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1 **Anti-inflammatory activity of *Crateva adansonii* DC on keratinocytes infected by**
2 ***Staphylococcus aureus*: From traditional practice to scientific approach using HPTLC-**
3 **densitometry**

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41

42 **ABSTRACT**

43 ***Ethnopharmacological relevance:*** Leaves of *Crateva adansonii* DC (Capparidaceae), a small
44 bush found in Togo, are widely used in traditional medicine to cure infectious abscesses.

45 Traditional healers of Lomé harvest only budding leaves early in the morning, in specific area in
46 order to prepare their drugs.

47 ***Aim of the study:*** The main goal was to validate the ancestral picking practices, and to assess the
48 activity of *C. adansonii* medicine towards infectious abscesses.

49 ***Materials and methods:*** A phytochemical screening of various *C. adansonii* leaf samples was
50 performed using an original HPTLC-densitometry protocol and major flavonoids were identified

51 and quantified. *C. adansonii* samples were collected in different neighborhoods of Lomé, at
52 different harvesting-times and at different ages. Radical scavenging capacity, using DPPH assay,
53 was used to quickly screen all extracts. Extracts were tested for anti-*Staphylococcus aureus*
54 activity and anti-inflammatory effect on human primary keratinocytes infected by *S. aureus*. IL6,
55 IL8 and TNF α expression and production were assessed by RT-PCR and ELISA assays.

56 **Results:** Using antioxidant activity as selection criteria, optimal extracts were obtained with
57 budding leaves, collected at 5:00 am in Djidjolé neighborhood. This extract showed the strongest
58 anti-inflammatory effect on *S. aureus*-infected keratinocytes by reducing IL6, IL8 and TNF α
59 expression and production. None of the extracts inhibited the growth of *S. aureus*.

60 **Conclusions:** Those results validate the traditional practices and the potential of *C. adansonii* as
61 anti-inflammatory drug. Our findings suggest that traditional healers should add to *C. adansonii*
62 leaves an antibacterial plant of Togo Pharmacopeia, in order to improve abscess healing.

63
64 **Keywords:** Traditional Medicine Africa; Inflammation; Antioxidant; Chromatography; Skin.

65
66 **Abbreviations :** AUC, area under the curve; BPE, Bovine pituitary extract; DPPH, •2,2-
67 diphenyl-1-picrylhydrazyl free radical; CH₂Cl₂, methylene chloride; DNI, direct normal
68 irradiation; EDTA, ethylene diamine tetraacetic acid; EGF, epidermal growth factor; EtOAc,
69 ethyl acetate; HPTLC, High Performance Thin Layer Chromatography; ICH, International
70 Conference of Harmonization; IL6, Interleukine 6; L8, Interleukine 8; K-SFM, Keratinocyte-
71 Serum Free Medium; LOD, limit of detection; LOQ, limit of quantification; MAPK, mitogen
72 activated protein kinase; MeOH, methanol; MOI, multiplicity of infection; MTT, 3-(4,5-
73 methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; *n*-BuOH, *n*-butanol; NF- κ B, nuclear
74 factor-kappa B; PBS, phosphate buffer saline; PVGIS: PhotoVoltaic Geographical Information
75 System; r^2 , correlation coefficient; RDSC, Radical DPPH Scavenging Capacity; rpm, round per

76 minutes; RSD, Relative standard deviation; RT-PCR, real time polymerase chain reaction; SEM,
77 standard error of the mean; TE, trolox equivalent; TLC, Thin Layer Chromatography; TNF α ,
78 Tumor Necrosis Factor α ; v/v, volume/volume; XTT, 3'-{1-[(phenylamino)carbonyl]-3,4-
79 tetrazolium }-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate; w/w, weigh/weigh.

80

81 **1. Introduction**

82 *Crateva adansonii* DC also known as *Crateva guineensis* Schumach & Thonn, belongs to the
83 family Cappariaceae. This species is confined to Africa but bears very close affinity to the
84 Asian *Crateva religiosa* G. Forst with which it has been equated by some authorities (Igoli et
85 al., 2014). This moderately sized deciduous tree is found throughout the tropics especially
86 along the river banks. The leaves are trifoliolate and ovate to oblong in shape. The flowers are
87 white or creamy and occur at the terminal corymbs when the tree is completely without
88 leaves. The edible fruits which are 3.5-5 mm large are spherical in shape. The barks are gray,
89 smooth, and sometimes horizontally wrinkled (Akanji et al., 2013; Nkeiruka and Samuel,
90 2015).

91 Traditionally, barks, leaves, flowers, and root barks are extensively used in folk medicine for
92 the cure of many disease conditions. In Senegal, the roots are used in the treatment of syphilis,
93 jaundice, and yellow fever (Igoli et al., 2014). In India and Nigeria, the bark is useful in skin
94 diseases and snakebites (Gupta et al., 2006; Akanji et al., 2013) while flowers are astringent
95 (Maruthupandian and Mohan, 2010). The powdered bark is useful in kidney, bladder and
96 urinary tract disorders, including urinary tract infections with burning micturition or
97 urolithiasis. Concerning the validation of ethnomedicinal potential of *Crateva adansonii* DC
98 for urinary tract disorder, an anti-urolithiasis activity of bark petroleum ether extract was
99 observed in rats (Gupta et al., 2006). The bark or leaves decoction is widely used as a
100 stomachic and tonic, while leaves also figure in treatments of intermittent fevers and

101 inflammation, e.g. rheumatic pains, swellings and asthma (Borikini et al., 2012; Igoli et al.,
102 2012; Gitte et al., 2012; Gupta et al., 2006).

103 The *in vitro* antimicrobial and antifungal activities of *Crateva adansonii* DC extracts of leaves
104 have been previously reported (Sahoo et al., 2008; Agboke et al., 2011), while crude hexane
105 and ethyl acetate extracts or isolated phytoconstituents of leaves presented moderate anti-
106 trypanosomal activity *in vitro* (Igoli et al., 2012, Igoli et al., 2014). Leaves extracts also
107 inhibited *in vitro* xanthine oxidase activity that might be helpful in preventing or slowing the
108 progress of gout (Abdullahi et al., 2012).

109 Phytochemically, the leaves contain flavonoids, tannins, saponins, cardiac glycosides,
110 terpenes and alkaloids (Abdullahi et al., 2012; Borikini et al., 2012). Lately, aurantiamide
111 acetate, pyropheophorbide A, ethyl pyropheophorbide A and purpurin-18 ethyl ester were also
112 isolated from leaves of *C. adansonii* DC (Igoli et al., 2014).

113 As a part of our ongoing research on traditional herbs, we turned our attention to the
114 ethnomedicinal use of *C. adansonii* DC in Togo. Indeed, the Togolese Pharmacopoeia relates
115 the use of this plant mainly for the treatment of abscesses, wounds, skin diseases, as dressing
116 paste on the lesional parts. Leaves of *C. adansonii* are crushed just before use to form a
117 plaster. The plaster is then put directly on the abscess. The exposure to the plaster is usually
118 brief, i.e. less than 5 min, due to an important prickly activity. Picking leaves of *C. adansonii*
119 DC is an art from *Egbétôs*, i.e. traditional healers in Mina language. According to their
120 empirical knowledge, herbalists harvest only budding leaves very early in the morning.
121 However, no scientific evidence supports either this specific harvest practice or the medicinal
122 use of this plant in management of skin disorders.

123 Wound healing is a natural body reaction leading to the restoration of structural and
124 functional integrity of injured tissues. Inflammation, proliferation and remodeling are three

125 overlapping and continuous wound healing phases. The healing properties of phytomedicines
126 have been widely used by traditional healers in many countries (Süntar et al., 2012; Reddy et
127 al., 2013). The medicinal value of these plants lies in their various health-sustaining
128 constituents, which act on different intracellular signaling pathways e.g. NF- κ B, MAPK
129 (Nicolaus et al., 2017; Bui et al., 2014). These constituents include various members of
130 chemical families like alkaloids, essential oils, flavonoids, tannins, terpenoids, saponins, and
131 phenolic compounds (Balekar et al., 2012; Arct et al., 2008).

132 The main goal of the present study was to validate traditional harvesting practices and to
133 investigate the biological effects of *C. adansonii* DC leaves. Thus, we studied the *in vitro*
134 antioxidant and anti-inflammatory activities of *C. adansonii* DC leaves extracts using DPPH
135 scavenging assay and human primary keratinocyte cultures infected by *Staphylococcus*
136 *aureus*.

137 **2. Materials and Methods**

138 *2.1. Chemicals*

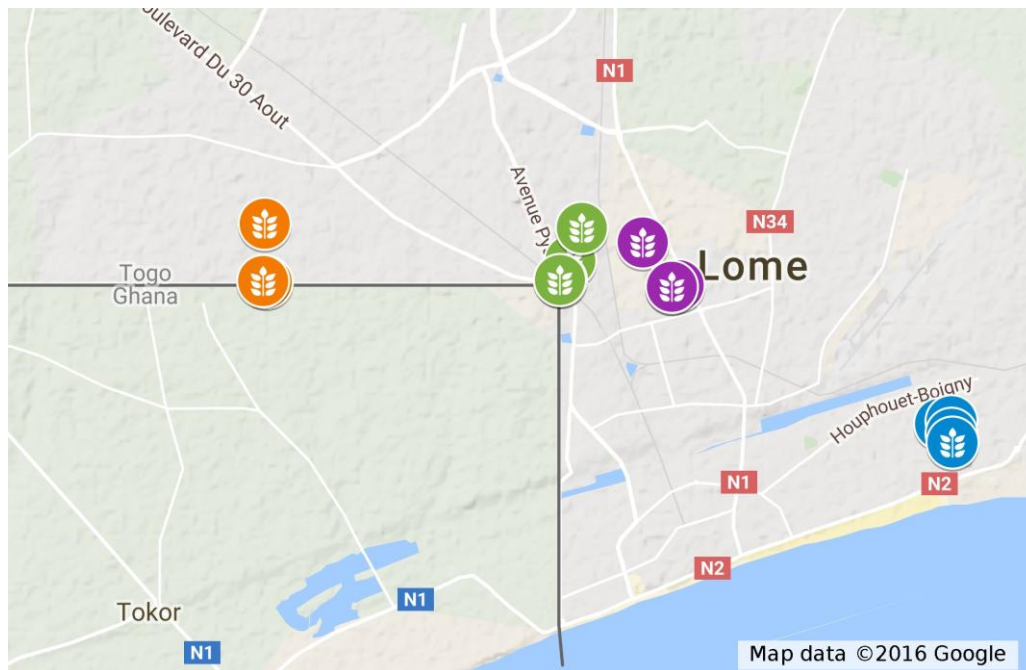
139 Quercitrin and isoquercitrin standards were purchased from Extrasynthèse (Genay, France).
140 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH[•]) was purchased from Sigma Aldrich (St
141 Louis, MO, USA). Trolox standard reagent was purchased from Acros Organics (Geel,
142 Belgium). Acetic acid, formic acid, petroleum ether, methylene chloride (CH₂Cl₂), ethyl
143 acetate (EtOAc), methanol (MeOH) were purchased from Carlo Erba (Val de Reuil, France).
144 Water was purified by a Milli-Q-system (Millipore Corporation, Bedford, MA, USA).

145

146 *2.2. Plants*

147 *2.2.1. Samples collection*

148 The leaves of *C. adansonii* were collected in September 2013, in different areas of Lomé, the
149 capital of Togo: Adidogomé, Bé, Campus of the University of Lomé, and Djidjolé (Figure 1).
150 GPS coordinates of samples harvesting places were detailed in Supplementary material.



151

152 Figure 1: Localization of the different collection points of leaves of *C. adansonii* in Lomé:

153 Adidogomé in orange, University of Lomé in purple, Bé in blue and Djidjolé in green.




154 Leaves from three or four different trees were harvested in each place and then pooled

155 together before extraction. Characteristics of budding, young and old leaves of *C. adansonii*

156 are found in Table 1.

157

158 Table 1: *C. adansonii* leaves characteristics depending on their age.

	Budding leaves	Young leaves	Old leaves
Sampling site	Apex of the stem	Just under the apex	Base of the stem
Leaves size	0-2 cm \pm 1 cm	2-5 cm \pm 2 cm	\geq 5 cm
Pictures			

159

160 The botanical identification of collected material was performed by botanists from the
 161 Laboratory of Botany and Vegetable Ecology of the Science Faculty, University of Lomé.

162 Voucher specimens were deposited at the National Herbarium of Togo (University of Lomé)
 163 under number 15078. The plant names have been verified with www.theplantlist.org.

164 (Accessing date: 19/10/2016).

165 The collected leaves of *C. adansonii* were dried at room temperature (20°C) in the laboratory
 166 for two weeks. Samples were chopped into smaller pieces and then grounded into powder
 167 using a blender.

168 2.2.2. Preparation of extracts

169 The dried budding leaves of *C. adansonii* were powdered. Maceration of 1g of powder in 30
 170 ml of solvent (acetone, ethyl acetate, petroleum ether, water, methanol or ethanol 70°) was
 171 sonicated for 30 min in an ultrasonic bath RK-100H (Bandelin electronic, Berlin, Germany) at
 172 room temperature. The supernatant was recovered after 5 min of centrifugation at 4000 rpm.

173 Solid/liquid extraction step was repeated three times. These extractions were done in
174 triplicate. The solvents were evaporated to dryness under reduced pressure.

175 *2.3. High-performance thin layer chromatography*

176 A Camag (Camag, Muttenz, Switzerland) HPTLC instrumental set-up consisting of a sample
177 applicator Linomat 5, a Reprostar 3, a TLC scanner 3 and Digistore 2 Documentation system
178 was used for the analyses. The data were managed by software platform Wincats 1.4.2 Planar
179 chromatography Manager. HPTLC Silica gel 60F254, 20x10 cm (Merck Darmstadt,
180 Germany) were employed and developed in Camag Automatic Developing Chamber ADC2.

181 *2.3.1. Preparation of standard and sample solutions*

182 Quercetin 3-*O*- β -D-glucoside standard stock solution (1 mg/ml) was prepared by dissolving
183 the appropriate amount in MeOH. Stock solution was stored at -18°C , in the dark. For
184 calibration curve, five standard solutions (0.2, 0.15, 0.1, 0.05, 0.025 mg/ml) were obtained by
185 diluting the stock solution in MeOH. 5 μL of each solution were applied, corresponding to 1,
186 0.75, 0.5, 0.25 and 0.125 μg of quercetin 3-*O*- β -D-glucoside per spot.

187 The dried *C. adansonii* leaf extracts were solubilized in methanol/water (50:50, v/v) in order
188 to obtain solutions at 2.5 or 5 or 10 $\text{mg}\cdot\text{mL}^{-1}$ and 5 μL of each solution were applied.

189 *2.3.2. Chromatographic procedure*

190 Standard and sample solutions were applied bandwise (band length 5 mm, 80 nL /s delivery
191 speed under nitrogen 6 bars pressure, track distance 8.5 mm and distance from the edge 10
192 mm). Standard solutions were applied in duplicate; sample solutions were applied in
193 triplicate. Plates were developed with ethyl acetate/formic acid/acetic acid/water
194 (100:11:11:26, v/v/v/v) as the mobile phase (80 mm), at room temperature, after 15 min plates
195 preconditioning with the mobile phase and tank saturation (humidity 45.5 to 52 %). Plates

196 were either pulverized by NEU solution and dried at 110°C during 2 min on a Camag TLC
197 plate's heater 3, or only dried for 15 min when plates were intended for scanner densitometric
198 measurements or for immersion in DPPH[•] reagent solution.

199 *2.3.3. Quantification by densitometry*

200 Visual inspection and documentation of chromatogram were carried out under white light and
201 365 nm. Densitometric measurements were performed in absorbance mode at 265 nm
202 (maximum absorbance wavelength of Isoquercitrin). Evaluation was assumed by area under
203 the curve (AUC) measurement. Results were obtained in µg of standard per spot and
204 converted in equivalent of isoquercitrin using applied volume and sample solution
205 concentration (mg.mL⁻¹).

206 Statistical validation was performed following International Conference on Harmonization
207 (ICH) guidelines Q2R1 and using accuracy profiles (Boudesocque et al., 2013).

208 *2.3.4. Direct antioxidant bioautography*

209 Bioautography derivatization was performed according to the Camag application Note F-38
210 procedure.

211 *2.4. DPPH[•] Antioxidant assay*

212 *2.4.1. Protocol*

213 The free radical scavenging capacity of samples was determined using the Radical DPPH[•]
214 (2,2-diphenyl-1-picrylhydrazyl free radical) Scavenging Capacity (RDSC) assay adapted from
215 Cheng et al. (2006), with few modifications. The DPPH[•], the standard antioxidant compound
216 (Trolox), and the dried leaves extracts were dissolved in an acetone/water (50:50, v/v) mixture
217 and stored at 4°C. 100 µl of leaves extracts (0.1 or 0.2 mg.mL⁻¹) or Trolox (25 µM and 50

218 μM) were added to 100 μl of a DPPH^{*} solution (0,208 mM) in 96-well plate. The absorbance
219 (A) of each reaction mixture at 515 nm was measured every minute during 40 min using a
220 Multiskan GO microplate reader (Thermofisher Scientific, Villebon Courtaboeuf, France).
221 The radical scavenging capacity was calculated for each extract as described in Cheng et al.
222 (2006), after 21 min of contact and was expressed as μmol of Trolox Equivalent (TE) per
223 gram of extract.

224 2.5. Statistical analysis

225 A statistical analysis of HPTLC and DPPH assay datas was performed using one way
226 ANOVA analysis of variance test followed by a Tuckey comparison between groups. The *p*
227 values ≤ 0.05 were considered as significant.

228 2.6. Identification of major flavonoids

229 2.6.1. Extraction and isolation

230 Old leaves of *C. adansonii* collected in Djidjolé neighborhood at noon (50 g) were powdered
231 and macerated with methanol (0.5 L) at room temperature for 72h. After filtration, the filtrate
232 was concentrated under reduced pressure and the residue was suspended in MeOH-H₂O
233 (20:80, v/v). The methanolic aqueous layer was defatted three times with *n*-hexane (total of
234 150 mL) and then extracted with *n*-butanol (*n*-BuOH) (200 mL, 2.08 g). After concentration
235 under reduced pressure, the 153 mg of the butanolic extract was subjected to preparative TLC,
236 developed with ethyl acetate/formic acid/acetic acid/water (100:11:11:30, v/v/v/v) to yield
237 compounds **1** (22 mg) and **2** (18 mg). Compound **3** was obtained by purification of 1 g of
238 butanolic extract using centrifugal partition chromatography (CPC). Centrifugal partition
239 chromatography (CPC) separation was performed on a FCPC® Preparative 200 Kromaton
240 Technologies apparatus (Rousselet Robotel, Annonay, France) using a rotor made of 20
241 circular partition disks (840 partition twin-cells; total column capacity: 205 mL, dead volume:

242 32.3 mL). Rotation speed could be adjusted from 200 to 3000 rpm, thus producing a
243 centrifugal force field in the partition cells of about $120 \times g$ at 1000 rpm and $480 \times g$ at 2000
244 rpm. The solvents were pumped by a preparative Ecom Beta 50 Gradient pump binary low-
245 pressure gradient pump (Praha, Czech Republic). The samples were introduced into the
246 column through a PEEK dual mode preparative scale sample injector 3725i (Rheodyne,
247 Rohnert Park, CA, USA) equipped with 20 mL sample loop. Effluent content was monitored
248 by an Ecom Flash 06 DAD 600 detector equipped with a preparative flow cell (45 μ L internal
249 volume, optical path 0.3 mm). Fractions were collected by an Advantec Super Fraction
250 collector (Otowa, Japan). CPC experiment was performed using the biphasic solvent system
251 ethylacetate/*n*-butanol/methanol/water (5:2:1:7, v/v/v/v), in descending mode, at 1800 rpm
252 with a mobile phase flow rate of 8 mL/min with 10 % of co-current. Fractions were monitored
253 at UV 210, 254 and 365 nm, and collected every 30 s. Stationary phase retention rate was 72
254 % and the back pressure was 34 bars. Compound **3** (10 mg) was eluted from 58 to 67 min
255 after sample injection in the column.

256 2.6.2. Compound characterization

257 Isolated compounds (**1** to **3**) were identified after application of spectroscopic methods ^1H and
258 ^{13}C NMR data in CD_3OD and MS and comparison with the literature. NMR data were
259 available in Supplementary material.

260 2.6.2.1. Compound **1**. *Quercetin-3-O- α -L-rhamnopyranoside* or *Quercitrin*

261 Physical and spectroscopic data were in accordance with literature (Fossen et al., 1999)

262 2.6.2.2. Compound **2**. *Quercetin-3-O- β -D-glucopyranoside* or *Isoquercitrin*

263 Physical and spectroscopic data were in accordance with literature (Fernandez et al., 2005)

264 2.6.2.3. Compound **3**. *Quercetin-3-O-(2''-O- β -D-xylopyranosyl- α -L-rhamnopyranoside)*

265 Physical and spectroscopic data were in accordance with literature (Nielsen et al., 2005)

266 2.7. Biological investigations

267 2.7.1. Primary human epidermis keratinocyte culture.

268 Keratinocytes were obtained from surgical samples of abdominal or breast skin of healthy
269 patients. The use of human skin samples for research studies was approved by the Ethical
270 Committee of the Poitiers Hospital and patients gave their written informed consent. Skin
271 samples were incubated overnight at 4°C in a dispase solution (25 U/ml; Invitrogen Life
272 Technologies). Epidermal sheets were removed from the dermis, and keratinocytes were
273 dissociated by trypsin digestion (trypsin-EDTA; Invitrogen Life Technologies) for 15 min at
274 37°C. The cell suspension was then filtered through a 280-µm sterile filter and centrifuged at
275 300 g for 10 min. Primary human keratinocytes were grown in Keratinocyte-Serum Free
276 Medium (K-SFM; Gibco) supplemented with 25 µg/ml of bovine pituitary extract (BPE;
277 Gibco), 0.25 ng/ml of epidermal growth factor (EGF; Gibco) and 50 µg/ml of gentamicin
278 (Sigma). The cultures were incubated at 37°C in a humidified atmosphere with 5% CO₂ until
279 confluency. Primary keratinocytes were stored frozen in nitrogen until use. Finally,
280 keratinocytes were seeded in 24-well culture plates at a density of 2 x 10⁴ cells/well in K-
281 SFM supplemented with BPE and EGF. Keratinocytes were cultured to 80% of confluence
282 and then starved for 24 h in keratinocyte serum-free medium without addition of growth
283 factors and gentamicin before infection.

284 2.7.2. Effect of *C. adansonii* extracts on keratinocytes infected with *S. aureus*.

285 *S. aureus* (ATCC 29213) was routinely grown on Muller Hinton agar plate (Oxoid) and
286 incubated for 24 h at 37°C. For cell infection assays, bacterial suspension was prepared in the
287 cell culture medium using 24 h bacterial cultures. Keratinocytes were incubated in presence or
288 absence of increasing concentrations of the extracts, ranging from 5 to 100 µg/mL, for 1 h

289 before being infected by *S. aureus*. Bacteria were added to cell culture supernatants at a
290 multiplicity of infection (MOI) of 1 bacterium per cell and incubated for 24 h at 37°C in a
291 humidified atmosphere of 5% CO₂. Bacterial concentration was determined by measuring the
292 optical density of the suspension at 600 nm and by bacterial counts controlled by serial 10
293 fold dilutions of the bacterial suspensions plated on Muller Hinton agar plate. Culture
294 supernatants were collected, centrifuged (400 g, 5 min, 20°C) and stored at -20°C until used.
295 Cells lysates were stored at -80°C before RNA extraction. Cultures without bacteria were
296 used as controls.

297 2.7.3. Effect of *C. adansonii* extracts on *S. aureus* viability and growth.

298 The effect of *C. adansonii* extracts on *S. aureus* viability was investigated using the
299 tetrazolium sodium 3'-{1-[(phenylamino)carbonyl]-3,4-tetrazolium}-bis(4-methoxy-6-
300 nitro)benzene sulfonic acid hydrate (XTT; Sigma-Aldrich) reduction assay. Briefly, XTT was
301 dissolved in phosphate-buffered saline (PBS) at 1 mg/mL and menadione was prepared in
302 acetone at 5 mM. The XTT/menadione reagent was prepared fresh and contained 10 parts
303 XTT/1 part menadione. 100 µL of a *S. aureus* suspension at a final concentration of 10⁵
304 cells/mL were added to the wells of a sterile 96-well plate and treated with extracts (0–200
305 µg/mL) for 4 h prior to adding 50 µL of XTT/menadione. After 2 h at 37°C, the absorbance at
306 490 nm was read using a microplate reader. Growth inhibition assays were performed with
307 sterile 96-well plates in a final volume of 200 µL. One hundred microliters of bacterial
308 cultures at a final concentration of 10⁵ cells/mL in Mueller-Hinton broth were added to
309 100 µL of serially diluted *C. adansonii* extracts (200 to 12.5 µg/mL). After 24 h of incubation,
310 the bacterial cell growth was assessed by measuring the optical density of cultures at 600 nm
311 and by counting bacterial colony forming units on Mueller-Hinton agar plates.

312 2.7.4. Cytotoxicity assay.

313 Cytotoxicity of inhibitors was evaluated using the MTT (3-(4,5-methylthiazol-2-yl)-2,5-
314 diphenyltetrazolium bromide) assay (Cell proliferation kit I, Roche) on primary keratinocytes,
315 as recommended by the manufacturer.

316 *2.7.5. RNA extraction.*

317 Total RNA extraction from primary keratinocytes was performed using the Nucleo-Spin XS
318 RNA extraction kit (Macherey-Nagel) according to the manufacturer's instructions. RNA was
319 eluted in 30 µl of RNase-free water supplemented with 40 units of RNaseout® (Invitrogen).
320 RNA concentrations and purity were determined using the Nanodrop 2000 spectrophotometer
321 (Thermo Scientific).

322 *2.7.6. Reverse transcription and Real-time PCR (RT-PCR) analysis.*

323 Total RNA (2 µg) was reverse transcribed using SuperScript II kit (Invitrogen) according to
324 the manufacturer's instructions. Quantitative RT-PCR was performed in 96-well plates using
325 LightCycler-FastStart DNA Master^{Plus} SYBR GREEN I kit (Roche) on LightCycler 480
326 (Roche). Reaction mixture consisted of 1X DNA Master Mix (Applied Biosystems), 1 µM
327 forward and reverse primers designed using Primer 3 software and 12.5 ng of cDNA template
328 in a total volume of 10 µl. PCR conditions were as follows: 5 min at 95 °C, 40 amplification
329 cycles comprising 20 s at 95 °C, 15 s at 64 °C and 20 s at 72 °C. Samples were normalized
330 with regard to two independent control housekeeping genes (*Glyceraldehyde-Phospho-*
331 *Dehydrogenase* and *β2-microglobulin*) and reported according to the $\Delta\Delta CT$ method as RNA
332 fold increase: $2^{-\Delta\Delta CT} = 2^{\Delta CT_{stimulated} - \Delta CT_{unstimulated}}$.

333 *2.7.7. Enzyme-linked immunosorbent assay.*

334 Levels of IL-6, IL8 and TNF α in cell culture supernatants were determined in duplicates using
335 Human ELISA development kits (R&D systems for TNF α , and PeproTech for IL-6, IL8) in
336 accordance with the manufacturers' specifications.

337 2.7.8. Statistical analysis.

338 The data presented constitute the average obtained from 5 independently performed
339 experiments with a standard error of the mean (SEM). Statistical analysis of significance was
340 calculated using Kruskal-Wallis one-way ANOVA analysis of variance test followed by
341 Dunn's comparison between groups. The p values ≤ 0.05 were considered as significant.

342 3. Results and discussion

343 Leaves of *C. adansonii* are used as a folk medicine in Togo almost exclusively for external
344 purposes. In particular, leaves or an aqueous paste of leaves can be spread directly on wounds.
345 The Togolese traditional healers relate a specific harvesting method for this ethnomedicinal
346 use, picking only budding leaves early in the morning.

347 Many plants that have traditionally been used in the treatment of wounds, or have been shown
348 to have a wound healing property, also possess high level of antioxidant property (Süntar et
349 al., 2012). Antioxidants counter the excess proteases and reactive oxygen species (ROS) often
350 formed by neutrophil accumulation in the injured site and protect protease inhibitors from
351 oxidative damage.

352 In order to validate the wound healing potential of *C. adansonii* and the requirement of a
353 specific leaf picking method, we decided to perform a DPPH radical scavenging kinetic
354 measurements of the leaf extracts depending on the harvesting area, the leaves' age and the
355 hour of harvesting. The Radical DPPH Scavenging Capacity (RDSC) was selected as

356 preliminary screening criteria, as a good indicator of potential anti-inflammatory activity, to
357 compare all extracts.

358 3.1. Optimization of the extraction solvent

359 In a first attempt, we had to determine the best extraction solvent to recover the highest
360 antioxidant fraction from *C. adansonii*. Six solvents were used to extract the plant powder of
361 budding leaves collected in Djidjolé at 12:00: petroleum ether, acetone, ethyl acetate,
362 methanol, ethanol 70 % (v/v) and water.

363 Table 2: RDSC and extraction yields obtained with extraction solvents

Extract	Extraction yield %	% RSD	RDSC	
			($\mu\text{mol TE/g}$ of extract)	% RSD
Petroleum ether	1.9	2.7	17.3 ^a	4.4
Ethylacetate	10.3	3.9	37.3 ^a	2.9
Acetone	5.4	4.8	24.8 ^a	4.1
Methanol	22.5	3.6	457.7 ^b	2.5
Ethanol 70 %	61.5	0.4	490.9 ^b	5.0
Water	38.3	0.5	416.3 ^c	2.7

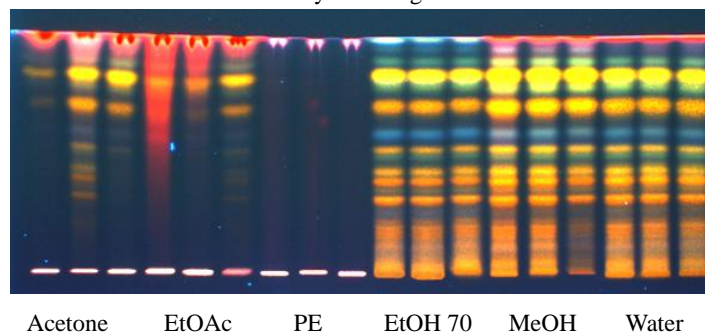
364 ^{a,b,c} average RDSC which did not share any letter were considered as significantly different ($p < 0.05$)

365 The RDSC of the different extracts were evaluated and expressed as μmol of Trolox
366 Equivalent (TE) per gram of extract (Table 2). The less polar solvents, petroleum ether, ethyl
367 acetate and acetone, appeared inefficient to extract antioxidant components, as low RDSC
368 (lower than 50 $\mu\text{mol TE/g}$) were observed with these extracts. Those three extracts were not
369 significantly different according to p value (> 0.05). The water, methanol and ethanol 70%
370 (v/v) extracts conversely exhibited high RDSC between 420 to 490 $\mu\text{mol TE/g}$.

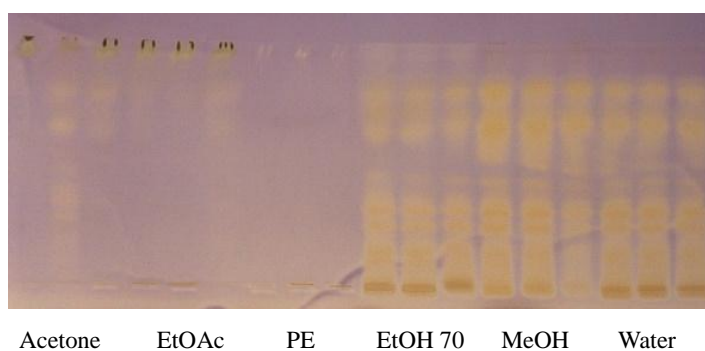
371 Secondary metabolites fingerprint of each extract was explored using HPTLC and 3 different
372 mobile phases: ethylacetate/acetic acid/formic acid/water (100:11:11:26, v/v/v/v) for
373 flavonoids analysis (Figures 2A and 2B), toluene/EtOAc (93:7, v/v) for lipophilic metabolites
374 (Figure 2C and S1A) and ethylformiate/toluene/formic acid (50:50:15, v/v/v) for medium
375 polarity compounds (Figure 2D and S1B) (Wagner and Bladt, 1996). For each
376 chromatographic conditions, an additional direct bioautography using DPPH was performed.

377 Using the flavonoid-dedicated mobile phase (Figure 2A), the most complex and flavonoid
378 rich fractions were obtained using water, methanol and ethanol 70% (v/v) as extraction
379 solvents. Similarly, the most intense antioxidant activities (Figure 2B) were observed after
380 DPPH direct bioautography with the same extracts and corresponded to the flavonoid patterns
381 previously observed at 365 nm, as we could expect. Among those three extracts, the
382 methanolic one exhibited the most representative profile of all metabolites. DPPH
383 bioautography performed on medium polarity and apolar metabolites plates led to an absence
384 of antioxidant activity detection (See Figure S1 Supplementary material). In conclusion, the
385 methanolic extract has several advantages: 1) the highest antioxidant activity (Figure 2B)
386 using HPTLC eluted with the flavonoids system combined with direct DPPH bioautography;
387 2) the most complete metabolite composition (flavonoids, apolar and medium polarity
388 components); 3) methanol is rapidly and easily evaporated. Thus this solvent was selected for
389 further extractions.

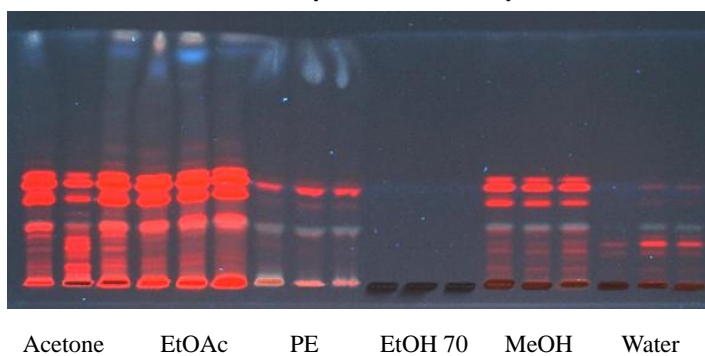
A. UV 365 nm after derivatization by Neu reagent



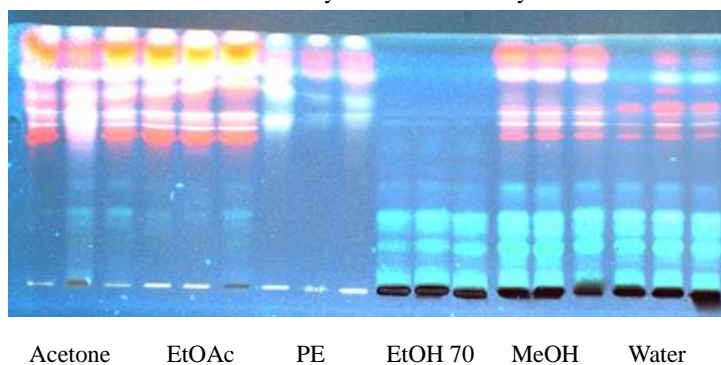
B. Bioautography by DPPH reagent



C. UV 365 nm after derivatization by sulfuric anisaldehyde



D. UV 365 nm after derivatization by sulfuric anisaldehyde



390

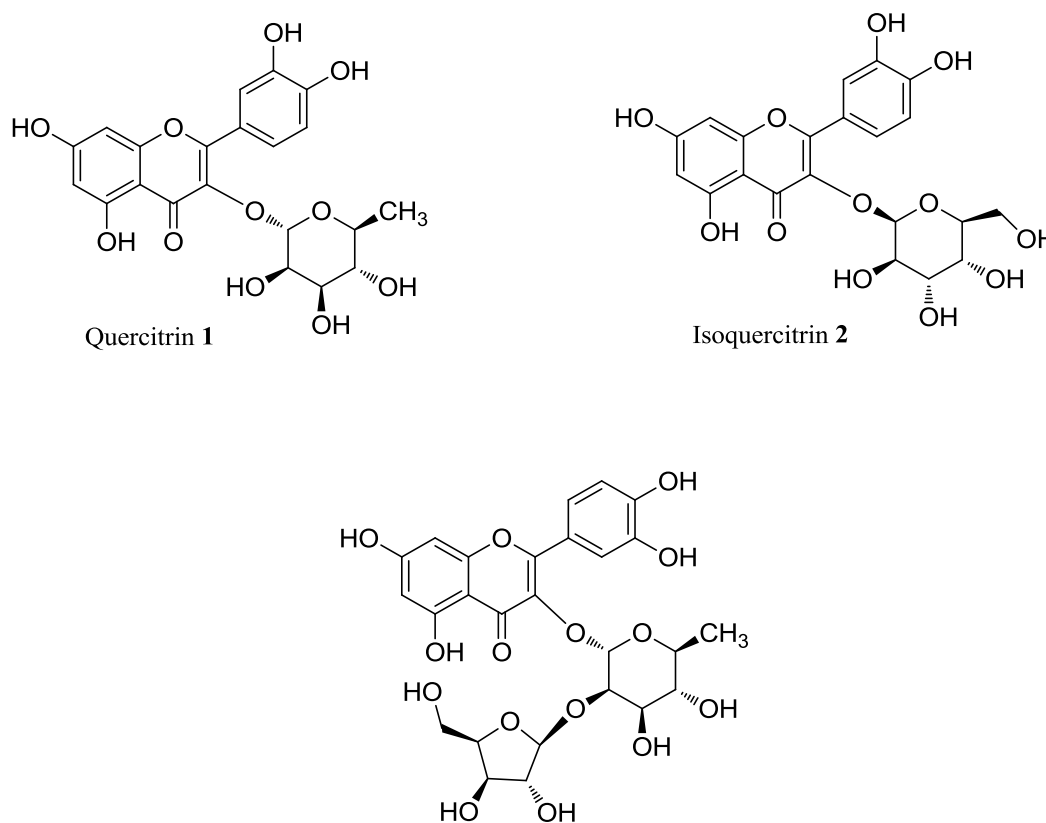
391 Figure 2 : Separation on HPTLC plates using ethyl acetate/acetic acid/formic acid/water

392 (100:11:11:26, v/v), at 365 nm after pulverization of Neu's reagent(A) and detection after

393 DPPH direct bioautography (B); using toluene/EtOAc (93:7, v/v) as mobile phase and
394 detection at 365 nm (C); using ethylformiate /toluene/formic acid (50:50:15, v/v/v) as mobile
395 phase and detection at 365 nm (D).

396 2. Isolation and identification of major compounds from the crude extract

397 The methanolic extract of the leaves of *C. adansonii* was resuspended in methanol-H₂O
398 (20:80, v/v) and then partitioned with *n*-butanol. The butanolic extract was fractionated by
399 preparative TLC to give the known flavonosides quercitrin **1**, isoquercitrin **2** (Figure 3). In
400 order to obtain quercetin-3-*O*-(β -D-xylopyranosyl- α -L-rhamnopyranoside) **3** (Figure 3), a
401 CPC fractionation of butanolic extract was required.



402 quercetin-3-*O*-[β -D-xylopyranosyl-(1 \rightarrow 2)- α -L-rhamnopyranoside] **3**

403 Figure 3: Structure of the three major isolated flavonosides.

404 These compounds were identified by comparing their physical and spectroscopic data to those
405 reported in the literature, and were selected as quality markers of *C. adansonii* extracts.

406 3. Validation of ancestral picking practices

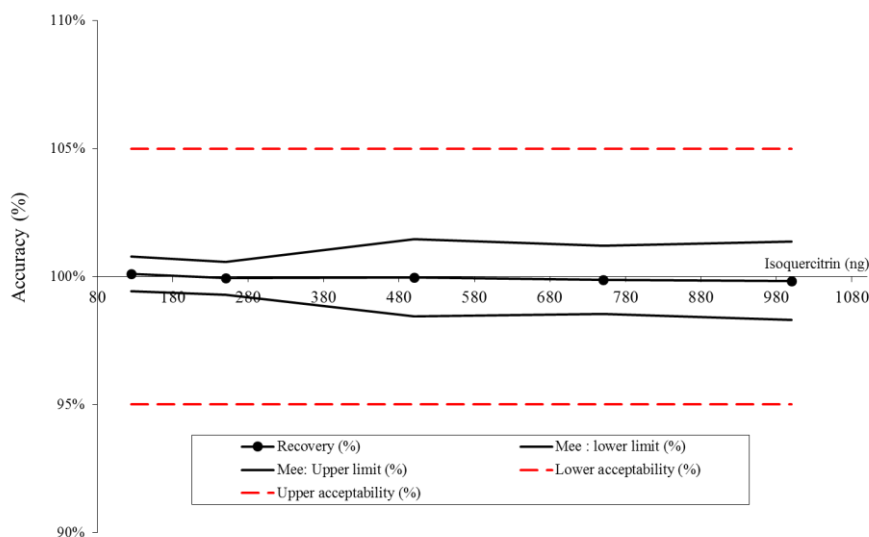
407 3.1. General methodology

408 For all conditions tested, the resulted extracts were compared using RDSC for antioxidant
409 activity and HPTLC-densitometry quantitation of the three major flavonoids: quercitrin **1**,
410 isoquercitrin **2**, quercetin-3-*O*-(β -D-xylopyranosyl- α -L-rhamnopyranoside) **3**.

411 HPTLC-densitometry quantitation assay was performed using a standard isoquercitrin
412 calibration curve and UV detection at 265 nm. Hence all major flavonoids in *C. adansonii*
413 extract exhibited the same UV spectra with absorption maximum at 265 nm and a quercetin
414 aglycone, and were thus titrated as isoquercitrin equivalent, using the isoquercitrin calibration
415 curve. This new assay was validated according to International Conference on Harmonization
416 (ICH) guidelines for analytical methods and using accuracy profile. Accuracy profile is a
417 graphical representation of accuracy, tolerance interval and acceptance limits, fixed by the
418 user (Feinberg, 2007).

419 Different dilutions of isoquercitrin stock solution were applied to HPTLC plates in order to
420 prepare five points linear calibration curve, ranging from 125 to 1000 ng. The correlation
421 coefficient (r^2) for isoquercitrin was 0.999, which indicated the high degree of correlation and
422 the good linearity of the method. LOD and LOQ were estimated according to signal-to-noise
423 ratio, of 3:1 for LOD and 6:1 for LOQ. LOD was found to be 31.25 ng whereas LOQ was
424 found to be 75 ng. All validation data were summarized Table S2, in Supplementary material.

425 Accuracy profile of isoquercitrin was built up (Figure 4), with acceptability limits fixed at
426 5%, value usually found in literature for densitometric measurements (Boudesocque et al.,
427 2013).



428

429 Figure 4: Accuracy profile of isoquercitrin

430 Tolerance intervals were contained in the 5% limits in the range of 125 to 1000 ng and the
431 method was validated.

432 3.2. Impact of the harvesting place

433 It is well known that harvesting place has an impact on secondary metabolites content of plant
434 material. In Lomé, traditional healers preferentially picked leaves in some neighborhood of
435 the city. To explore the real impact of the harvesting place, budding leaves were collected at
436 12:00 in various neighborhood of Lomé: Adidogomé, Bé, Campus of the University of Lomé,
437 and Djidjolé (Figure 1). Adidogomé is in the urban area of Lomé, Bé is located close to the
438 biggest lake of Lomé, and the Campus and Djidjolé are both located in the city center.

439 The trees grown in Djidjolé neighborhood exhibited a four-fold higher antioxidant activity
440 (RDSC about 425 $\mu\text{mol TE/g}$) than the trees in the Campus, and twice higher than trees

441 grown in Adidogomé or Bé neighborhoods, which were not significantly different ($p > 0.05$)
442 (Table 3). The major flavonosides content followed the same evolution, with higher amounts
443 observed in Djidjolé leaves extract (Table 3). All flavonoid amounts were significantly
444 different from each other ($p < 0.05$), indicating that flavonoids were potentially more
445 discriminating than global RDSC. The possible explanation for the differences between
446 harvesting places might be multifactorial; the tree age, the soil composition as well as the sun
447 exposure should be involved. Considering sun exposure, the direct normal irradiation (DNI)
448 of each place, according to GPS location, was estimated using the PhotoVoltaic Geographical
449 Information System (PVGIS) (European Council Joint Research Center). According to the
450 PVGIS, Djidjolé and Adidogomé showed closed DNI around 4800 W/m²/day while Lomé
451 University and Bé exhibited higher DNI around 5050 W/m²/day. No correlation was found
452 between those data and antioxidant activity or flavonoids amounts. It was then reasonable to
453 state that the DNI had a limited impact. The tree age in each harvesting place was also
454 collected. In Adidogomé, the *C. adansonii* trees were the youngest (10 years old), followed by
455 the trees growing in Djidjolé (13 years old) and in the Campus (15 years old). The oldest trees
456 were found in Bé (20 years old). Again no correlation could be highlighted between tree age
457 and RDSC or flavonosides rates. The last factor explored in this study was the soil pH at each
458 place. The soil pH were found to be 6.08 in the Campus, 7.38 in Bé, 7.81 in Adidogomé and
459 8.4 in Djidjolé. The lowest soil pH was found at the place exhibited the lowest antioxidant
460 activity and flavonosides amounts (Campus), while the highest soil pH was found at the place
461 exhibited the highest antioxidant activity and flavonosides amounts (Djidjolé). Moreover, Bé
462 and Adidogomé exhibited very closed soil pH as were their RDSC and flavonosides' rates. It
463 was then reasonable to state that soil pH soil affect the production of antioxidant metabolites,
464 especially flavonosides.

465

466 Table 3: Radical DPPH scavenging capacity of the extracts obtained with *C. adansonii* leaves harvested at different times, ages and places and
 467 major flavonosides content values in mass percentage, obtained by HPTLC-densitometry assay.

Extract	RDSC $\mu\text{mol TE/g}$ of extract (%RSD)	Quercitrin* (%) (% RSD)	Isoquercitrin (%) (% RSD)	Compound 3* (%) (% RSD)
<i>Harvesting place (budding leaves, 12:00)</i>				
Adidogomé	191.1 (3.9) ^a	1.24 (0.48) ^a	1.07 (0.68) ^a	0.22 (0.10) ^a
Bé	190.0 (3.8) ^a	1.59 (0.22) ^b	1.27 (0.25) ^b	0.24 (1.12) ^a
Campus	118.6 (4.0) ^b	0.31 (1.48) ^c	0.58 (0.85) ^c	0.22 (1.16) ^a
Djidjolé	426.2 (1.9) ^c	3.26 (0.64) ^d	3.69 (0.52) ^d	0.71 (0.11) ^b
<i>Leaves ages (Djidjolé,, 12:00)</i>				
Budding leaves	448.8 (3.5) ^a	3.29 (0.38) ^a	3.86 (0.28) ^a	0.61 (1.23) ^a
Young leaves	101.5 (1.9) ^b	2.64 (0.52) ^b	2.55 (0.56) ^b	0.62 (2.09) ^a
Old leaves	115.0 (1.0) ^b	1.16 (0.51) ^c	1.13 (0.65) ^c	0.27 (2.50) ^b
<i>Harvesting time (budding leaves, Lomé University Campus)</i>				
05:00	146.1 (3.2) ^a	0.67 (0.48) ^a	0.83 (0.54) ^a	0.37 (1.42) ^a
12:00	122.1 (3.9) ^b	0.36 (1.29) ^b	0.57 (0.75) ^b	0.22 (1.83) ^b
17:00	103.2 (3.8) ^c	0.17 (0.38) ^c	0.29 (1.09) ^c	0.19 (2.21) ^c
22:00	129.6 (3.0) ^b	0.59 (1.53) ^d	0.78 (0.37) ^d	0.27 (0.50) ^d

468 ^{a,b,c} average RDSC or flavonoid rate which did not share any letter were significantly different ($p < 0.05$), comparison occurred in each
 469 condition

470 * % (w/w) in isoquercitrin equivalent;

471

472 3.3. Impact of the leaves age

473 In order to investigate the recommendations of the traditional healers to harvest only budding
474 leaves, budding, young and old leaves were picked in Djidjolé neighborhood at noon,
475 extracted and analyzed.

476 As we can see in Table 3, there was a dramatic difference of antioxidant activity between
477 budding and young/old leaves. The budding leaves exhibited a RDSC about 4 fold higher
478 (450 $\mu\text{mol TE/g}$) than the two others, which were not significantly different ($p > 0.05$). In
479 Table 3, we could also noticed that even though the budding leaves showed higher rates of
480 quercitrin and isoquercitrin, the gap between budding and young leaves was smaller than for
481 antioxidant activity. This led to the two following hypothesis: 1. the flavonosides were not the
482 only metabolites responsible for the global antioxidant effect; 2. a small decrease of major
483 flavonosides amount led to a dramatic decrease of antioxidant activity, highlighting a possible
484 synergy of action inside this class of metabolites. Nevertheless, again, the traditional healers'
485 recommendation was validated.

486 3.4. Impact of the harvesting hour

487 Budding leaves of *C. adansonii* were sampled at different times of the day (05:00, 12:00,
488 17:00, and 22:00) in the campus of the University of Lomé, for convenient purpose.

489 Using RDSC DPPH assay, the highest antioxidant activities were obtained for the samples
490 harvested at 05:00 (146 $\mu\text{mol TE/g}$), while the lowest activity was measured in the middle of
491 the afternoon at 17:00 (103 $\mu\text{mol TE/g}$) (Table 3). The antioxidant activity decreased all day
492 long from the beginning of the day to rise again during night.

493 Using HPTLC-densitometry assay (Table 3), the major flavonosides were also contained in
494 higher rates in nighttime extracts, in accordance with the antioxidant activity variation.

495 Those results were in agreement with the classical circadian biosynthesis of flavonoids,
496 especially chalcone synthase circadian activation (Harmer et al., 2000) and validated the
497 traditional harvesting method that recommends to pick the leaves early in the morning close
498 to the dawn.

499 At this point, all recommendations from traditional healers were validated, according to
500 RDSC and flavonosides content of *C. adansonii* leaves. The optimal harvest should take place
501 just before dawn, in Djidjolé neighborhood, and be limited to budding leaves.

502 To complete this study, the potential beneficial effects of *C. adansonii* leaf extracts on skin
503 infections were investigated, as the main used of *C. adansonii* in Togo was to treat infected
504 wounds.

505 *4. Effect on the inflammation induced by S. aureus on primary keratinocytes and S. aureus* 506 *viability*

507 *S. aureus* is a leading cause of skin and soft tissue infections such as abscesses, furuncles, and
508 cellulitis. Keratinocytes, the predominant cell type in the epidermis which is the outermost
509 layer of the skin, play an important role in innate immune response during infections and
510 wound healing. In order to explore the potential beneficial effects of *C. adansonii* extracts on
511 infected skin, two different mechanisms of action were explored: anti-infectious activity
512 towards *S. aureus*, and anti-inflammatory activity. The effects of the extracts on the *S. aureus*
513 viability as well as the inflammatory response of primary keratinocytes induced by *S. aureus*
514 were evaluated.

515 *4.1. Selection of C. adansonii extracts*

516 To elucidate the activity of *C. adansonii* extracts and to investigate the impact of picking
517 practices on this activity, three extracts were selected: 1) budding leaves harvested at 05:00,

518 as potential maximum activity; 2) old leaves harvested at 17:00, as potential minimum
 519 activity; 3) budding leaves harvested at 12:00, to check if RDSC gap was a good indicator of
 520 biological activity gap. All leaves were collected in Djidjolé neighborhood to avoid
 521 misinterpretation due to harvesting place.

522 *In vitro* data of these three extracts (RDSC and flavonosides rates) were described in Table 4.

523 Table 4: RDSC and flavonoid rates of *C. adansonii* extracts, biologically tested

Extract	RDSC ($\mu\text{mol TE/g}$) (% RSD)	Quercitrin* (%) (% RSD)	Isoquercitrin (%) (% RSD)	Compound 3* (%) (% RSD)
Budding leaves 05:00	513.3 (0.5) ^a	4.21 (0.9) ^a	4.66 (0.4) ^a	0.98 (1.6) ^a
Budding leaves 12:00	426.2 (1.9) ^b	3.29 (0.4) ^b	3.86 (0.3) ^b	0.61 (1.2) ^b
Old leaves 17:00	130.5 (1.9) ^c	0.39 (0.5) ^c	0.37 (1.3) ^c	0.40 (2.0) ^c

524 ^{a,b,c} average RDSC or flavonoid rate which did not share any letter were significantly different ($p < 0.05$)

525 * % (w/w) in isoquercitrin equivalent

526 As observed previously with the sample collected in the Campus, the budding leaves collected
 527 at 5:00 exhibited higher RDSC and higher flavonosides amounts than budding leaves
 528 collected at noon. The difference between these samples was more significant than the one
 529 observed with Campus samples, probably due to their higher activity. Also as expected from
 530 our previous dataset, the old leaves collected at 17:00 showed the lowest RDSC and
 531 flavonosides amounts.

532 4.2. Anti-bacterial effect

533 The XTT reduction assay indicated that the extracts of budding leaves harvested at 5:00 and
 534 12:00 (BL5 and BL12 respectively) reduced the viability of *S. aureus* at a concentration of
 535 100 $\mu\text{g/mL}$ (Figure S6). In contrast, the extract of old leaves harvested at 17:00 (OL17) had
 536 no effect on *S. aureus* viability. In addition, none of the extracts inhibited the growth of *S.*

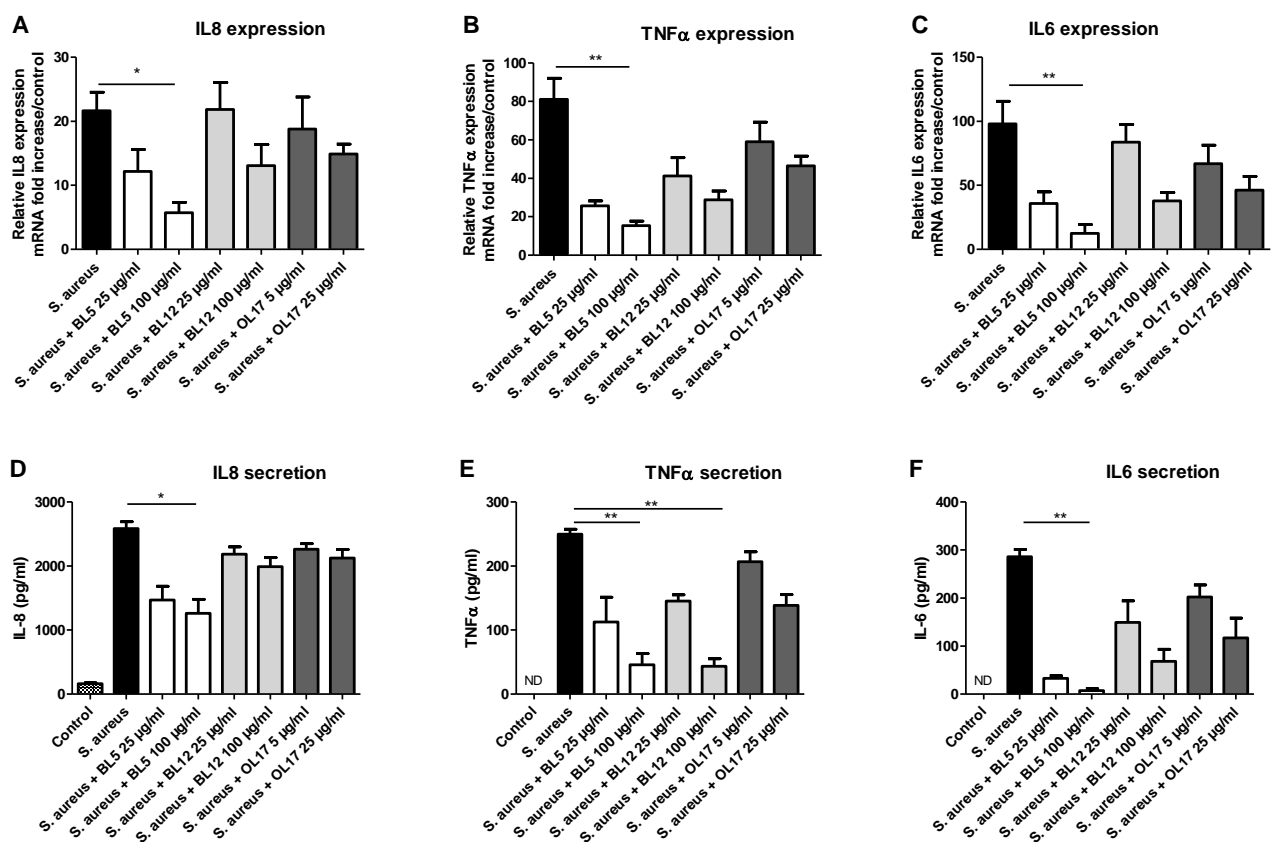
537 *aureus* in liquid medium (Figure S7). These results suggest that *C. adansonii* extracts did not
538 exert potent antibacterial activity against *S. aureus*.

539 4.3. Anti-inflammatory activity

540 *S. aureus* is known to induce keratinocyte production of IL8, a neutrophil-activating
541 chemokine contributing to establishment of a chemotactic gradient during inflammation, and
542 TNF α , a potent pro-inflammatory cytokine (Tankersley et al., 2014). The effect of *C.*
543 *adansonii* extracts was investigated on IL8 and TNF α expression and production by
544 keratinocytes. Preliminary experiments have shown that budding leaf extracts at a final
545 concentration of 100 $\mu\text{g/mL}$ had no impact on primary keratinocyte viability in contrast to the
546 old leaves extract which showed cytotoxic effect at concentrations higher than 25 $\mu\text{g/mL}$, *e.g.*
547 the cell viability was reduced by about 20% at a final concentration of 50 $\mu\text{g/mL}$ (Figure S8).
548 Consequently, extracts of budding leaves (BL5 and BL12) were tested at final concentrations
549 of 25 and 100 $\mu\text{g/mL}$ and extract of old leaves (OL17) was tested at final concentrations of 5
550 and 25 $\mu\text{g/mL}$. As recently reported by Zingue et al. (2016), *C. adansonii* bark extract
551 exhibited cytotoxicity towards breast carcinoma cell lines suggesting that old leaves may
552 contain potential similar cytotoxic compounds than bark. Besides, the extracts showed no
553 effect on the basal levels of cytokine produced by keratinocytes (data not shown).

554 The BL5 extract exhibited a potent anti-inflammatory effect on keratinocytes infected by *S.*
555 *aureus*. Indeed, IL6, IL8 and TNF α expression and secretion induced by *S. aureus* were
556 significantly inhibited in presence of 100 $\mu\text{g/mL}$ of BL5 (Figure 5A to F). A significant
557 inhibition of TNF α secretion but not of IL6 and IL8 secretion was also observed in presence
558 of 100 $\mu\text{g/mL}$ of BL12 (Figure 5D and F). On other hands, OL17 extract had no significant
559 impact on IL6, IL8 and TNF α levels secreted by *S. aureus*-stimulated keratinocytes. The anti-
560 inflammatory effects of *C. adansonii* extracts were more pronounced with the BL5 extract

561 which had the highest flavonoid content (Table 4). These compounds, including quercitrin or
 562 isoquercitrin, possess anti-inflammatory properties through inhibition of the NF- κ B and AP-1
 563 pathways (Comalada et al., 2005; Kim et al., 2013; Valentová et al., 2014), and might be
 564 involved in the biological effect of BL5 observed on *S. aureus* induced inflammatory
 565 reactions. It can be hypothesized that a combination of several compounds is responsible for
 566 the anti-inflammatory activities of the BL extracts as usually observed with various herbal
 567 medicines (Efferth and Koch, 2011).



568
 569 Figure 5. IL6, IL8 and TNF α mRNA expression and secretion after infection of primary
 570 keratinocytes by *S. aureus* for 24 h in the presence or absence of *C. adansonii* extracts. Cells
 571 were infected by *S. aureus* at a MOI of 1 for 24 h after 2 h incubation with or without *C.*
 572 *adansonii* extracts. qRT-PCR analysis was carried out on total RNA of 5 independent cultures
 573 from different individuals. mRNA expression levels are expressed as the fold increase above

574 unstimulated cultures. Protein concentrations (pg/mL) were measured in culture supernatants
575 of 5 independent cell cultures from different individuals by ELISA assays. Data are
576 represented as mean + SEM. * $p < 0.05$, ** $p < 0.01$ compared to control. ND: not detected.

577 During wound healing a key step is the transition from the inflammatory to the proliferative
578 phase (Landén et al., 2016). Prolonged inflammation is detrimental and may result in
579 deregulated differentiation and activation of keratinocytes, impeding the progress through the
580 normal stages of wound healing (Mustoe et al., 2006). Our results suggest that *C. adansonii*
581 extracts, especially BL5, may have beneficial effects on *S. aureus* cutaneous infection through
582 moderation of inflammatory response. Indeed, it was reported that local inflammation
583 exacerbated the severity of *S. aureus* skin infection (Montgomery et al., 2013). In addition, it
584 has been suggested that suppression of inflammation may be beneficial for promoting healing
585 of chronic wounds and reducing scarring (Hofman et al., 2007; Landén et al., 2016). The anti-
586 inflammatory properties of the BL5 extract, by reducing the release of TNF α , might benefit
587 the healing of infected wounds (Cowin et al., 2006; Donath, 2014). Recent preclinical
588 research demonstrated that therapies moderating the inflammatory response to infection
589 without attempting to kill microbes could be a successful approach (Spellberg et al., 2013). In
590 other hands, therapeutic strategies combining antibacterial and anti-inflammatory activities
591 may be efficient to manage *S. aureus*-induced skin lesions (Mohamed and Seleem, 2014).
592 Thus, this study suggests that *C. adansonii* leaves preparations may be beneficial in the
593 management of infected skin lesions either alone or in combination with antibacterial
594 compounds or plant extract.

595 **5. Conclusion**

596 The results of this study validate the traditional practices and the potential of *C. adansonii* as
597 anti-inflammatory drug. The optimal harvest of *C. adansonii* leaves was found to be the

598 picking of budding leaves early in the morning. In Lomé, the Djidjolé neighborhood was
599 found to be the best place to pick leaves, with the highest antioxidant activity. This point
600 should be submitted to further investigations in order to understand which environmental
601 factors were implied. Our preliminary data highlighted a possible role of the soil pH, with an
602 increased activity correlating with an increase of pH value, whereas sun exposure and tree age
603 did not seem to play a role. The biological investigation of *C adansonii* extract demonstrated
604 that the optimal picking recommended by traditional healers led to the best anti-inflammatory
605 activities on IL6, IL-8 and TNF- α . Interestingly, old leaves were less active on inflammatory
606 process but also exhibited a higher toxicity towards keratinocytes than budding leaves.

607 Considering the antibacterial activity, our findings suggested that traditional healers should
608 add to *C. adansonii* leaves an antibacterial plant of Togo Pharmacopeia, in order to improve
609 abscess healing.

610

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617

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