference strains consisting of
93 Gram-negative bacteria and Gram-positive bacteria. All bacterial strains were cultured
94 in Muller Hinton agar at 37 ℃ overnight.

95 **Agar disk diffusion method**. The results of the antimicrobial activity of WU-FA-00
96 and WU-FA-01 against six different microorganisms are summarized in Table 1. Two
97 different concentrations were examined in this method. The sizes of the inhibition
98 zone indicated that the tested compounds with Gram-positive bacterial strains were
99 larger than those with Gram-negative strains, and both compounds showed dose
100 dependence. The inhibition zone diameter was in the range of 10.37±1.23 to
101 24.22±1.66 mm for Gram-positive strains. However, both WU-FA-00 and WU-FA-01
102 showed no inhibitory effect against the Gram-negative strains. Furthermore, the
103 screening of the antimicrobial potential of the two compounds revealed that reducing

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104 the double bond to a single bond at positions C-24 and C-25 could retain their
105 antimicrobial activities, specifically against the Gram-positive strains.

106 **<Insert Table 1 Here>**

107 **Broth microdilution method**. A microtiter plate dilution method was conducted to
108 determine the minimal inhibitory concentration (MIC) and the minimal bactericidal
109 concentration (MBC) in a 96-well plate. At the end of the incubation period, the plates
110 were evaluated for the presence or absence of bacterial growth. Each sample
111 concentration was tested four times against each microorganism. WU-FA-00, the
112 parent compound, was employed as a positive control against bacterial growth. The
113 final concentration of DMSO in the 96-plate well had no effect on bacterial growth.

114 WU-FA-00 and WU-FA-01, the two tested compounds, were found to be active
115 against the microorganisms studied, especially the Gram-positive bacteria. The MIC
116 and MBC values of the two compounds were determined according to the results of
117 the micro-dilution method (Table 2). The results suggested that WU-FA-01
118 (MIC=100-625 ng/mL, MBC=200-1250 ng/mL) showed activity similar to its parent
119 compound WU-FA-00 (MIC=100-625 ng/mL, MBC=312.5-1250 ng/mL) and
120 indicated that the double bond at C-24 and C-25 positions in WU-FA-00 structure has
121 little effect on its antibacterial activity. On the other hand, both WU-FA-00 and
122 WU-FA-01 were more effective against Gram-positive strains of *Staphylococcus* than
123 the Gram-negative strains, and this result is in accordance with the previous agar disk
124 diffusion studies and implied that WU-FA-01 could be developed as an active
125 antibacterial agent.

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126 **<Insert Table 2 Here>**

127 **Killing kinetic studies**. The time killing studies were carried out over a period of 24 h;
128 bacteria were exposed to the tested compound at four different concentrations, which
129 were determined according to their MICs. Figure 2 displays the time-kill curves of the
130 tested compounds for *Staphylococcus aureus* (ATCC 6538), *Staphylococcus albus*
131 (ATCC 29213), and *Staphylococcus epidermidis* (ATCC 12228). As shown in Figure
132 2, the MICs of the tested compounds were sufficient to inhibit almost all of the
133 bacterial growth but with a slight increase after 20 h during this assay. Similar to the
134 MBC results, no remarkable difference in the bacterial counts were found after
135 incubation for 24 h at MICs, and the results confirmed that the MBCs were highly
136 effective for killing bacteria. Furthermore, the bacterial population incubated with
137 DMSO or with the test compounds, which were lower than that of their MICs,
138 indicated less inhibitory action upon all selected bacterial strains. Moreover, Figure 2
139 also indicates that there is no difference in terms of killing kinetic between the two
140 compounds against all of the chosen Gram-positive microorganisms.

141 **<Insert Fig. 2 Here>**

142 **Inhibitory effects of WU-FA-00 and WU-FA-01 on TPA-induced edema in a**
143 **mouse ear model**. A TPA-induced ear edema mouse model was utilized to evaluate
144 the *in vivo* anti-inflammatory activities of WU-FA-00 and WU-FA-01. It has been
145 reported that TPA, which was normally adopted in this investigation model, is a
146 well-known promoter of skin inflammation. The average weight of the ear punches is
147 an important indicator that reflects the degree of skin edema when compared with the

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148 vehicle control group. As shown in Figure 3, the weight of mouse ear punches were
149 significantly increased to 160.90% after 6 h when 20 μL TPA (0.125 μg/μL in acetone)
150 was topically applied compared to the acetone-treated control group. Topical
151 application of 2, 4 and 8 μg/mL of WU-FA-00 after TPA treatment modestly inhibited
152 TPA-induced ear edema by 39.04%, 73.46%, and 83.83%, respectively, compared
153 with the TPA group. However, 2, 4 and 8 μg/μL of WU-FA-01 significantly decreased
154 the TPA-induced ear edema by 48.16%, 113.97% and 137.32%, respectively, in a
155 dose-dependent manner. Furthermore, the compound WU-FA-01 had a similar effect
156 on the positive control when it was used at a dose of 4 μg/mL (7.71 μmol/mL) with an
157 inhibition rate of 113.97%, whereas dexamethasone had an inhibition rate of 134.13%
158 at a dose of 2.5 μg/mL (6.37 μmol/mL). This result also suggested that WU-FA-01
159 had stronger protective effects than WU-FA-00 against TPA-induced skin
160 inflammation.

161 **<Insert Fig. 3 Here>**

162 **Inhibitory effects of WU-FA-00 and WU-FA-01 on the histological appearance of**
163 **mouse ears**. To investigate the role of WU-FA-00 and WU-FA-01 plays in the
164 histological appearance of a TPA-induced mouse ear model, both WU-FA-00 and
165 WU-FA-01 were evaluated by transdermal application. In this model, the right ears of
166 each group of mice were pretreated with 20 μL TPA (0.125 μg/mL in acetone), while
167 the controls were topically adopted with acetone. The treatment compounds (20 μL) at
168 three different concentrations were dissolved in acetone and used 5 min later.
169 Dexamethasone was used as a positive control at a concentration of 2.5 μg/mL (6.37

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170 μmol/mL) in acetone. After the ear tissues had been stained with H&E stain, as
171 shown in Figure 4, the histological appearances of the ear sections indicated that the
172 ears treated with acetone alone appeared normal in the epidermal layer without any
173 obvious lesion. However, the TPA alone group displayed significant swelling, which
174 was consistent with the results of the ear thickness and the ear punch weight (Figure
175 3). Moreover, the topical application of WU-FA-00 and WU-FA-01 could effectively
176 suppress signs of the inflammatory response, such as epidermal hyperplasia and dense
177 dermal leukocyte infiltration.

178 **<Insert Fig. 4 Here>**

179 **Inhibition of TPA-induced expression of TNF-α, IL-1β, COX-2**. To gain insight
180 into the molecular mechanisms by which WU-FA-00 and WU-FA-01 suppressed
181 TPA-induced skin inflammation, we examined the effects of both WU-FA-00 and
182 WU-FA-01 on the expression levels of pro-inflammation cytokines, including TNF-α,
183 IL-1β and COX-2, in mouse ears using immunohistochemical analysis. As shown in
184 Figure 5, the expression level of pro-inflammation cytokines (TNF-α, IL-1β and
185 COX-2) were dramatically elevated 6 h after topical stimulation with TPA, which was
186 apparently down-regulated in a dose-dependent manner by treatment with WU-FA-00
187 and WU-FA-01. However, the pro-inflammation cytokines levels of TNF-α, IL-1β and
188 COX-2 between the treated groups and control group in the TPA-induced mouse ear
189 model, were increased 20.37, 31.47 and 3.16-fold. Firstly, 2, 4 and 8 mg/mL of
190 WU-FA-00 retarded TPA-induced overexpression of TNF-α by 5.1%, 52.9% and
191 80.7%, while 2, 4 and 8 mg/mL of WU-FA-01 retarded TPA-induced overexpression

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192 of TNF-α by 20.5%, 56.5% and 82.5% relative to the TPA group, respectively (Figure
193 5A). Secondly, WU-FA-00 at 2, 4 and 8 mg/mL greatly reduced the overexpression of
194 IL-1β by 36.0%, 59.8% and 86.1%, while 2, 4 and 8 mg/mL of WU-FA-01 greatly
195 reduced the overexpression of IL-1β by 35.9%, 65.6% and 86.6%, respectively
196 (Figure 5B). Thirdly, 2, 4 and 8 mg/mL of WU-FA-00 retarded TPA-induced
197 overexpression of COX-2 by 8.3%, 26.7% and 45.8%, while 2, 4 and 8 mg/mL of
198 WU-FA-01 retarded TPA-induced overexpression of COX-2 by 12.1%, 31.6% and
199 56.6%, compared to the TPA group, respectively (Figure 5C). Therefore, the above
200 results indicate that WU-FA-00 and WU-FA-01 could markedly suppressed the
201 overexpression of pro-inflammation cytokines, which was in accordance with the
202 previous results of ear weight and ear thickness (Figure 3) and histological changes
203 (Figure 4) in this TPA-induced ear model.

204 **<Insert Fig. 5 Here>**

205 **Inhibition of TPA-induced expression of p65, IκB-α, and p-IκB-α**. The activation
206 of NF-κB is significant for the regulation of TNF-α, IL-1β and COX-2 overexpression
207 in the TPA-induced inflammatory model. Therefore, whether WU-FA-00 and
208 WU-FA-01 could affect the NF-κB signalling pathway was determined by
209 immunohistochemical analysis. As illustrated in Figure 6A, the results revealed that
210 p65 was markedly suppressed by the treatment of WU-FA-00 and WU-FA-01, in
211 which both WU-FA-00 and WU-FA-01 were more active at a concentration of 8
212 mg/mL. Moreover, the results also confirmed that the transcriptional activity was
213 markedly up-regulated in the TPA-induced model but was inhibited by WU-FA-00

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214 and WU-FA-01 at 8 mg/mL.

215 The signalling pathway of IKK is involved in the induction of pro-inflammation

216 cytokines *via* the modulation of NF-κB. Thus, it is necessary to gain insights into the

217 IκB-α/p-IκB-α pathway in this TPA-induced model. From the immunohistochemical

218 analysis in Figure 6B and Figure 6C, the levels of IκB-α and p-IκB-α in the TPA

219 group were significantly increased. However, they could be suppressed by WU-FA-00

220 and WU-FA-01 in a dose-dependent manner, especially at a higher concentration of 8

221 mg/mL. These results imply that both WU-FA-00 and WU-FA-01 might block the

222 activation of NF-κB through interfering with p65 and IκB-α/p-IκB-α to inhibit the

223 expression of the TPA-induced pro-inflammation cytokines of TNF-α, IL-1β and

224 COX-2.

225 **<Insert Fig. 6 Here>**

226 **Discussion**

227 Both WU-FA-00 and WU-FA-01 not only possessed excellent *in vitro*

228 antimicrobial activities for Gram-positive *Staphylococcus* strains but also exhibited

229 effective inhibition effects in the TPA-induced mouse ear model. Thus, both

230 WU-FA-00 and WU-FA-01 could be considered as inhibitors of inflammation

231 induced by bacterial infection. Moreover, it is possible that inflammation is frequently

232 triggered by bacterial infection. The inhibitory effect of WU-FA-01 against

233 microorganisms and TPA-induced skin inflammation is similar to its parent

234 compound. The inhibitory effect of both compounds was associated with the

235 suppression of TPA-stimulated pro-inflammation cytokines of TNF-α, IL-1β and

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236 COX-2. This study provides a further understanding of the anti-inflammatory
237 properties of WU-FA-00 and WU-FA-01. Therefore, the results of this study implied
238 that fusidic acid and the dihydro- analouge could be developed as di-functional agents,
239 which possess both antimicrobial and anti-inflammatory activities.

240 ## Methods and materials

241 **Chemicals**. Fusidic acid (FA) was purchased from Macklin Co., Ltd., with over 98%
242 purity. 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was ordered from Sigma-Aldrich
243 Chemical Co. (Saint Louis MO). TPA, FA and its derivative were dissolved in
244 acetone to produce the desired concentrations of each compound. TNF-α and IL-1β
245 antibodies were purchased from Bioss biotechnology Co. (Beijing, China) and
246 Beyotime Biotechnology Co. (Beijing China). The silica gel (200-300 mesh) used in
247 the column chromatography was supplied by Inno-chem Co., Ltd. (Beijing China).
248 All other reagents and solvents were purchased from Adamas Reagent Ltd. (Shanghai
249 China) or other commercial suppliers in their analytically or chemically pure forms
250 and used without purification. TLC was performed on pre-coated silica gel F_{254} plates
251 (0.25 mm; E. Merck); the starting material and the product were detected by either
252 viewing under UV light or treating with an ethanolic solution of *p*-anisaldehyde spray
253 followed by heating. The antimicrobial activity was determined by using a
254 Multi-model Plate Reader (Infinite 200).

255 **Preparation of WU-FA-01.** A 100-mL glassware was flamed-dried and allowed to
256 cool in a desiccator before use. FA (1.0 g, 1.94 mmol) was dissolved in 50 mL of
257 ethanol. 5% palladium on calcium carbonate (0.1 g, 0.19 mmol) was added to the

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258 reaction. Moreover, the reaction mixture was subjected to a vacuum-nitrogen purge
259 and left to stir under a hydrogen atmosphere for 3 h. TLC was eluted in the mixture of
260 Ethyl acetate: Petroleum ether=1:2 (V:V) and stained in *p*-anisaldehyde. R_f values of
261 the starting material were 0.14, and the product was 0.17. Then, the reaction mixture
262 was filtered through a pad of Celite and washed with ethyl acetate. The solvent was
263 removed under vacuum to obtain a white solid. Yield: >98%.
264 **Microorganisms and culture conditions**. Six bacterial strains were used for the
265 bioassays, including three Gram-positive species, *Staphylococcus aureus* (ATCC
266 6538), *Staphylococcus albus* (ATCC 29213), *Staphylococcus epidermidis* (ATCC
267 12228), *Listeria monocytogenes* (ATCC 19115), and *Streptococcus pneumoniae*
268 (ATCC 49619), and one Gram-negative species, *Escherichia coli* (CMCC 44102). All
269 bacteria were maintained on Mueller-Hinton agar, and the cultures were stored at 4 ℃
270 and sub-cultured every week.
271 **Agar disk diffusion method**. The antimicrobial activity of WU-FA-00 and
272 WU-FA-01 were determined according to the standard agar disk diffusion method
273 with a slight modification (24-26). A 0.5 McFarland (1×10^7 to 1×10^8 CFU/mL)
274 concentration of the bacterial suspension was uniformly inoculated onto Mueller
275 Hinton agar (MHA) solidified in 120 mm Petri dishes. Once the dishes were prepared,
276 6 mm-diameter discs of filter paper containing 5 μL of the examined compound,
277 which had been diluted ten times with dimethyl sulfoxide (DMSO), were pressed
278 gently against the surface of the agar. Discs containing WU-FA-00 were used as the
279 positive control, while DMSO was used as the negative control. The dishes were

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280 incubated in a constant temperature incubator at 37 ℃ for 24 h. The inhibition zone
281 (IZ) diameter was measured by a vernier caliper. All of the experiments were
282 performed in triplicate.

283 **Broth microdilution method**. The minimum inhibitory concentration (MIC) and the
284 minimum bactericidal concentration (MBC) were determined by a microdilution
285 method in 96-microwell plates according to Clinical and Laboratory Standards
286 Institute (CLSI), with a slight modification (27, 28). A dilution series of the test
287 compounds were obtained with DMSO as the solvent by two-fold serial dilution. The
288 final concentrations of the test compound were 1~400 μg/mL. Each well received 5
289 μL of a specific concentration of the compound and 195 μL of Mueller Hinton broth
290 inoculated with the test microorganism (1.5×10^5 CFU/mL); the final concentration of
291 the test compound reached 0.025~10 μg/mL. WU-FA-00 and DMSO were treated as a
292 positive control and a negative control, respectively. The microplates were incubated
293 in a bacteriological oven for 24 h at 37 ℃, and the drug susceptibility results were
294 monitored by measuring the absorbance at 600 nm using a Multimodel Plate Reader
295 (Infinite 200). The lowest concentration without visible growth was defined as the
296 MIC.

297 The minimum bactericidal concentrations (MBCs) were determined based on the
298 MIC results (29, 30): serial sub-cultivation of a 5 μL aliquot near the MIC in
299 microtiter plates containing 195 μL of Mueller Hinton broth per well; incubation for
300 24 h at 37 ℃. The lowest concentration of antimicrobial agent that killed at least 99.9%
301 of the starting inoculum was defined as the MBC endpoint, which was determined as

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302 the lowest concentration with no visible growth by measuring the absorbance at 600
303 nm using a Multimodel Plate Reader (Infinite 200). All experiments were conducted
304 in triplicate.
305 **Killing kinetic studies**. The killing kinetic assay on the Gram-positive strains (27, 31,
306 32), including *Staphylococcus aureus* (ATCC 6538), *Staphylococcus albus* (ATCC
307 29213), and *Staphylococcus epidermidis* (ATCC 12228), was performed against
308 WU-FA-00 and WU-FA-01 in 96-microwell plates, respectively, and four different
309 concentrations (0, 25, 100, 312.5 ng/mL) of each compound were tested. The
310 microplates were incubated for 24 h at 37 ℃, and the growth of bacteria was
311 monitored by measuring the absorbance at 600 nm using a Multimodel Plate Reader
312 (Infinite 200) every 1 h.
313 **Animals, diets and treatments**. Female Kunming mice approximately 22-25 g were
314 used in the TPA-induced *in vivo* model. All animals were supplied by the
315 Experimental Animal Centre of Guangdong Province. They were maintained at 25±1℃
316 with standard mouse chow diet and tap water *ad libitum* and were kept on a regular
317 light-dark cycle with 50% relative humidity. All the animal experiments were
318 performed according to the Ethical Regulations on Animal Research of Southern
319 Medical University (Approval Documents: SCXK/20130002).
320 **TPA-induced skin inflammation in mouse**. The mice were divided into nine groups:
321 each group consisted of six mice, including a blank group, a TPA group, a
322 dexamethasone group, and six groups for WU-FA-00 and WU-FA-01. In the mouse
323 ear edema model, 20 μL of acetone vehicle was topically applied to the right ear, and

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324 20 μL of the treatment compounds at three different concentrations, which were
325 dissolved in acetone, were used 5 min later after 20 μL of TPA (0.125 μg/mL in
326 acetone) was previously applied to induce the inflammation model (33, 34).
327 Dexamethasone at a concentration of 2.5 μg/mL (6.37 μmol/mL) in acetone was used
328 as the positive control. Then, all of the mice were maintained at a standard condition
329 and sacrificed 6 h after TPA treatment. Two ear punches (9 mm in diameter) from the
330 right and left ears were then harvested immediately and weighted; the left ear was
331 used for comparison. All experiments were carried out in compliance with the
332 relevant laws and institutional guidelines, which were all approved by the Southern
333 Medical University (Approval Documents: SCXK/20130002).
334 **Histological appearance of mouse ears**. The right ear punches were fixed in 10%
335 neutral buffered formalin, decalcified in EDTA buffer, subjected to a series
336 progression of dehydration and embedded in paraffin. Sections of 9 mm were cut by
337 using a microtome and were mounted on colourfrost microslides (VWR scientific,
338 Edmonton, Alberta, Canada). The sections were dried overnight and stained with
339 haematoxylin and eosin (H&E) in accordance with the classical methods of histology.
340 Images of the sections representing each treatment group were observed under a
341 microscope (Olympus, Japan) to evaluate the damage of ear tissue.
342 **Scoring the expression of biomarkers**. Each histologic type of lesion in the
343 TPA-induced ear model was scored independently by two experienced investigators
344 who were not aware of the identity of the specimens (×200) (33, 35). The staining
345 intensity was scored as follows: 0, no staining; 1+, faint; 2+, moderate; and 3+, strong.

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346 1+, 2+ and 3+ were recorded as 1, 2 and 3 points, respectively. The staining extent
347 was graded as follows: 0, no staining; 1+, ≤25% of cell positive; 2+, 26% to 50% of
348 cells positive; and 3+, ≥51% of cells positive.
349 **Immunohistochemical detection of TNF-α, IL-1β, COX-2, p65, IκB-α, and**
350 **p-IκB-α expression**. The ear punch tissues were fixed in formalin, and paraffinised
351 sections of 5 μm thickness were incubated with 1.2% H_2O_2 in PBS to quench the
352 endogenous peroxidase activity in order to minimize nonspecific staining. Then, the
353 sections were washed three times (5 min each) with 1X TBST (0.05% Tween-20).
354 Subsequently, the primary antibody of a proliferating cell nuclear antigen was diluted
355 100 times, applied to each section and left overnight at 4 ℃. The sections were
356 washed with PBS and incubated with a biotin-conjugated horseradish peroxidase
357 antibody (1:200) for 1 h at room temperature. Finally, peroxidase was detected using
358 the 3, 3-diaminobenzidine tetrahydrochloride reaction, which produced a brown label
359 in the epidermal tissue. The cells that stained positive for TNF-α, IL-1β and COX-2
360 were counted in the section of the mouse ear using the Image-Pro Plus (Version 6.0)
361 software (33). The results were expressed as the number of stained cells.
362 Immunohistochemical analysis of p65, IκB-α and p-IκB-α were also conducted to
363 gain insight into the signalling pathway of WU-FA-00 and WU-FA-01 in the
364 TPA-induced mouse ear edema model.
365 **Statistical analysis**. The results are expressed as the mean ± standard error (SE) or
366 standard deviation (SD). Statistical comparisons among groups were performed by
367 using Dunnett's multiple test. Statistical significance was defined by a *P* value of <

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368 0.05.

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489 # Author Contributions

490 Performing the experimental work and drafting the manuscript: (PPW, XPC).
491 Performing the bioactivity test: (PPW, HH, TRW, BRT, YYZ). Performing the
492 experimental statistical analysis (PPW, XPC, MG, DLL, JZ, ZJS). The director as well
493 as the designer of the manuscript: (WDH, XTX, KZ). The project coordinator: (WDH,
494 SAW, PMO, SQZ).

495 # Competing of interests

496 The authors declare no competing financial interests.

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Table 1 Antibacterial activity of WU-FA-00 and WU-FA-01 expressed in the inhibition zone (mm).

Compound	WU-FA-00		WU-FA-01	
Structure				
Concentration (μg/mL)	25	12.5	25	12.5
Dosage (ng)	125	62.5	125	62.5
Bacterium	Inhibition Zone (mm)			
Staphylococcus aureus (ATCC 6538)	23.09±0.37	20.60±0.40	23.08±1.13	20.05±0.30
Staphylococcus albus (ATCC 29213)	21.48±0.36	18.75±0.44	19.93±1.03	17.10±0.14
Staphylococcus epidermidis (ATCC 12228)	24.22±1.66	20.04±0.35	22.91±0.84	17.42±0.40
Listeria monocytogenes (ATCC 19115)	11.63±1.33	10.44±0.82	11.49±0.41	10.55±1.20
Streptococcus pneumoniae (ATCC 49619)	8.22±0.79	7.37±1.23	9.39±0.90	7.52±1.11
Escherichia coli (CMCC 44102)	ND	ND	ND	ND

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502 ND, not determined.

503 **Table 2 Antibacterial activities of WU-FA-00 and WU-FA-01 expressed in MIC**

504 **and MBC (ng/mL).**

Bacteria		Compounds			
		WU-FA-00		WU-FA-01	
		MIC	MBC	MIC	MBC
		ng/mL		ng/mL	
Gram-positive bacteria	*Staphylococcus aureus* (ATCC 6538)	100	312.5	100	200
	Staphylococcus albus (ATCC 29213)	625	1250	625	1250
	Staphylococcus epidermidis (ATCC 12228)	625	1250	625	1250
	Listeria monocytogenes (ATCC 19115)	＞1250	＞1250	＞1250	＞1250
	Streptococcus pneumoniae (ATCC 49619)	＞1250	＞1250	＞1250	＞1250
Gram-negative bacteria	*Escherichia coli* (CMCC 44102)	ND	ND	ND	ND

505 MIC (ng/mL), minimum inhibitory concentration, i.e., the lowest concentration of the compound

506 that completely inhibits the growth of bacteria.

507 MBC (ng/mL), minimum bacterial concentration, i.e., the lowest concentration of the compound

508 that completely kills the bacteria.

509 ND, not determined.

WU-FA-00 — 1) 5% palladiumon calcium carbonate 2) H_2 / Ethanol → WU-FA-01

510

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511 **Scheme 1 Synthesis of the hydrogenation derivative of fusidic acid (WU-FA-01).**

512 Figure legends

513 **Fig. 1 Chemical structure and conformation of fusidic acid (WU-FA-00).**

514 **Fig. 2 Time-kill curves for the three Gram-positive strains**. Including
515 *Staphylococcus aureus* (ATCC 6538) (A and D), *Staphylococcus epidermidis* (ATCC
516 12228) (B and E), and *Staphylococcus albus* (ATCC 29213) (C and F), exposed to
517 four different concentrations of WU-FA-00 (Figure 2A, 2B, 2C) and WU-FA-01
518 (Figure 2D, 2E, 2F) according to their respective MICs (n=4).

519 **Fig. 3 Inhibition effects of WU-FA-00 and WU-FA-01 on TPA-induced edema in**
520 **mouse ears.** The right ears of all animals (n=6) were topically treated with 20 μL of
521 acetone (vehicle control) or WU-FA-00/WU-FA-01 (2, 4 or 8 μg/μL) in 20 μL of
522 acetone after the application of TPA (2 μg/μL) in 20 μL of acetone. The mice were
523 sacrificed 6 h after the TPA treatment. Both ear punches (9 mm in diameter) were
524 immediately taken, and then, they were weighted and measured. The data from each
525 group are expressed as the mean ± S.D. Compared to the TPA induced model group
526 (n=6), $^{*}P<0.05$, $^{**}P<0.01$ (Dunnett's multiple comparison test).

527 **Fig. 4** H&E **staining for histological changes of TPA-induced mouse ears treated**
528 **with an acetone control, TPA, WU-FA-00 and WU-FA-01 at different**
529 **concentrations.** The data from each group are expressed as the mean ± S.D.
530 Compared to the TPA-induced model group (n=6), $^{*}P<0.05$, $^{**}P<0.01$ (Dunnett's
531 multiple comparison test). Magnification 200×.

532 **Fig. 5 Effects of WU-FA-00 and WU-FA-01 treatment on pro-inflammation**

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533 **cytokines levels of TNF-α (Figure 5A), IL-1β (Figure 5B) and COX-2 (Figure 5C)**
534 **in a mouse ear model.** Mouse ears treated with acetone, TPA, WU-FA-00 and
535 WU-FA-01 at different concentrations were analysed by immunohistochemical
536 staining. The data are shown as the mean ± SD. Compared to the TPA-induced model
537 group (n=6), $^{*}P < 0.05$, $^{**}P < 0.01$ (Dunnett's multiple comparison test).
538 Magnification 200×.

539 **Fig. 6 Inhibitory effects of WU-FA-00 and WU-FA-01 on the phosphorylation of**
540 **p65 (Figure 6A), IκB-α (Figure 6B) and p-IκB-α (Figure 6C) in the TPA-induced**
541 **ear model.** Mouse ears treated with acetone, TPA, WU-FA-00 and WU-FA-01 at
542 different concentrations were analysed by immunohistochemical staining. The data
543 are shown as the mean ± SD. Compared to the TPA-induced model group (n=6), $^{*}P <$
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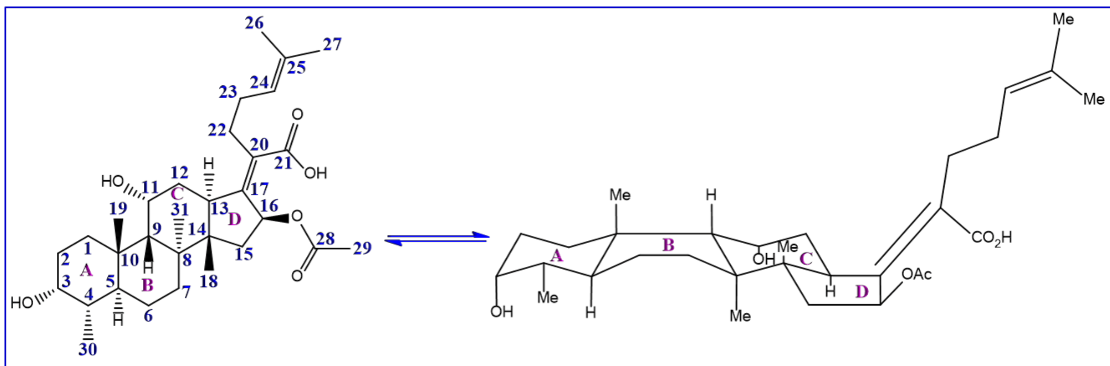
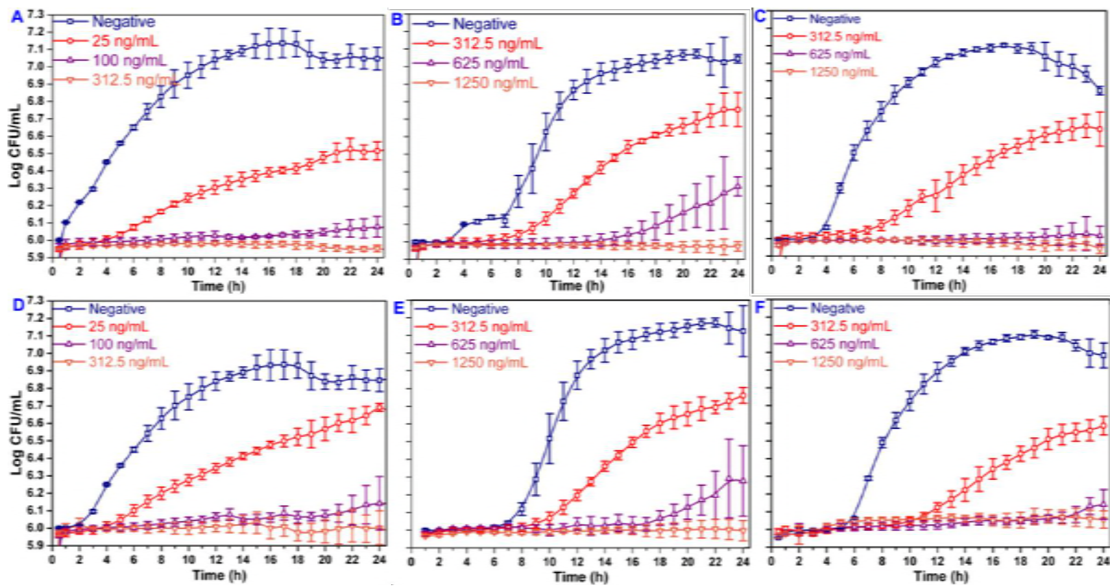
546 # Figures



547

548 **Fig. 1 Chemical structure and conformation of fusidic acid (WU-FA-00).**



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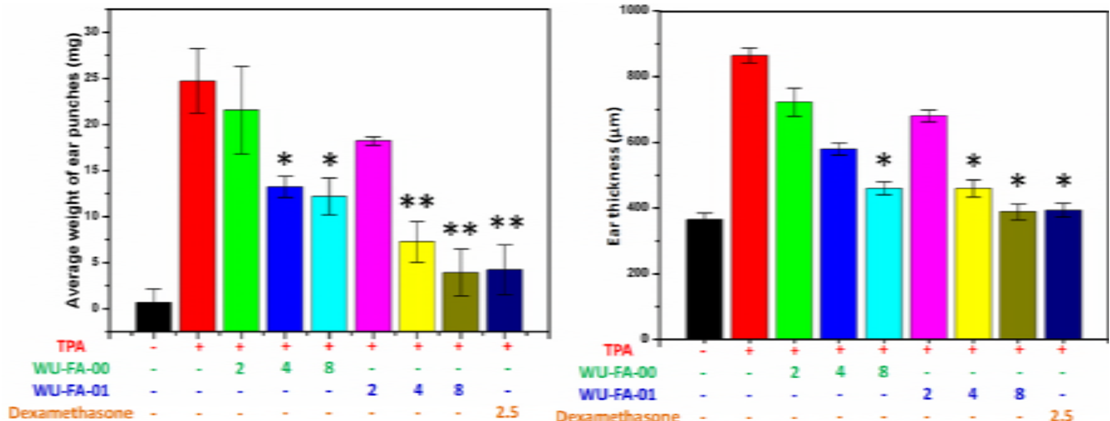
555 

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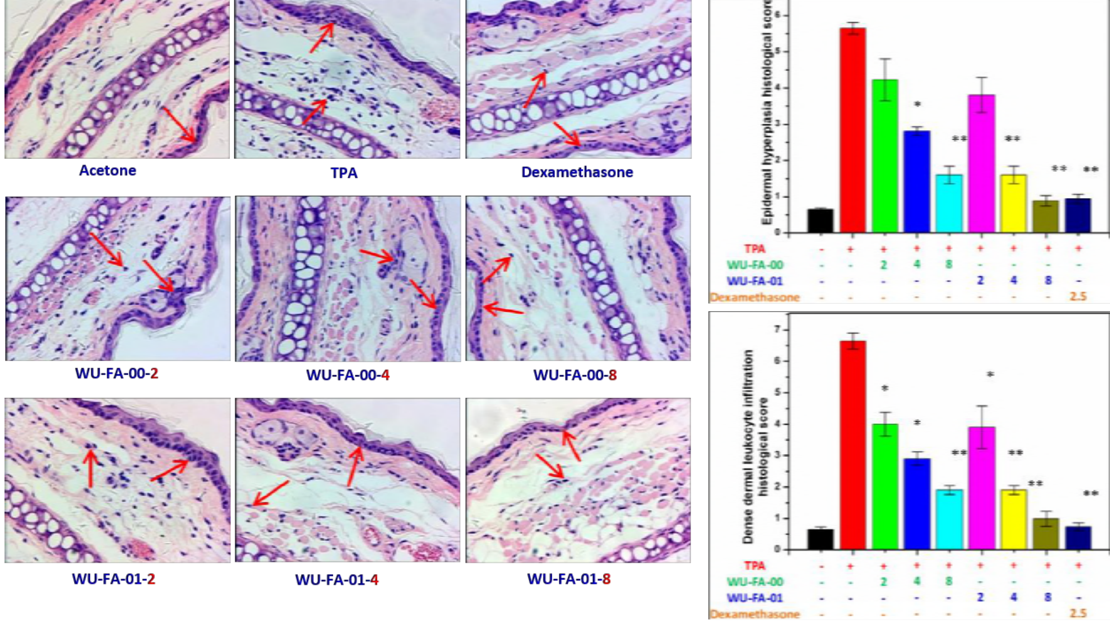
564 

565 **Fig. 4** H&E **staining for histological changes of TPA-induced mouse ears treated**

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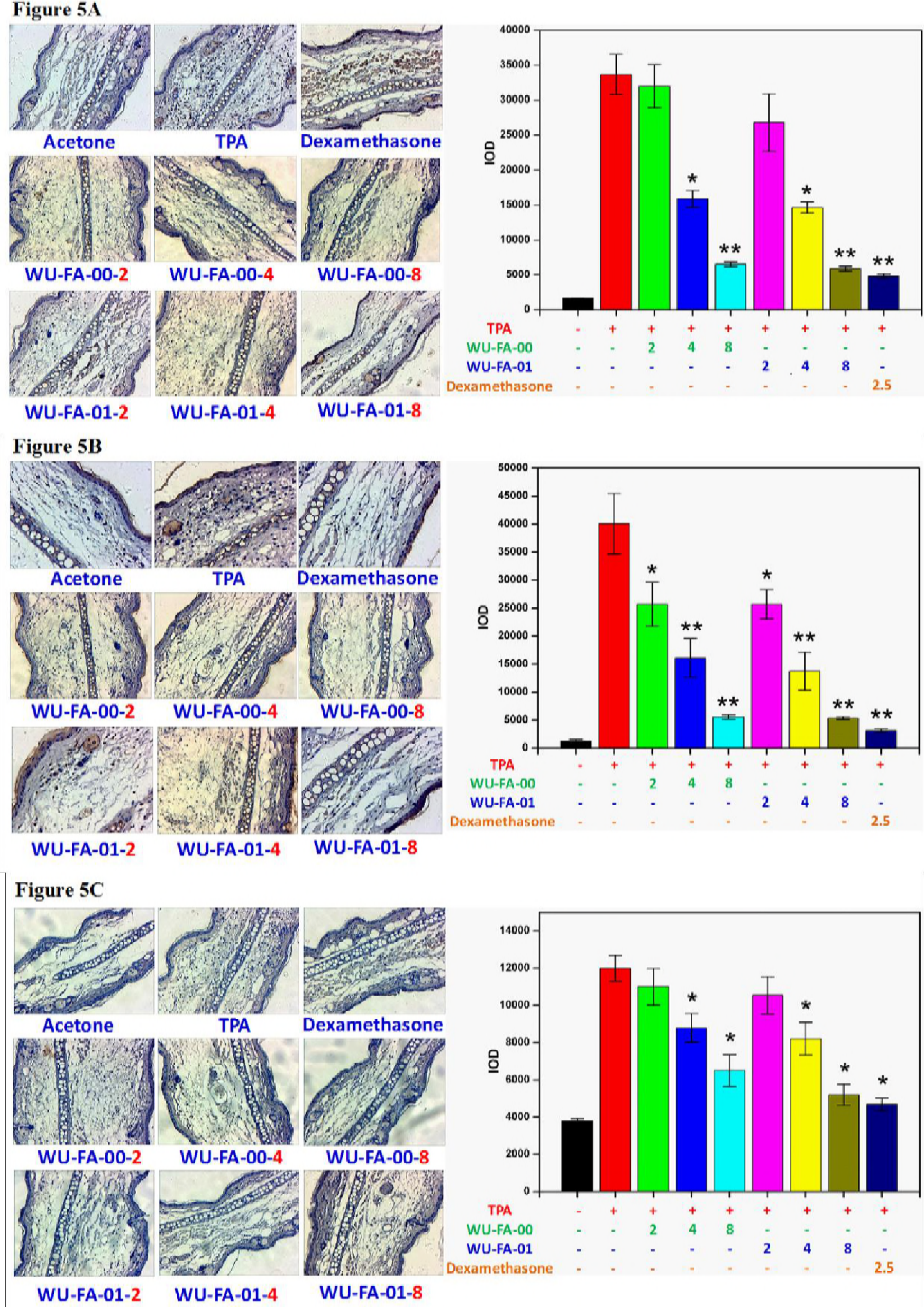

570

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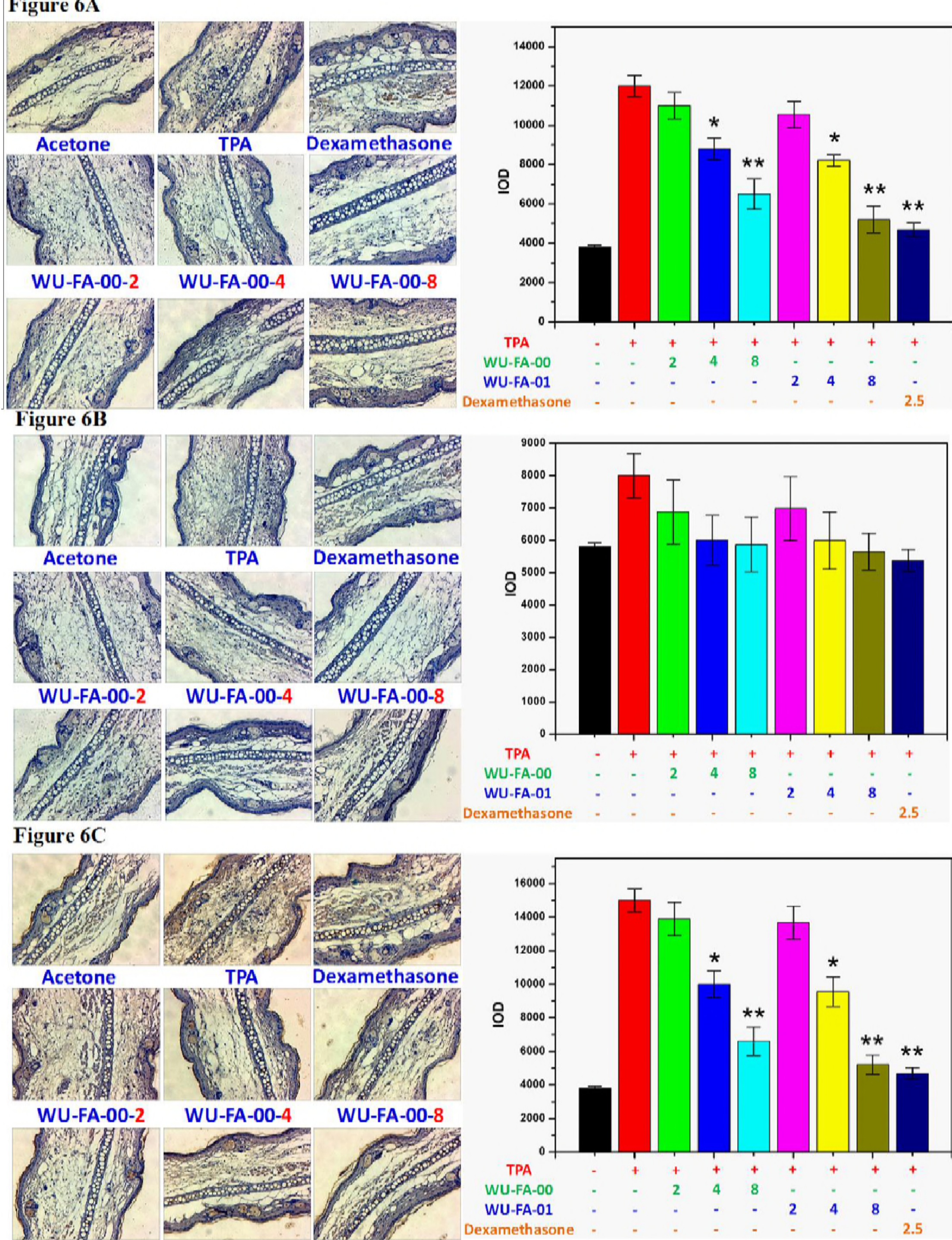
577 Magnification 200×.



578

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580 **p65 (Figure 6A), IκB-α (Figure 6B) and p-IκB-α (Figure 6C) in the TPA-induced**
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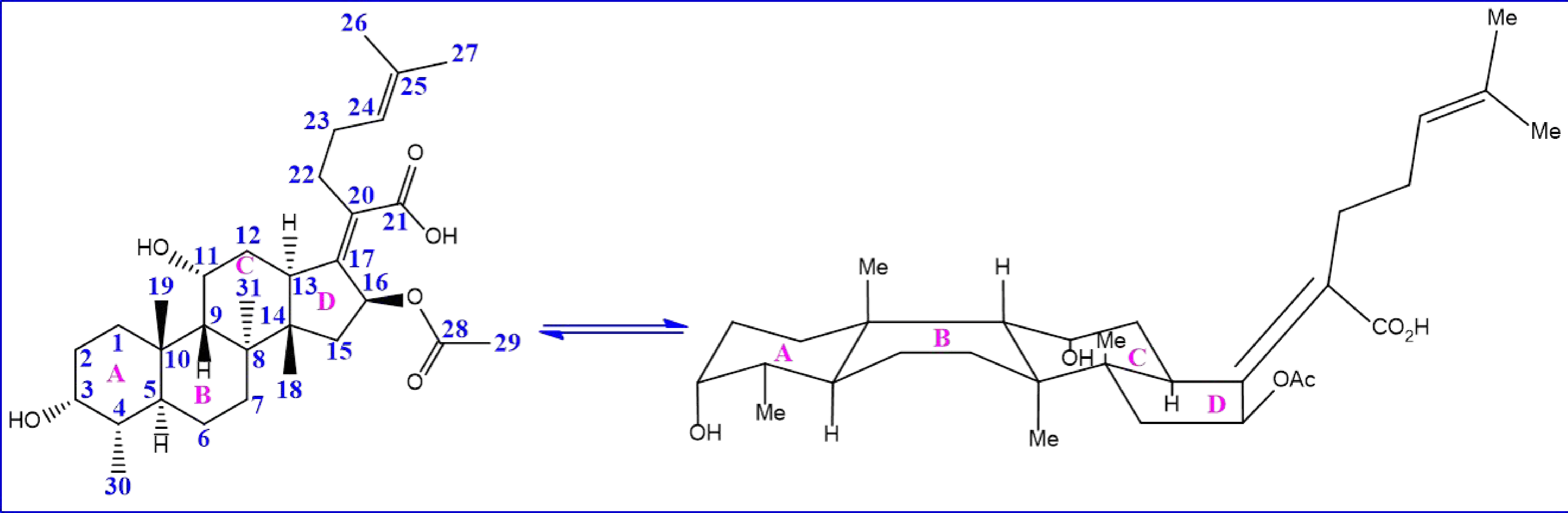

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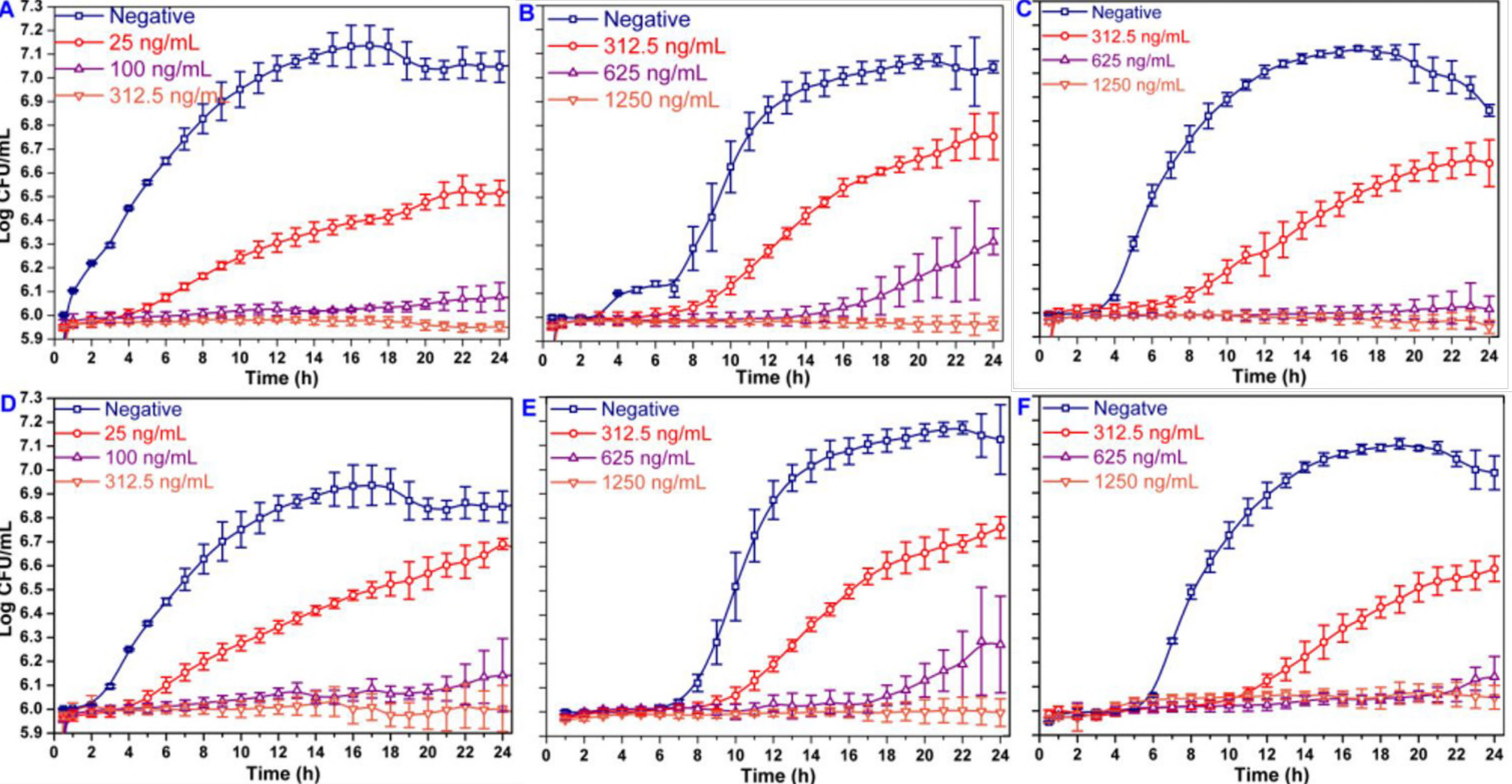
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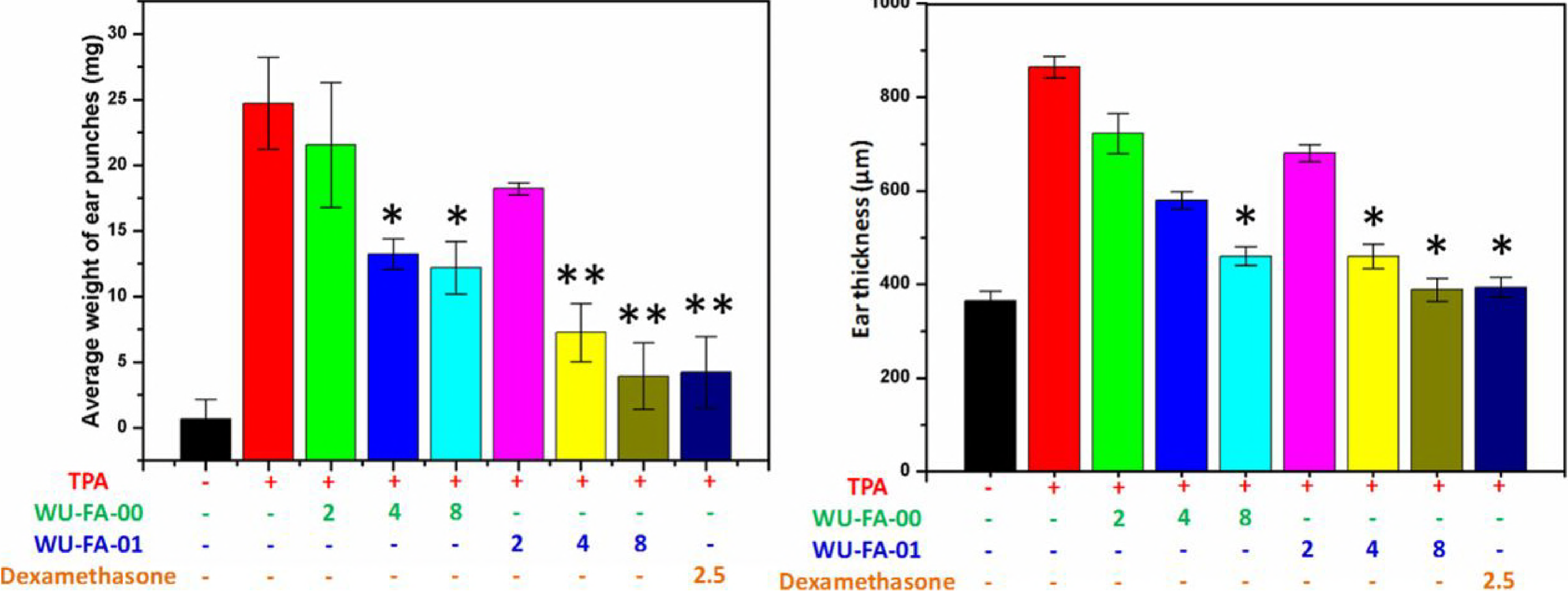

Average weight of ear punches (mg)
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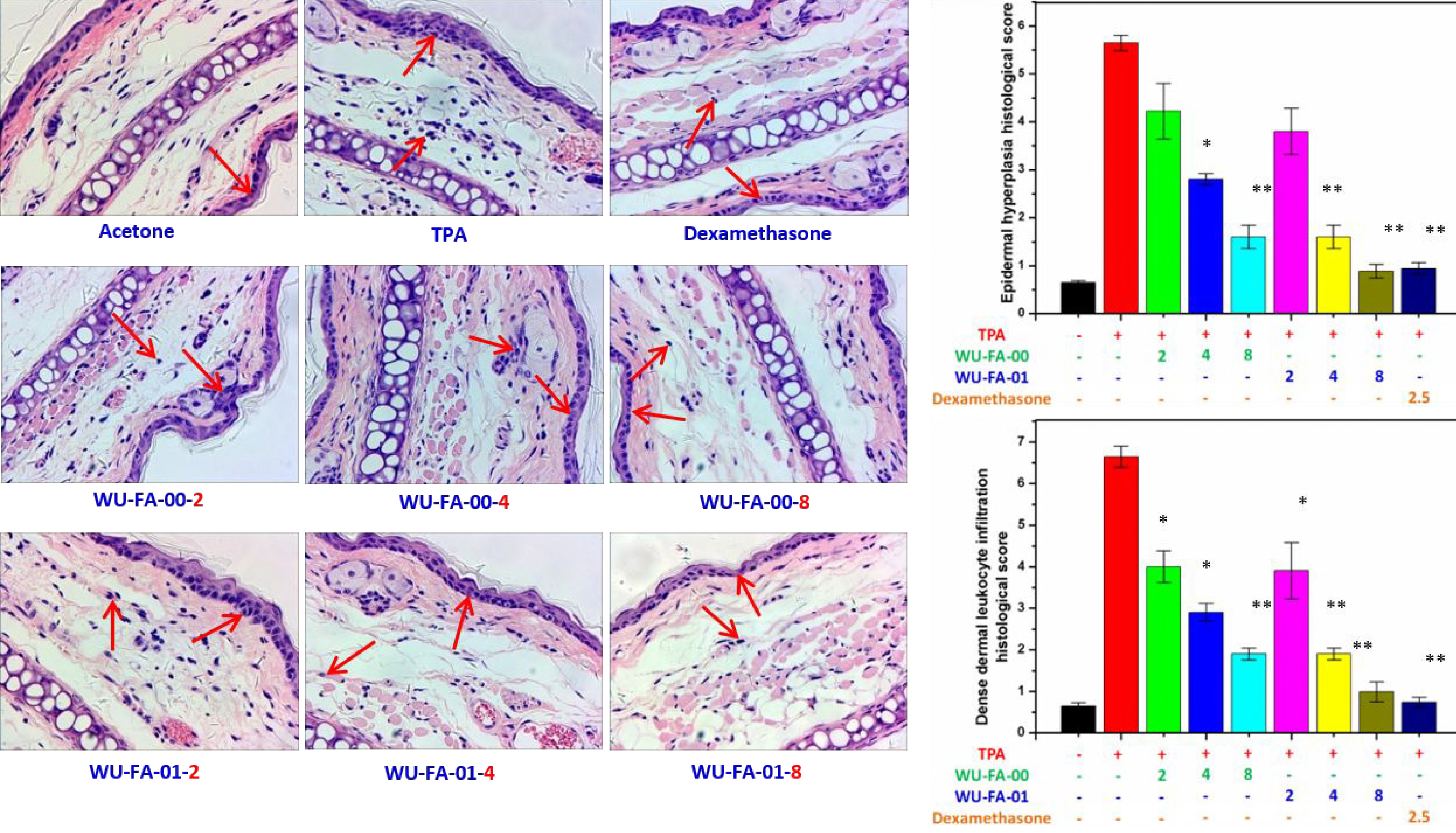
* *
* *
TPA - + + + + + + + +
WU-FA-00 - - 2 4 8 - - - -
WU-FA-01 - - - - - 2 4 8 -
Dexamethasone - - - - - - - - 2.5
Ear thickness (µm)
0
200
400
600
800
1000
*
*
*
*
TPA - + + + + + + + +
WU-FA-00 - - 2 4 8 - - - -
WU-FA-01 - - - - - 2 4 8 -
Dexamethasone - - - - - - - - 2.5



Acetone
TPA
Dexamethasone
WU-FA-00-2
WU-FA-00-4
WU-FA-00-8
WU-FA-01-2
WU-FA-01-4
WU-FA-01-8
Epidermal hyperplasia histological score
Dense dermal leukocyte infiltration histological score
TPA
WU-FA-00
WU-FA-01
Dexamethasone
2.5

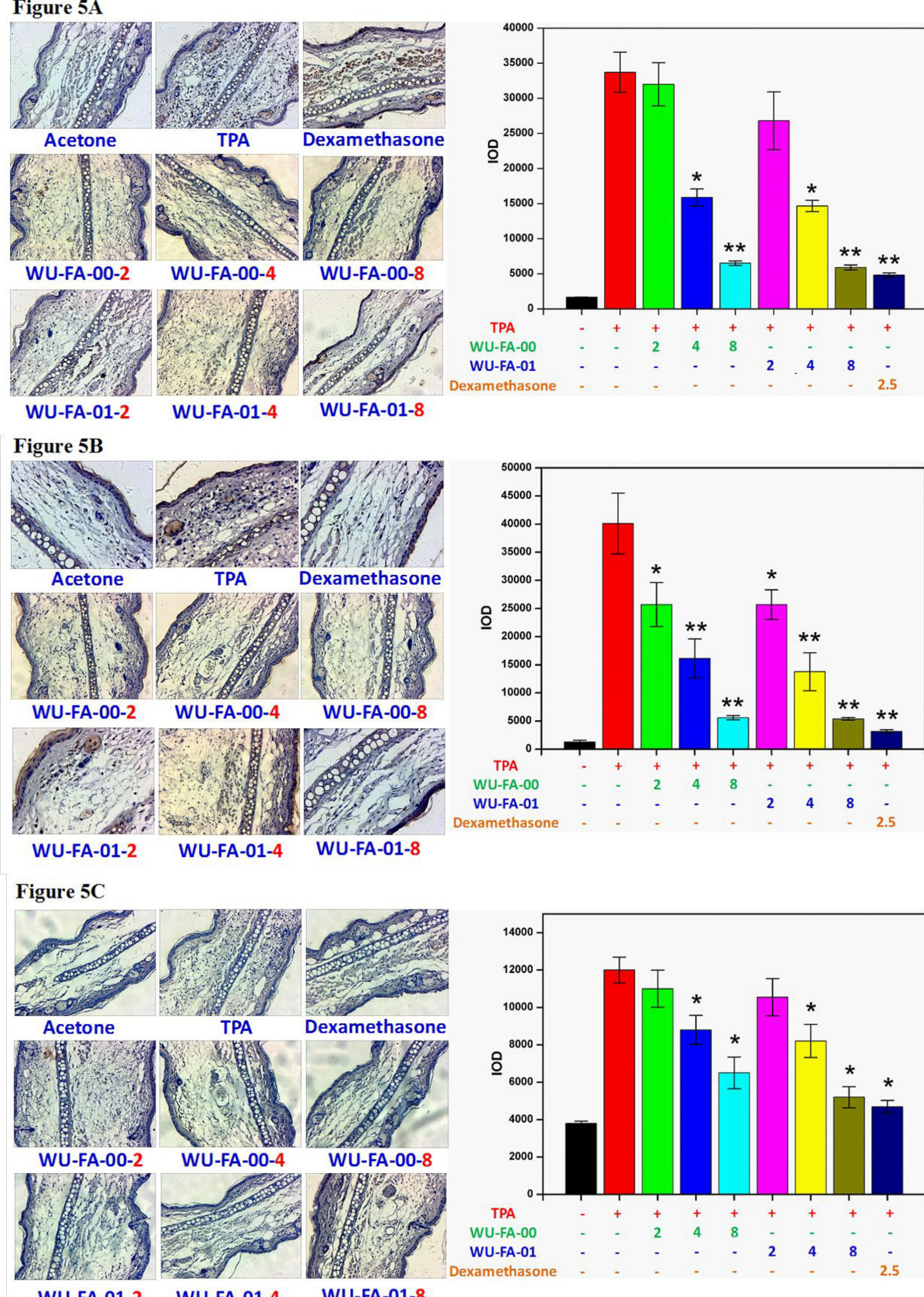


Figure 5A
Acetone
TPA
Dexamethasone
WU-FA-00-2
WU-FA-00-4
WU-FA-00-8
WU-FA-01-2
WU-FA-01-4
WU-FA-01-8
IOD
TPA - + + + + + + + +
WU-FA-00 - - 2 4 8 - - - -
WU-FA-01 - - - - - 2 4 8 -
Dexamethasone - - - - - - - - 2.5
Figure 5B
Acetone
TPA
Dexamethasone
WU-FA-00-2
WU-FA-00-4
WU-FA-00-8
WU-FA-01-2
WU-FA-01-4
WU-FA-01-8
IOD
TPA - + + + + + + + +
WU-FA-00 - - 2 4 8 - - - -
WU-FA-01 - - - - - 2 4 8 -
Dexamethasone - - - - - - - - 2.5
Figure 5C
Acetone
TPA
Dexamethasone
WU-FA-00-2
WU-FA-00-4
WU-FA-00-8
WU-FA-01-2
WU-FA-01-4
WU-FA-01-8
IOD
TPA - + + + + + + + +
WU-FA-00 - - 2 4 8 - - - -
WU-FA-01 - - - - - 2 4 8 -
Dexamethasone - - - - - - - - 2.5

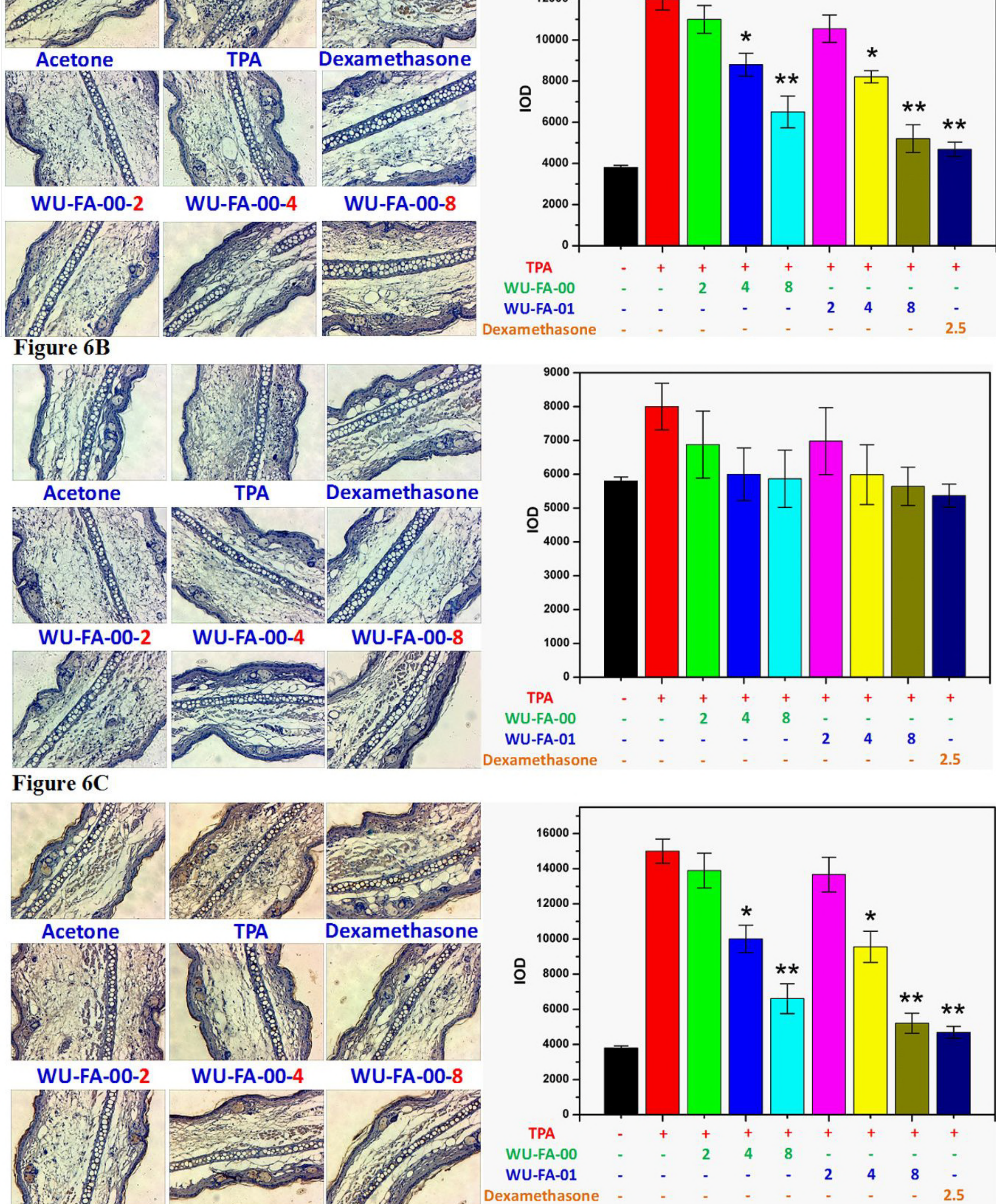
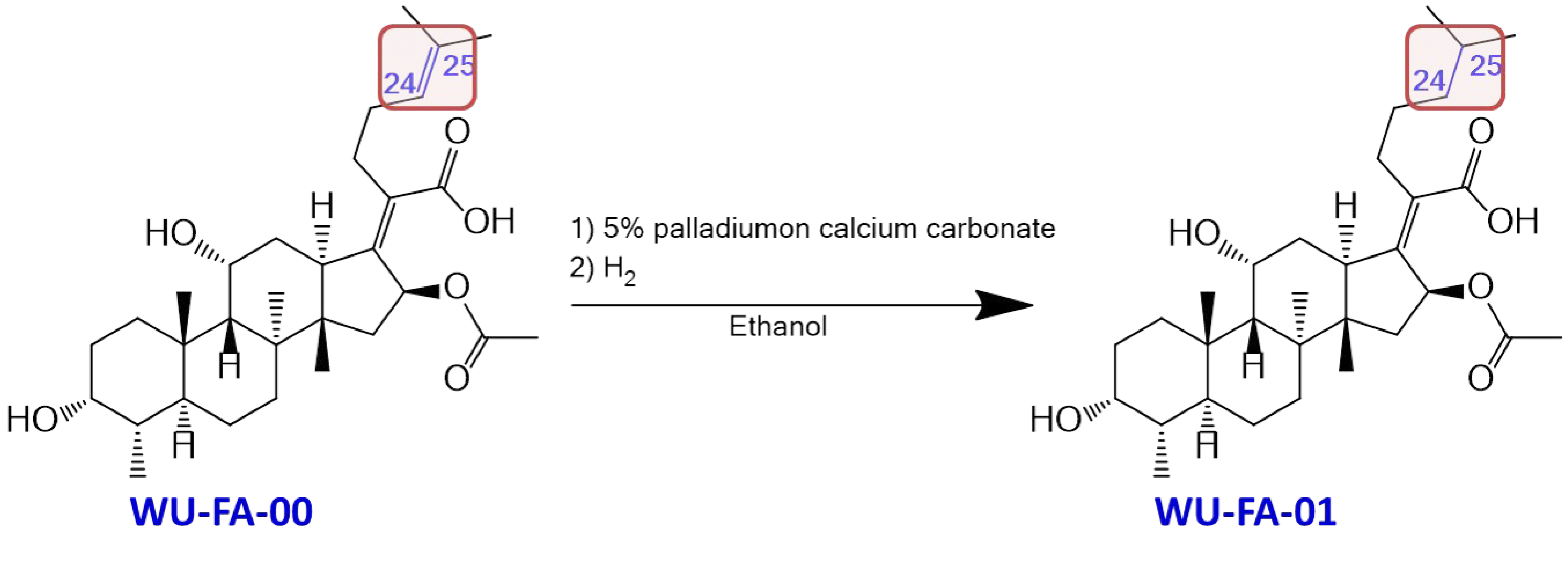


Figure 6A
Acetone
TPA
Dexamethasone
WU-FA-00-2
WU-FA-00-4
WU-FA-00-8
IOD
14000
12000
10000
8000
6000
4000
2000
0
*
**
*
**
**
TPA - + + + + + + + +
WU-FA-00 - - 2 4 8 - - - -
WU-FA-01 - - - - - 2 4 8 -
Dexamethasone - - - - - - - - 2.5
Figure 6B
Acetone
TPA
Dexamethasone
WU-FA-00-2
WU-FA-00-4
WU-FA-00-8
IOD
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
TPA - + + + + + + + +
WU-FA-00 - - 2 4 8 - - - -
WU-FA-01 - - - - - 2 4 8 -
Dexamethasone - - - - - - - - 2.5
Figure 6C
Acetone
TPA
Dexamethasone
WU-FA-00-2
WU-FA-00-4
WU-FA-00-8
IOD
16000
14000
12000
10000
8000
6000
4000
2000
0
*
**
*
**
**
TPA - + + + + + + + +
WU-FA-00 - - 2 4 8 - - - -
WU-FA-01 - - - - - 2 4 8 -
Dexamethasone - - - - - - - - 2.5



1) 5% palladiumon calcium carbonate
2) H2
Ethanol
WU-FA-00
WU-FA-01
24
25
HO
OH
O
H

Table 1 Antibacterial activity of WU-FA-00 and WU-FA-01 expressed in the inhibition zone (mm).

Compound	WU-FA-00		WU-FA-01	
Structure				
Concentration (μg/mL)	25	12.5	25	12.5
Dosage (ng)	125	62.5	125	62.5
Bacterium	Inhibition Zone (mm)			
Staphylococcus aureus (ATCC 6538)	23.09±0.37	20.60±0.40	23.08±1.13	20.05±0.30
Staphylococcus albus (ATCC 29213)	21.48±0.36	18.75±0.44	19.93±1.03	17.10±0.14
Staphylococcus epidermidis (ATCC 12228)	24.22±1.66	20.04±0.35	22.91±0.84	17.42±0.40
Listeria monocytogenes (ATCC 19115)	11.63±1.33	10.44±0.82	11.49±0.41	10.55±1.20
Streptococcus pneumoniae (ATCC 49619)	8.22±0.79	7.37±1.23	9.39±0.90	7.52±1.11
Escherichia coli (CMCC 44102)	ND	ND	ND	ND

ND, not determined.

Table 2 Antibacterial activities of WU-FA-00 and WU-FA-01 expressed in MIC and MBC (ng/mL).

Bacteria		Compounds			
		WU-FA-00		WU-FA-01	
		MIC	MBC	MIC	MBC
		ng/mL		ng/mL	
Gram-positive bacteria	*Staphylococcus aureus* (ATCC 6538)	100	312.5	100	200
	Staphylococcus albus (ATCC 29213)	625	1250	625	1250
	Staphylococcus epidermidis (ATCC 12228)	625	1250	625	1250
	Listeria monocytogenes (ATCC 19115)	＞1250	＞1250	＞1250	＞1250
	Streptococcus pneumoniae (ATCC 49619)	＞1250	＞1250	＞1250	＞1250
Gram-negative bacteria	*Escherichia coli* (CMCC 44102)	ND	ND	ND	ND

MIC (ng/mL), minimum inhibitory concentration, i.e., the lowest concentration of the compound that completely inhibits the growth of bacteria.

MBC (ng/mL), minimum bacterial concentration, i.e., the lowest concentration of the compound that completely kills the bacteria.

ND, not determined.