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1 **Phytosteranes and phytosterols : Bioactive compounds in aerial parts of *Acacia***
2 ***cyanophylla* Lindl.**

3

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23 **Note:** C Messaoud and C Vigor contribute equally to this work.

24 ⁱ

ⁱ **Abbreviation** : ALA, α -linolenic acid; PUFA, Polyunsaturated fatty acid; PhytoPs, Phytosteranes; PhytoFs, Phytosterols; ROS, reactive oxygen species; fw, fresh weight; BHT, butylated hydroxytoluene; LC-MS/MS, liquid chromatography–mass spectrometry; SPE, solid phase extraction; HPLC, high-pressure liquid chromatography; LOD, the limit of detection; LOQ, the limit of quantification; IS, Internal Standard; MRM, monitoring reaction mode; EY, extraction yield; ME, matrix effect; SD, standard deviation; QTRAP, triple-quadrupole combined to ion trap mass spectrometer.

Abstract

The relevance of oxylipins as biomarkers of oxidative stress has been established in recent years. Phytosteranes and phytofurans are plant metabolites derived from peroxidation of α -linolenic acid (ALA) induced by ROS. Previous findings have suggested new valuable biological properties for these new active compounds in the frame of diverse pathophysiological situations and health constraints. Lipidomic profiling of different aerial parts of the same *Acacia cyanophylla* Lindl. specimen, was evaluated for the first time here, using LC-MS/MS technology. Analysis revealed the existence of six PhytoPs and three PhytoFs. Stems have the highest amount of these metabolites with 179.35 ng/g and 320.79 ng/g respectively. This first complete profile paves the way to explore *Acacia cyanophylla* Lindl. as a source of plant oxylipins for therapeutic or pharmaceutical uses.

Keywords:

Acacia cyanophylla Lindl., plant non-enzymatic oxylipins, micro-LC-MS/MS.

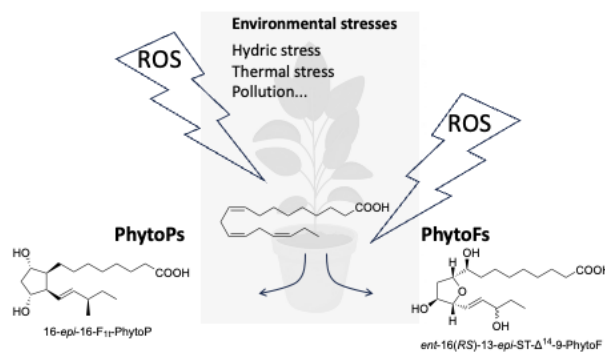
25 **1. Introduction**

26 Plants have been a major source of several important chemical compounds and therapeutic
27 agents for the cure of human diseases since a long time. Realizing their importance, different
28 parts of plant are extensively explored as new sources of phytochemicals. *Acacia* is the
29 second largest genus in the *Fabaceae* family with more than 1350 species worldwide in warm
30 sub-arid and arid parts of the world.¹ It tolerates different type of soils and lives in pure stands
31 or combined with other species. *Acacia* genus is characterized by distinctive leaves which
32 take the form of small finely divided leaflets that give the leafstalk. Flowers are small, often
33 fragrant and arranged in compact globular or cylindrical clusters. They are usually yellow but
34 occasionally white and have many stamens apiece, giving each one a fuzzy appearance. Pods
35 and seeds are highly variable in appearance, depending on the species.²

36 Although the main use of *Acacia* is as a fodder source and in soil stabilization, many studies
37 proved that *Acacia* species contains a variety of bioactive components which are responsible
38 for numerous biological and pharmacological uses. They have long been used in folk
39 medicine and have been shown to possess hypoglycemic, anti-bacterial, anti-hypertensive,
40 analgesic, and anti-atherosclerotic due to their strong antioxidant and free radical scavenging
41 activities.³ Other authors reported its spasmogenic and vasoconstrictor actions⁴ and also its
42 cytotoxic activities.⁵ This important therapeutic value is related to the high level of bioactive
43 compounds in *Acacia* species.¹

44 Usually, biosynthesis of specific metabolites is limited to specific stages of plant development
45 and periods of stress.⁶ Plant cells produce phytochemicals to maintain their survival and
46 proper function. In most cases, they are a result of the interactions between plant and
47 environment as form of protection against predators, pathogens, or environmental stress.
48 Some others are related to the reproductive mechanism of the plant. Phytochemicals can be
49 found in various parts, including stems, leaves, roots, seeds, fruits, and flowers. Their

50 quantitative and qualitative profile could be influenced by several environmental factors such
 51 as temperature, humidity, light intensity, water, mineral salts and CO₂.⁶ For this reason, in
 52 traditional medicine, each part of the plant does not necessarily have the same therapeutic
 53 action, and usually, the part that contains the most active ingredients is the most used.
 54 Phytoprostanes (PhytoPs) and phytofurans (PhytoFs), are some plant oxylipins formed by a
 55 non-enzymatic peroxidation of α-linolenic acid (ALA). They are biomarkers of plant
 56 oxidative degradation and biologically active molecules involved in many plant defense
 57 mechanisms.⁷ Imbusch and Mueller are the first who have identified these new active
 58 compounds in various plant matrices.^{8,9} The possible structures resulting from the oxidation of
 59 ALA are A₁-PhytoPs, B₁-PhytoPs, D₁-PhytoPs, and L₁-PhytoPs. It seems that A₁-PhytoPs and
 60 B₁-PhytoPs are generally found in low abundance in plants, whereas D₁-PhytoPs and F₁-
 61 PhytoPs are found in higher quantities.¹⁰
 62 PhytoPs and PhytoFs are good indicators of oxidative stress.¹¹ When a plant is exposed to
 63 unfavorable environmental conditions such as water deficit, salinity, extreme temperatures, or
 64 excessive light, the production of reactive oxygen species (ROS) becomes excessive and can
 65 be deleterious to different biomolecules. As unsaturated lipids are the most sensitive to ROS
 66 attack, they are the first to undergo a lipid oxidation reaction which leads to an increase in the
 67 production of PhytoPs and PhytoFs (Figure 1).¹²



68

69

Fig.1. Oxylipins from ALA non-enzymatic oxidation

70

71 These compounds have been also, associated with a variety of biological activities in
72 mammals. As lipid mediators,^{13,14} PhytoPs and PhytoFs play a significant role in prevention
73 of some diseases. They are implicated in protection of cells against the damage produced
74 under oxidative stress conditions,¹⁵ they play an important role in the immune and neuronal
75 function regulation,¹⁶⁻¹⁸ and developing anti-inflammatory and apoptosis-inducing
76 activities.^{19,20} These actions are being recently associated with the structural similarities of
77 PhytoPs and PhytoFs vs. human oxylipins.⁷ LC-MS/MS approach allows the separation,
78 identification and quantification of this isoprostanoids derivatives with selectivity and
79 sensitivity.¹⁹ PhytoP and PhytoF were detected, for the first time, in a new study on *Acacia*
80 *cyanophylla* seeds collected in Tunisia from 7 ecotypes.²⁰ This first and complete profile
81 proves that *Acacia cyanophylla* is a new source of bioactive compounds for therapeutic and
82 pharmaceutical applications. To the best of our knowledge, until now, the chemical
83 composition of different part of *Acacia cyanophylla* has not been reported. Accordingly, this
84 present work aims to identify and quantify plant oxylipins in stem, leaf, pod, and flower using
85 liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS).

86 2. Material and methods

87 2.1. Plant Materials and Sampling

88 Stems, leaves, and flowers of *Acacia cyanophylla*. Lindl were collected during the blooming
89 stage in spring (Mars 2018) and fully mature fruits (pods and seeds) were harvested in
90 summer (June 2018) from the region of Tunis situated in the north of Tunisia, at random from
91 at least five trees spaced 10 meters apart. All samples were treated as one during extraction
92 procedures. Biological replicates were used to limit variation due to the sampling process.
93 Botanical identification of species was carried out by Professor Mohamed Larbi Khouja.
94 Voucher specimens (AC_S 2018, AC_L 2018, AC_P 2018, AC_F 2018 and AC_SE 2018)
95 were deposited in the Herbarium of the Laboratory of nanobiotechnology and valorization of
96 medicinal phyto-resources of the National Institute of Applied Science and Technology of
97 Tunis (INSAT). During series of observations, the phenological state is assessed for each
98 individual. This involves highlighting the leafing, flowering, and fruiting phases according to
99 a global visual method. Harvesting is a very important step; it must be carried out at the most
100 favorable time to maintain the effectiveness of the active compounds. All samples were shade
101 dried, powdered, and stored in a tightly closed container for further use.

102 2.2. Chemicals and Reagents

103 All the oxylipin analytical standards, coming from oxidation of the C18 n-3 ALA, as well as
104 the internal standard (IS) mixture C19-16-F_{1t}-PhytoP and C21-15-F_{2t}-IsoP, used to determine
105 the calibration curve ratio, were synthesized according to our previously described
106 procedures. PhytoP analytical standards are as follows: *ent*-9-L₁-PhytoP, *ent*-16-B₁-PhytoP,
107 16(*RS*)-16-A₁-PhytoP, 16-F_{1t}-PhytoP, 16-*epi*-16-F_{1t}-PhytoP, 9-F_{1t}-PhytoP and 9-*epi*-9-F_{1t}-
108 PhytoP. PhytoF analytical standards are *ent*-16(*RS*)-9-*epi*-ST- Δ^{14} -10-PhytoF, *ent*-9(*RS*)-12-
109 *epi*-ST- Δ^{10} -13-PhytoF and *ent*-16(*RS*)-13-*epi*-ST- Δ^{14} -9-PhytoF. Only the analytical standard,
110 16(*RS*)-16-A₁-PhytoP was purchased from Cayman Chemicals (Ann Arbor, MI, USA). Liquid

111 chromatography–mass spectrometry (LC-MS) grade solvent (methanol, acetonitrile), and
112 HPLC grade solvent (chloroform) were purchased from Fisher Scientific (Loughborough,
113 UK). Hexane, acetic acid, formic acid, absolute ethanol, ammonia, and potassium hydroxide
114 (KOH) were obtained from Sigma-Aldrich (Saint Quentin Fallavier, France). Ethyl acetate
115 (HPLC grade), and butylated hydroxytoluene (BHT) were supplied by VWR (EC), and
116 ACROS ORGANIC (Geel, Belgium), respectively. Water used in the present work, was
117 provided by Fisher Scientific (Loughborough, UK). Solid-Phase Extraction SPE Oasis MAX
118 3cc cartridges (60 mg) were obtained from Waters (Milford, MA, USA).

119 *2.3 Preparation of Samples for Lipidomic Analysis*

120 A protocol like what was described for our previous work on *Acacia cyanophylla* Lindl. seeds
121 was applied for lipidomic analysis.²⁰ Extraction of PhytoPs and PhytoFs from different parts
122 of *Acacia cyanophylla* Lindl. was performed by SPE, following our methodology previously
123 optimized and validated. 200 mg of powder was added in grinding matrix tubes (Lysing
124 matrix D, MP Biochemicals, Illkirch, France) with 1 mL of MeOH, 25 μ L of butylated
125 hydroxytoluene (BHT), and 4 μ L of Internal Standard (each IS at 1 μ g/mL). Tubes were placed
126 in a FastPrep-24 (MP Biochemicals), and samples were ground for 30 seconds, 6.5 m/s, (1
127 cycle) at room temperature. After that, suspensions were transferred into a 15 mL centrifuge
128 tube. The lysis tubes were rinsed at first with 1mL MeOH, and then with 1.5 mL of phosphate
129 buffer (pH 2) saturated in NaCl. Samples were shaken again for 1h (100 rpm-45sec / 90°-
130 15sec), at room temperature. Subsequently, the mixture was centrifuged at 5000 rpm for 5
131 min at 25°C, the supernatant was separated, and added by 4 mL of cold chloroform. This
132 solution was vortexed for 30 seconds and then centrifuged at 2000 rpm for 5 min at cold
133 temperature. Afterward, the aqueous phase was eliminated, and the organic phase was
134 recovered in a Pyrex tubes. The solvent was dried under a stream of nitrogen at 40°C for 1 h.
135 Thereafter, lipids were hydrolyzed with 950 μ L of KOH (1M / H₂O) for 30 min at 40°C. After

136 incubation, and before running the SPE separation, SPE Oasis MAX cartridges were
137 conditioned with 2 mL of MeOH and equilibrated with 2 mL of formic acid (20 mM, pH 4.5).
138 After sample loading, and to remove other compounds, the cartridges were successively
139 washed with : 2 mL of NH₃ 2 % (v/v), 2 mL of MeOH / formic acid 20 mM (30:70, v/v), 2
140 mL of hexane, and finally 2 mL of hexane: ethyl acetate (70:30, v/v). The column was dried
141 for 1 to 2 minutes. Next, targeted metabolites were eluted with 2x1 mL of
142 hexane:ethanol:acetic acid mixture (70:29.4:0.6, v/v/v). Lastly, all samples were evaporated to
143 dryness under a nitrogen flow at 40°C. To recover the sample, 100 µL of H₂O/ ACN (83:17,
144 v/v) were added. The mixture was transferred in the filtering Eppendorf® 0.45 µm to be
145 centrifuged at 10 000 rpm for 1 min. The filtrate was then ready to be injected. Each sample
146 was extracted in triplicate.

147 *2.4 Extraction Yield and Matrix Effect*

148 For a better description of oxylipin profiles in different parts of *A. cyanophylla*, two analytical
149 parameters were determined: extraction yield (EY) and matrix effect (ME). These parameters
150 fully describe the impact of sample processing on the determination of the content of different
151 compounds since they allow the evaluation of product losses at the time of extraction, using
152 spiking experiments for instance. All samples were analyzed using the LC-MS/MS system
153 described below. For each analyte, the extraction yield from the SPE column and the matrix
154 effect at two different concentrations (32 and 256 pg/µL) which correspond to two calibration
155 range points were determined. The EY was calculated as the percentage difference between
156 peaks areas of standards in pre-spiked and post-spiked samples. To this aim, two different
157 types of samples were prepared. The first one where the analytes will be introduced into the
158 matrix before the column, it will be called BEFORE and the second one where the analytes
159 will be introduced into the matrix after the column (in the elution vial) it will be called
160 AFTER. For the two concentrations of standards (32 and 256 pg/µL of compounds with IS),

161 results were expressed in percentage ($EY = A_{\text{Before}} / A_{\text{After}} * 100$). The ME was
162 determined as the percentage difference between peak areas of standard added to the extracted
163 samples (post-spiked sample) and pure standards diluted into the mobile phase : $ME = A_{\text{After}}$
164 $/ A_{\text{Pure}} * 100$.

165 *2.5 Micro-LC-MS/MS Analysis*

166 All analyses were carried out using a micro liquid chromatography equipped with CTC
167 Analytics AG (Zwingen, Switzerland) on a HALO C18 analytical column (100 * 0.5 mm, 2.7
168 μm ; Eksigent Technologies, CA, USA) kept at 40 °C and the autosampler vial tray was kept
169 at 10 °C. The mobile phase consisted of a mixture of water with 0.1 % (v/v) formic acid
170 (solvent A) and acetonitrile:methanol (80:20, v/v; with 0.1 % (v/v) of formic acid) (solvent
171 B). The elution was performed at a flow rate of 0.03 mL/min using the following gradient
172 profile (min/%B): 0/17, 1.6/17, 2.85/21, 7.3/25, 8.8/28.5, 11/33.3, 15/40, 16.5/95 and 18.9/95
173 and then returned to the initial conditions. No sample contamination or sample-to-sample
174 carryover was observed, under these conditions. Mass spectrometry analyses were performed
175 on an AB SCIEX QTRAP 5500 (Sciex Applied Biosystems, ON, Canada). The source of
176 ionization was an electrospray (ESI), operated in the negative mode. The source voltage was
177 kept at - 4.5 kV and nitrogen was used as curtain gas.

178 The analysis was conducted by monitoring MRM transitions that means following couple
179 formed with precursor ion and product ion. The selected multiple ion monitoring (MRM) of
180 each compound was predetermined by MS/MS analysis to define two transitions for
181 quantification (T1) and specification (T2). Peak detection, integration and quantitative
182 analysis were performed by MultiQuant 3.0 software (Sciex Applied Biosystems). The
183 quantification of oxylipins was based on calibration curves obtained from the analyte to IS
184 area under the curve ratio. Linear regression of six concentrations of standards mixture (16,

185 32, 64, 128, 256 and 512 pg/ μ L) of each standard were calculated. The sensitivity of the
186 method was evaluated through the limit of detection (LOD) and the limit of quantification
187 (LOQ). These parameters defined as the lowest concentration with a signal-noise ratio above
188 3 and 10, respectively, are dependent on the type of isoprostanoids and vary from 0.16 to 0.63
189 pg injected (LOD) and 0.16 to 1.25 pg injected (LOQ).

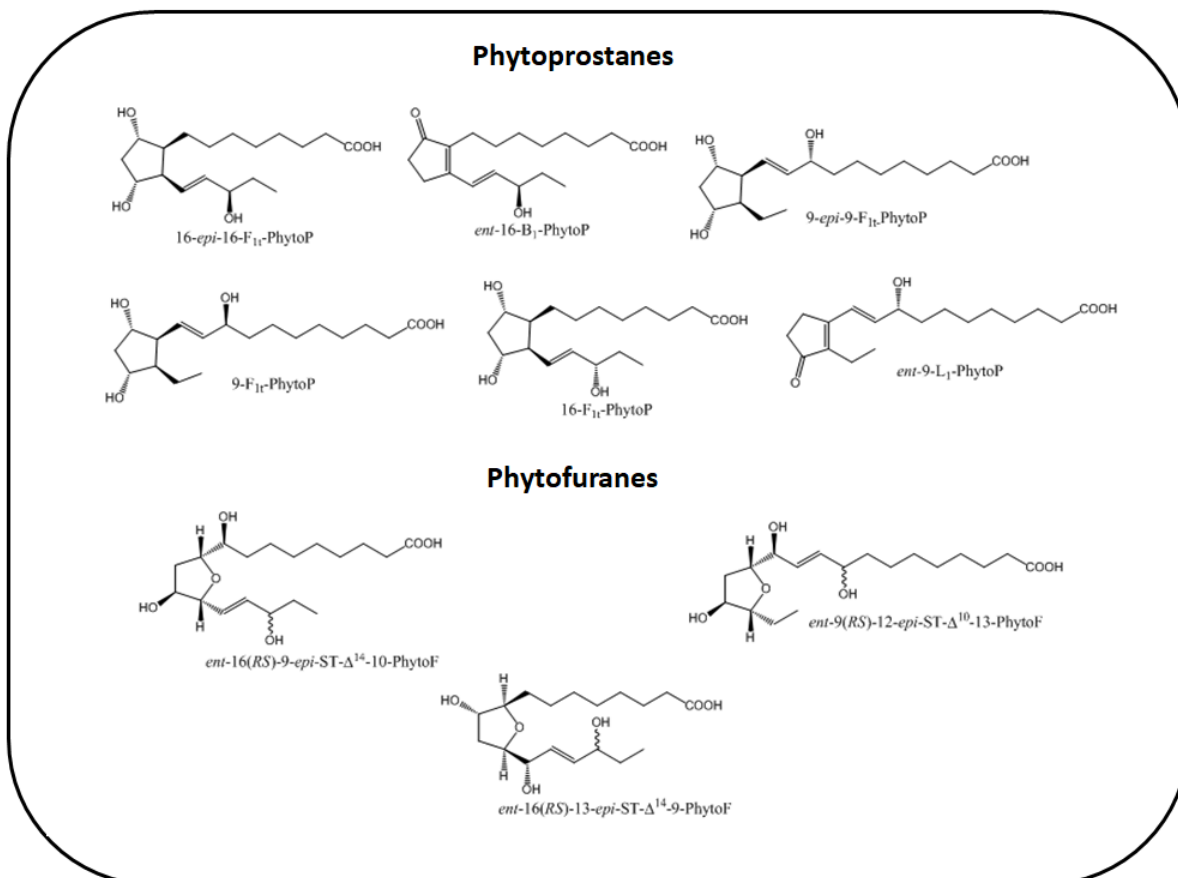
190 2.6 Statistical analysis

191 All samples are performed in triplicate (n=3), and results were provided as means \pm standard
192 deviation (SD). Analysis of variance (ANOVA) and Duncan's multiple range test were
193 performed using SPSS Version 20, to evaluate the significance of differences between
194 individual chemical contents of samples at the level of $P < 0.05$.

195 3. Results and discussion

196 3.1 Qualitative and quantitative PhytoP profile in aerial parts of *Acacia cyanophylla* Lindl.

197 PhytoPs were identified on the basis of their mass spectra, considering their molecular ion
198 (m/z 327.2 and 307.2), their fragmentations, and their elution order according to their
199 retention times.¹⁹ Six PhytoPs were identified and quantified for the first time in stems,
200 leaves, pods, flowers, and seeds of *Acacia cyanophylla* Lindl. : *ent*-16-B₁-PhytoP, 9-F_{1t}-
201 PhytoP, 9-*epi*-9-F_{1t}-PhytoP, *ent*-9-L₁-PhytoP, 16-*epi*-16-F_{1t}-PhytoP, and 16-F_{1t}-PhytoP. Their
202 chemical structures were shown in figure 2. The identified PhytoPs belonging to two series (9
203 and 16) of the F_{1t}-PhytoP, B₁-PhytoP and L₁-PhytoP classes, were previously detected in other
204 plant materials.^{7,12} In our study, MRM transitions allowed to readily classify PhytoPs or
205 PhytoFs belonging to different series. F₁-PhytoPs represented an interesting case. This class
206 of compounds was also found to occur in leaves, flowers and roots of taxonomically distinct
207 plant species, as main class.⁸



208

209

Fig.2. Chemical structures and names of plant oxylipins

210 F₁₁-PhytoPs were characterized by the MRM transition (m/z) 327.2 → 283.2, which is

211 common to 9-F₁₁-PhytoP (+9-*epi*-9-F₁₁-PhytoP) and to 16-F₁₁-PhytoP (+16-*epi*-16-F₁₁-PhytoP).

212 This first transition m/z allowed the metabolites to be quantified. A second MRM transition

213 was considered, to distinguish between these compounds. The 9-F₁₁-PhytoP and 9-*epi*-9-F₁₁-

214 PhytoP presented the second MRM transition (m/z) 327.2 → 171.2, whereas 16-F₁₁-PhytoP

215 and 16-*epi*-16-F₁₁-PhytoP were detected by following the MRM transition (m/z) 327.2 →

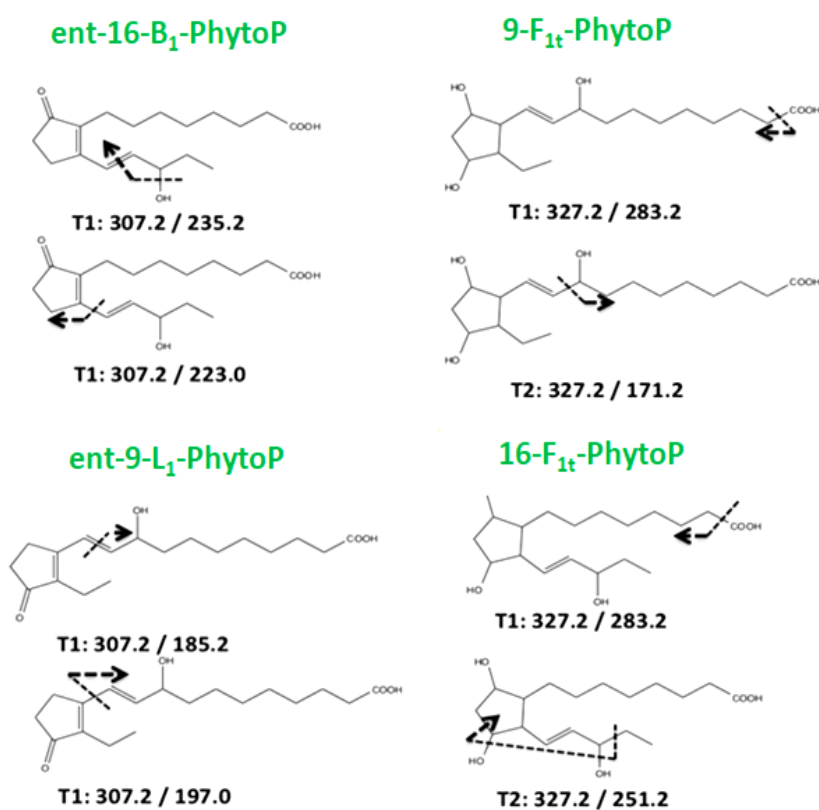
216 151.2. Based on the combination of transitions 1 and 2, the 9-F₁₁-PhytoP and 16-*epi*-16-F₁₁-

217 PhytoP have been quantified. For B₁-PhytoP, and L₁-PhytoPs, the main transition of each

218 series was already specific. A precursor ion at m/z 307 and a product ion at m/z 235 were

219 observed for B₁-PhytoP, whereas a precursor ion at m/z 307 and a product ion at m/z 185 for

220 L₁-PhytoP (Figure 3).

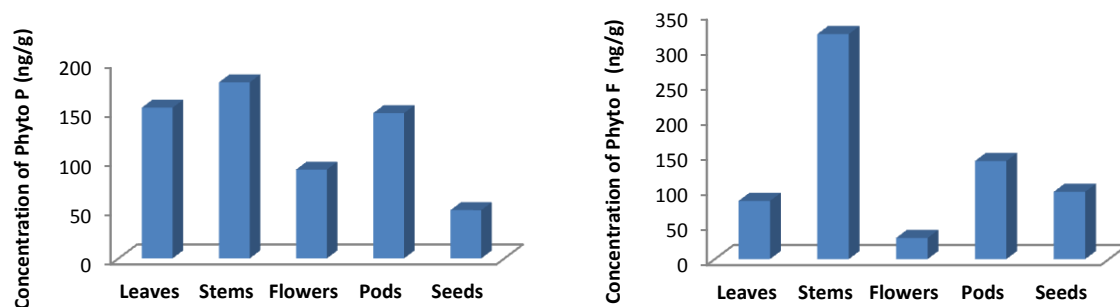


221

222 **Fig.3.** Phytoprostanoid characteristic fragmentations. T1: transition 1; T2: transition 2

223 All aerial parts of *Acacia cyanophylla* Lindl. had the same qualitative profile with a difference
 224 in the concentration. The total/analyzed contents of PhytoPs varied from 49.1 to 179.35 ng/g.
 225 The highest concentration was obtained in vegetative organs : stems (179.35 ng/g) and leaves
 226 (153.51 ng/g), followed by pods (148.03 ng/g), flowers (90.46 ng/g) and seeds (49.1 ng/g)
 227 (table 1, figure 4). Quantitative profile showed that the *ent-9-L₁-PhytoP* was the major
 228 compound detected for all parts with a level ranging from 9.75 ng/g in flowers to 51.79 ng/g
 229 in stems. Contrary to our result, this compound was reported as minor PhytoPs in melon
 230 leaves.¹² The 16-F_{1t}-PhytoP was the compound with the lowest content varying from 3.32
 231 ng/g in seeds to 18.37 ng/g in leaves of *Acacia cyanophylla* Lindl. This same metabolite was
 232 identified as the most abundant PhytoP in melon leaves (1160.0 ng/g).

233 Furthermore, the 9-F_{1t}-PhytoP was reported as the second major compound in aerial parts of
 234 *Acacia cyanophylla* Lindl. and was detected at high concentrations from 6.58 ng/g in seeds to
 235 45.00 ng/g in stems. In some plant material as almonds²¹ and some macroalgae species,²² the
 236 9-series of F_{1t}-PhytoPs represented the major PhytoPs.



237 **Fig. 4.** Total concentration of PhytoPs and PhytoFs in different organs of *Acacia cyanophylla*
 238 Lindl. determined by micro LC-MS/MS and expressed in ng/g.
 239

240 Variance analyses indicated a highly significant difference ($P < 0.001$) between the plant
 241 parts in levels of PhytoPs. As observed with the qualitative profile, the quantification of
 242 phytoprostanes in this present work, revealed significant variability depending on the plant
 243 part. Stems contained the highest concentration oscillating from 10.54 to 51.79 ng/g. The 16-
 244 F_{1t}-PhytoP was the compound with the lowest content and *ent*-9-L₁-PhytoP having the highest
 245 one (Table 1).

246 In seeds these same compounds were detected also as major and minor metabolites : *ent*-9-L₁-
 247 PhytoP (18.33 ng/g) and 16-F_{1t}-PhytoP (3.32 ng /g). The 16-*epi*-16-F_{1t}-PhytoP is dominant in
 248 leaves, followed by *ent*-9-L₁-PhytoP (32.78 ng/g). This same ranking of compounds was
 249 found in pods with 47.34 ng/g for 16-*epi*-16-F_{1t}-PhytoP, and 30.52 ng/g for *ent*-9-L₁-PhytoP.
 250 In flowers, the 9-F_{1t}-PhytoP is the dominant compound with 30.02 ng/g whereas the *ent*-16-
 251 B₁-PhytoP is the lowest one (9.28 ng/g).

Table 1. PhytoP Levels (ng/g) in *A. cyanophylla*

| Organs | 16-F _{1t} -PhytoP | 9- <i>epi</i> -9-F _{1t} -PhytoP | 16- <i>epi</i> -16-F _{1t} -PhytoP | 9-F _{1t} -PhytoP | <i>ent</i> -16-B _{1t} -PhytoP | <i>ent</i> -9-L _{1t} -PhytoP | Total |
|---------|------------------------------|--|--|------------------------------|--|---------------------------------------|-------------------------------|
| Leaves | 18.3 ± 0.12 ^a | 26.99 ± 0.15 ^a | 35.14 ± 0.02 ^b | 19.54 ± 0.01 ^c | 20.69 ± 0.01 ^c | 32.78 ± 0.03 ^b | 153.51 ± 0.34 ^b |
| Stems | 10.54 ± 0.01 ^c | 13.05 ± 0.12 ^c | 25.48 ± 0.04 ^c | 45.00 ± 0.03 ^a | 33.49 ± 0.04 ^a | 51.79 ± 0.07 ^a | 179.35 ± 0.31 ^a |
| Flowers | 13.03 ± 0.02 ^b | 17.77 ± 0.15 ^b | 10.61 ± 0.11 ^d | 30.02 ± 0.28 ^b | 9.28 ± 0.08 ^e | 9.75 ± 0.49 ^d | 90.46 ± 1.13 ^d |
| Pods | 5.49 ± 0.24 ^d | 10.10 ± 0.14 ^d | 47.34 ± 0.01 ^a | 28.63 ± 0.01 ^b | 25.95 ± 0.06 ^b | 30.52 ± 0.05 ^b | 148.03 ± 0.51 ^c |
| Seeds | 3.32 ± 0.27 ^e | 5.89 ± 0.10 ^e | 4.11 ± 0.05 ^e | 6.58 ± 0.09 ^d | 10.87 ± 0.13 ^d | 18.33 ± 0.49 ^c | 49.1 ± 1.13 ^c |

The values are expressed as means ± standard deviations, n=3 for each analysis. Different letters in each column represent statistically different mean values ($P < 0.05$, Duncan's multiple range test).

252 3.2 Qualitative and quantitative PhytoF profile in aerial parts of *Acacia cyanophylla* Lindl.

253 PhytoPs were not the only compounds identified in *A. cyanophylla* organs. Three PhytoFs
 254 were detected for the first time : *ent*-9(*RS*)-12-*epi*-ST- Δ^{10} -13-PhytoF, *ent*-16(*RS*)-13-*epi*-ST-
 255 Δ^{14} -9-PhytoF, and *ent*-16(*RS*)-9-*epi*-ST- Δ^{14} -10-PhytoF. These metabolites were found by
 256 following their specific MRM transitions: m/z 343.2 → m/z 237.1 for the first transition, m/z
 257 343.2 → m/z 201 for the second transition and m/z 343.2 → m/z 209 for the last transition.

258 As shown in Figure 4 and Table 2, concentration of PhytoFs was significantly different from
 259 one part of plant to another ($P < 0.001$). Total contents of these compounds ranged from 30.14
 260 ng/g to 320 ng/g. Stems contain the highest concentration, followed by pods (139.77 ng/g),
 261 seeds (95.89 ng/g), and leaves (82.87 ng/g). The lowest concentration was found in flowers.
 262 In comparison with other matrix, these levels are important. For example, in dry fermented
 263 cocoa beans, level of PhytoF does not exceed 13.13 ng/g.²³ Same, for white and brown rice
 264 flours (0.69 and 4.2 ng/g respectively).²⁴ But less important, than those quantified in dry
 265 leaves of melon plants (4400 ng/g).¹²

Table 2. PhytoF Levels (ng/g) in *A. cyanophylla*

| Organs | <i>Ent-9(RS)-12-epi-ST-Δ^{10}-13-PhytoF</i> | <i>Ent-16(RS)-13-epi-ST-Δ^{14}-9-PhytoF</i> | <i>Ent-16(RS)-9-epi-ST-Δ^{14}-10-PhytoF</i> | Total |
|---------|---|---|---|--------------------------------|
| Leaves | 43.03 \pm 0.02 ^a | 39.84 \pm 0.06 ^c | nd | 82.87 \pm 0.08 ^d |
| Stems | 38.29 \pm 0.05 ^b | 282.50 \pm 0.01 ^a | nd | 320.79 \pm 0.06 ^a |
| Flowers | 30.14 \pm 0.08 ^c | nd | nd | 30.14 \pm 0.08 ^e |
| Pods | 31.34 \pm 0.03 ^c | 108.43 \pm 0.04 ^b | nd | 139.77 \pm 0.07 ^b |
| Seeds | 32.18 \pm 2.64 ^c | 15.06 \pm 0.28 ^d | 48.65 \pm 0.33 ^a | 95.89 \pm 3.25 ^c |

The values are expressed as means \pm standard deviations, n=3 for each analysis. Different letters in each column represent statistically different mean values ($P < 0.05$, Duncan's multiple range test).

266 The qualitative profile showed that the *ent-16(RS)-13-epi-ST- Δ^{14} -9-PhytoF* was the most
267 abundant PhytoF detected for all parts, except flowers, with a concentration ranging from
268 15.06 ng/g in seeds to 282.50 ng/g in stems. This same compound was not detected in dry
269 melon leaves. The *ent-9(RS)-12-epi-ST- Δ^{10} -13-PhytoF* was reported as the second major
270 compound and it was found for all parts. The level of this compound, varied from 30.14 ng/g
271 in flowers to 43.03 ng/g in leaves. Whereas the *ent-16(RS)-9-epi-ST- Δ^{14} -10-PhytoF* was the
272 compound with the lowest content. It was detected only in seeds.

273 Surprisingly, large variations in PhytoF levels were observed in various samples. Total and
274 individual contents presented significant differences. Notably, stems contained the highest
275 concentration, although it did not contain the *ent-16(RS)-9-epi-ST- Δ^{14} -10-PhytoF* (Table 2).

276 The same for pods, leaves and flowers which contained just two compounds. In contrary, all
277 PhytoFs were found in seeds and the *ent-16(RS)-9-epi-ST- Δ^{14} -10-PhytoF* is the most
278 abundant metabolite detected (48.65 ng/g). Also, it is important to highlight that flowers
279 contain only one metabolite, the *ent-9(RS)-12-epi-ST- Δ^{10} -13-PhytoF*.

280 In this study, identification, and quantification of PhytoPs and PhytoFs from aerial parts of
281 *Acacia cyanophylla* Lindl. was reported for the first time. To get a rational understanding on
282 the level of these metabolites in our samples, it is required to evaluate their abundance in
283 other plant materials. As comparison with our results, PhytoPs have already been quantified

284 with lowest concentrations in green olives (6.0-87.9 ng/g fw),²⁵ three types of raw pecan nuts
285 (7.8 ng/g), macadamia (6.5 ng/g) and walnut (5.5 ng/g).²⁶ While, they stated a higher
286 concentration in brown macroalgae (*Ectocarpus siliculosus*) (310 ng/g)²⁷ and different plant
287 leaves, flowers and roots (43-1380 ng/g).⁸ Also, several plant materials have been
288 characterized by their content in total PhytoFs. Yonny *et al.* and Cuyamendous *et al.* have
289 reported the occurrence of PhytoFs in pine, nuts, chia, and melon leaves with levels ranged
290 from 0.30 to 4400.00 ng/g.^{12,28} This comparison of oxylipin levels in different plant matrices
291 placed the five parts studied of *Acacia cyanophylla* Lindl. in the average range of most
292 vegetables, which revealed this matrix as an especially interesting source of oxylipins.
293 Indeed, considering the described results, it is important to notice that levels of PhytoPs as
294 well as PhytoFs in aerial parts of *Acacia cyanophylla* Lindl., registered variations. The
295 concentration of PhytoFs has been detected with quantities significantly higher to those
296 described for PhytoPs, and even for the same metabolite, levels were different from one part
297 to another. These differences could be related to several factors including plant part, abiotic
298 stress and harvest time.²⁹ Also, growing conditions of plants as well as their chemical
299 composition with respect to ALA, the precursor of both PhytoPs and PhytoFs, could cause
300 variations.

301 Samples of *Acacia cyanophylla* Lindl., were found to have high level of PhytoP and PhytoF
302 contents. Stem, which showed the highest amount of these compounds, are the part of the
303 plant that the main function is to transport minerals and water to the leaves, where it is
304 transformed into useful products by the process of photosynthesis, and then these are
305 transported to other parts of the plant. During this photosynthetic process, the production of
306 ROS is intensified due to a limited use of the light absorbed upon photosynthetic electron
307 transport and fixation of CO₂. This fact has been confirmed in many previous studies.^{11,30} For
308 instance, a research conducted on rice, pursues to shed some light on the response of PhytoPs

309 and PhytoFs, as signaling molecules, in situations of nutritional stress directly related to
310 photosynthesis, at the level of photosystem II (PSII) and the intercellular ROS levels.¹¹ This
311 study supports our results and could explain the high concentration of oxylipins in stem and
312 leaves of *Acacia cyanophylla* Lindl.

313 Moreover, plants use oxygen to produce the energy necessary for their growth and
314 development. This oxidative phosphorylation is done in particular through electron transport
315 chains. The reduction of oxygen by the cytochrome of the respiratory chain is always
316 accompanied by the production of oxygenated radicals. When this reduction is incomplete,
317 highly reactive molecules derived from oxygen are produced such as superoxide anions $O_2^{\cdot-}$
318 or hydrogen peroxide H_2O_2 which in turn produce reactive oxygen species ROS. In this
319 situation, a large quantity of lipid peroxidation products is generated, including oxylipins.

320 In the same context, PhytoPs and PhytoFs could be produced in plant species under other
321 abiotic stress situations.^{12,20,27} The unfavorable environmental conditions like extreme
322 temperatures, disturb the balance between the generation of ROS and the scavenging capacity,
323 which entails the redox homeostasis in cells. These radicals are unstable and react rapidly
324 with other compounds in the separate cell compartments, causing losses of enzymatic
325 activities like that of Superoxide dismutases (SODs) or catalase, by degradation of essential
326 metabolites³¹ and increase the formation of lipid peroxidation products, including oxylipins.¹⁰

327 On this matter, a previous studies on olive oil have considered PhytoPs and PhytoFs as
328 biomarkers of olive tree water stress, as their content in extra virgin olive oil correlated with a
329 water deficit in olives tree plantations.²⁵ Similarly, an important variation of PhytoPs was
330 identified in melons leaves when the plant was exposed to thermal stress. Accordingly,
331 oxylipins could be considered as early biomarkers of water stress.¹² In our case, the presence
332 of these compounds would not necessarily be dependent on the hydric stress in *Acacia*
333 *cyanophylla* Lindl., as samples were harvested in a region with good growing conditions.

334 Another hypothesis can be considered is the harvest time. Stems, the part that contains the
335 highest concentration of oxylipins, was collected in the blooming stage (Mars). In this period,
336 plants show higher photosynthetic rates and reproductive efforts. Leaves and flowers were
337 harvested in Mars too, but their concentrations were not as important as for stems. The
338 minimum of PhytoP concentrations was observed in June concordant with the full ripening of
339 fruits (seeds). The decreasing of PhytoPs as well as PhytoFs during fruiting stages for the
340 vegetative organs could be interpreted as a result of stabilization of reproductive allocation
341 resources (in terms of biomass and nutriment).

342 Anyhow, the quantification of PhytoPs and PhytoFs could provide a unique sensitive
343 indicator of oxidant injury (crop conditions as well as postharvest factors). Further studies are
344 needed to investigate if hydric and/or thermal stress growing conditions have a real effect on
345 the amount of PhytoPs and PhytoFs in aerial parts of *Acacia cyanophylla* Lindl.. On the other
346 hand, it would be also interesting to investigate how the different edaphoclimatic conditions
347 influence the qualitative and quantitative profile of PhytoPs and PhytoFs.

348 *3.3 Analysis of Extraction Yield and Matrix Effect*

349 Identification of oxylipins in natural matrices is extremely sensitive, requiring specific
350 analytical methods for their quantification. Sample validation was an important step to verify
351 that the work was done under favorable conditions. As previously explained, the protocol
352 relying on the specific extraction of lipophilic compounds (Folch extraction), followed with a
353 step of SPE to eliminate or to reduce the amount of potentially interfering substances. PhytoPs
354 and PhytoFs were subsequently separated, using a micro-LC-MS/MS method validated by
355 previous studies.¹² Their recovery could be influenced by several factors as the efficiency of
356 separation of compounds during liquid/liquid extraction or the selectivity of the used SPE
357 cartridges.¹⁹ Identification of metabolites relied also, on retention times observed during
358 spiked experiments, determination of molecular masses and the analysis of specific MS/MS

359 transitions. Therefore, an important part of this work consisted in the validation of the
 360 methodology presented below by two parameters: Extraction Yield (EY) and Matrix Effect
 361 (ME). Samples were spiked with two different concentrations of a standard mixture (SM₃₂ =
 362 each standard of the mixture at 32 ng.ml⁻¹ or SM₂₅₆ = each compound at 256 ng.ml⁻¹) to
 363 determinate the efficiency of the sample processing (Table 3).

Table 3. Percent of Matrix Effect and Extraction Yield of PhytoPs and PhytoFs

| Compounds | Concentration (pg/ μ L) | Extraction yield (%) | Matrix Effect (%) |
|--|--------------------------------|-------------------------|----------------------|
| 16- <i>epi</i> -16-F _{1t} -PhytoP | 32 | 87.5 \pm 6.4 | 54.4 \pm 2.2 |
| | 256 | 61.2 \pm 3.2 | 49.7 \pm 3.7 |
| <i>ent</i> -16-B ₁ -PhytoP | 32 | 84.9 \pm 4.8 | 59.1 \pm 2.4 |
| | 256 | 71.2 \pm 3.4 | 57.3 \pm 3.2 |
| 9- <i>epi</i> -9-F _{1t} -PhytoP | 32 | 80.7 \pm 5.8 | 75.6 \pm 5.7 |
| | 256 | 65.3 \pm 8.7 | 73.8 \pm 4.1 |
| 9-F _{1t} -PhytoP | 32 | 87.4 \pm 5.3 | 100.2 \pm 5.8 |
| | 256 | 92.7 \pm 2.1 | 83.6 \pm 2.2 |
| 16-F _{1t} -PhytoP | 32 | 67.5 \pm 4.7 | 48.8 \pm 2.4 |
| | 256 | 70.8 \pm 3.9 | 51.0 \pm 1.9 |
| <i>ent</i> -9-L ₁ -PhytoP | 32 | 89.3 \pm 2.3 | 73.4 \pm 1.2 |
| | 256 | 78.8 \pm 1.9 | 68.1 \pm 4.8 |
| <i>ent</i> -16(<i>RS</i>)-9- <i>epi</i> -ST- Δ ¹⁴ -10-PhytoF | 32 | 92.7 \pm 6.3 | 64.2 \pm 6.0 |
| | 256 | 68.1 \pm 5.4 | 51.3 \pm 3.7 |
| <i>ent</i> -9(<i>RS</i>)-12- <i>epi</i> -ST- Δ ¹⁰ -13-PhytoF | 32 | 68.5 \pm 1.9 | 46.8 \pm 4.9 |
| | 256 | 73.1 \pm 2.3 | 52.5 \pm 8.1 |
| <i>ent</i> -16(<i>RS</i>)-13- <i>epi</i> -ST- Δ ¹⁴ -9-PhytoF | 32 | 106.3 \pm 11.2 | 71.9 \pm 8.6 |
| | 256 | 73.8 \pm 4.9 | 59.2 \pm 3.9 |

Results are expressed as mean \pm (SD) of a triplicate analysis performed on different samples.

364 In this study, PhytoPs and PhytoFs were exhibited a good recovery with EY ranged from 61
 365 % to 93 % and from 68 % to 106 %, respectively (Table 3). Some metabolites were observed
 366 with better recovery (e.g. *ent*-9-L₁-PhytoP; EY₃₂= 89.3 % and *ent*-16(*RS*)-9-*epi*-ST- Δ ¹⁴-
 367 PhytoF; EY₃₂= 92.7 %), than other (e.g. 9-*epi*-9-F_{1t}-PhytoP; EY₂₅₆= 65.3%). For some
 368 analytes, the extraction yield calculated was more than 100 % corresponding probably to the
 369 coelution of a compound that presents the same MRM transition.³² It is the case, for instance,
 370 for the *ent*-16(*RS*)-13-*epi*-ST- Δ ¹⁴-9-PhytoF with an EY of 106.3 % at 32 pg. μ L⁻¹. It's
 371 important also to notice that EYs were better at low than at high concentrations. To complete

372 this validation, the matrix effect, corresponding to an ion-suppression/enhancement of
373 coeluted matrix compounds, was evaluated. ME varied between 49 % and 100 % for the
374 different PhytoPs, whereas it was ranged between 47 % and 72 % for PhytoFs. Like EY, ME
375 is specific to each compound and at low concentration, a clearly marked effect was observed.
376 These two parameters prove the efficiency of the analytical method used for the detection of
377 metabolites in this present work.

378 **4. Conclusion**

379 In conclusion, this study confirmed the presence of PhytoPs and PhytoFs in aerial parts of
380 *Acacia cyanophylla* Lindl. for the first time. To the best of our knowledge, no information has
381 been published before on the content of naturally occurring oxylipins in this matrix.
382 Therefore, the results obtained can only be compared with those of previous work performed
383 on other edible and nonedible plant materials such as pecan, melon leaves, green olive and
384 macadamia. This first qualitative and quantitative profile was performed by using LC-
385 MS/MS, which is one of the most analytical techniques applied to identify oxylipins. The
386 methodology was optimized to its use in plant system, and the results obtained with it are
387 reliable according to the analytical parameter values found.

388 PhytoPs and PhytoFs were detected with levels statistically different. They are not only
389 considered as relevant oxidative stress biomarkers in plant, but also have demonstrated a
390 several promising biological activities (anti-inflammatory, apoptosis, immune function
391 regulation, neuroprotection).^{14,18,33} The concentrations retrieved from these compounds in
392 aerial parts of *Acacia cyanophylla* Lindl. suggest that this plant is a valuable source of
393 PhytoPs and PhytoFs. Due to high amounts quantified, it may be worth exploring this matrix
394 as a potential natural resource to produce oxylipins. Stresses can be used as a lever to act on
395 the concentrations of compounds of interest in the plant products. By using natural
396 compounds that do not affect the plant structure and function could be an interesting way to

397 produce oxylipins. Through this study and the results obtained we can now directly address to
398 the organ which contains the compound of interest, to extracted it and applied in phytotherapy
399 preparation. Although additional studies are necessary, this work could constitute a solid
400 scientific base for the search for new compounds with significant application in
401 pharmaceutical uses and open new insights, from *Acacia cyanophylla* Lindl. as a new source
402 of bioactive compounds.

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407 **Conflicts of Interest**

408 The authors declare no conflict of interest.

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