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1 ***Eucalyptus brevifolia* F. Muell and *Eucalyptus stricklandii* Maiden leaves extracts:**
2 **HPLC-DAD, GC-MS analysis and *in vitro* biological activities, combined with the**
3 **principal component analysis**
4

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24

25 **Highlights**

- 26 • The highest extraction yields were observed for the methanolic and aqueous
27 *Eucalyptus* leaves extracts.
- 28 • Phenolic acids, flavonoids and stilbenes are the most biochemical groups present in
29 *Eucalyptus* leaves extracts.
- 30 • Chemical compounds in the *Eucalyptus* leaves extracts were detected with HPLC-
31 DAD and GC-MS before and after derivatization.
- 32 • Terpenes, phenols, alcohols, sugars, acids, ketones and fatty alcohols represent the
33 abundant molecules revealed with GC-MS before and after derivatization.
- 34 • *Eucalyptus* leaves extracts are endowed of several biological activities with health
35 promoting benefits (antioxidant, anti-Alzheimer, anti-inflammatory, anti-xanthine
36 oxidase, cytotoxic effect).

37

38 **Abstract**

39 Natural products always have been relevant for the advancement of new pharmacological
40 products intended to prevent oxidative stress and many diseases since ancient times. Herein,
41 the chemical composition was investigated *via* chromatographic analysis of varying polarity
42 solvents (petroleum ether, ethyl acetate, methanol and distilled water) from Tunisian
43 *Eucalyptus brevifolia* F.M and *Eucalyptus stricklandii* M. leaves. *In vitro* provision of
44 biological activities through their antioxidant power (ABTS⁺), ability to inhibit 15-
45 lipoygenase (15-LOX), acetylcholinesterase (AChE), xanthine oxidase (XOD), as well as,
46 cytotoxic potency were undertaken. Chemical profiling showed that the methanolic extracts of
47 both species were the richest in total phenolic compounds. GC-MS analysis of the two
48 *Eucalyptus* species revealed that spathulenol, globulol, epiglobulol, and trans-Z- α -bisabolene-
49 epoxide were the main volatile compounds detected in the different extracts. In addition, the
50 derivatization procedure allowed the identification of ethylene glycol, and D-fructofuranose
51 as very abundant molecules. The HPLC-DAD has enabled the identification of eight new
52 molecules among them icariin (1.1 mg/g dr), and 5-hydroxy-3'-methoxyflavone (7.5 mg/g dr)
53 in the *E. stricklandii* MeOH and EtOAc extracts. Aqueous *E. brevifolia* extract possessed the
54 highest anti-AChE with an IC₅₀ (10 μ g/mL). Whereas, the MeOH *E. stricklandii* extract
55 possessed the highest anti 15-LOX with an IC₅₀ (9.7 μ g/mL), as well as, the most important
56 percentage of XOD inhibition 51.1%. All extracts revealed an antiproliferative potency
57 against human cancer cell lines HCT-116, MCF-7 and OVCAR, with an inhibition percentage
58 up to 96%. Taken together, both *Eucalyptus* species have a broad range of potential
59 applications in several fields, such pharmaceutical, medical and food industries as
60 nutraceutical supplements.

61

62 **Keywords:** *Eucalyptus*, HPLC, GC-MS derivatization, antioxidant, bioactivity, PCA.

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72 **List of "Abbreviations"**

73 ABTS⁺: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid; ACTHi: acetylthiocholine
74 iodide; AMDIS: automated mass spectral deconvolution and identification system;
75 BSTFA:N,O-*bis*(trimethylsilyl) trifluoroacetamide; CYHA: cyclohexane; DMEM:
76 Dulbecco's Modified Eagle Medium; DMSO: dimethyl sulfoxide; DNS: 3,5-dinitrosalicylic
77 acid; DTNB: dithio-*bis*-nitrobenzoate; EtOAc: ethyl acetate; GC-MS: gas chromatography-
78 mass spectrometry; HCT-116: human colon cancer; HPLC: high performance liquid
79 chromatography; MCF-7: human breast cancer; MeOH: methanol; MTT: 3-(4,5-dimethyl
80 thiazol-2-yl) 2,5-diphenyl tetrazolium bromide; Na₃PO₄: sodium phosphate; NDGA:
81 nordihydroguaiaretic acid; NIST: National Institute of Standards and Technology; OVCAR:
82 human ovarian cancer; PEth: petroleum ether; THF: tetrahydrofuran; TPC: total phenolics
83 content.

84

1. Introduction

Mainly an Australian genus, *Eucalyptus* is a monotypic one, which is considered as one of the world most important medicinal tree and widely planted genera (Elansary et al., 2017). The essential oil obtained from the *Eucalyptus* tree is used as an antiseptic, a perfume, an ingredient in cosmetics, a flavoring agent, and in aromatherapy (Sabo and Knezevic, 2019). Currently, there are about 900 different *Eucalyptus* species over the world, of which about 300 containing volatile oils used as medications in traditional medicine (Sharifi-Rad et al., 2017). In order to overcome the deforestation in the arid areas, Tunisian authorities decided to implant new species in the 1950s. This rehabilitation program, supervised by the Food and Agriculture Organization, included mainly the plantation of *Eucalyptus* species (*E. globulus*, *E. erythrocorys*, *E. camaldulensis*, etc.) (Elaieb et al., 2020). Thus, these species were very well adapted to the Tunisian conditions, and now they tend to become invasive wood species, which need to be economically valued (Ossipov et al., 2020). In the last years, *Eucalyptus* trees are increasingly used for their essential oils considered as natural sources of fragrance compounds for cosmetics and cosmeceuticals (Sharmeen et al., 2021). Furthermore, *Eucalyptus* trees are used for several fields. They represent the most important hard wood forestry crops worldwide and provide a major source of pulp-wood for high quality paper production (Ben Hassine et al., 2013). In particular, the genus *Eucalyptus* is known to be a rich source of bioactive compounds including, hydroxycinnamic acids, flavonoids, condensed tannins, terpenoids, and phloroglucinol derivatives (Elansary et al., 2017). *Eucalyptus brevifolia*, commonly known as snappy white gum, is a northern Australia endemic tree (Alsharif et al., 2020). The specific epithet "*brevifolia*" is from the Latin words; *brevis* meaning "short" and *folium* meaning "leaf", referring to the relatively short leaves (Von Mueller, 1859). *Eucalyptus stricklandii*, a species of small tree, commonly known as Strickland's gum, or also yellow flowered gum. It is endemic to Western Australia (Maiden, 1914). Nevertheless, with exception of the previous report on essential oils, there are no scientific reports have yet been carried out on the phytochemical value, antioxidant and biological characterization of *E. brevifolia* and *E. stricklandii* leaves extracts. The desire to return to natural ancient remedies has encouraged us to explore potential candidates from vast treasure of ethno medicinal knowledge. Consequently, the actual work was carried out to investigate, for the first time, chemical composition (TPC, HPLC-DAD and GC-MS) and biological activities (antioxidant, anti-AChE, anti-15-LOX, anti-XOD and cytotoxicity) of

117 two *Eucalyptus* species extracts; *E. brevifolia* F. Muell and *E. stricklandii* Maiden growing in
118 Tunisia.

119 **2. Material and Methods**

120 **2.1. Chemicals and reagents**

121 All chemicals used were of analytical reagent grade. All reagents were purchased from
122 Sigma-Aldrich-Fluka (Saint-Quentin, France).

123

124 **2.2. Plant material**

125 The leaves of *E. brevifolia* and *E. stricklandii* were picked from trees growing in the Hajeb
126 Layoun arboretum, located in Kairouan (center of Tunisia). They were stored at a dry place
127 for fifteen days. Specimens were identified at the Regional Station of the National Institute of
128 Research in Farming Studies, Waters and Forests. A voucher specimen (reference 0109) was
129 deposited at the Department of Biology. Dried leaves were subjected to preparation of the
130 various extracts.

131

132 **2.3. Extraction procedure**

133 The sequential extraction with solvents of increasing polarity was used to obtain the different
134 extracts. Solvents were: petroleum ether (PEth), ethyl acetate (EtOAc), methanol (MeOH) and
135 distilled water. Ten grams (10 g) of harvested leaves were grounded using a blender type
136 Moulinex AR 1105(France). The obtained powder of 2 μm of diameter were placed in (100
137 mL) of PEth for 16 h under frequent agitation at ambient pressure and temperature. The
138 mixture was filtered using Wattman paper (GF/A, circle format of 110 mm of diameter and
139 1.6 μm pore size). The solvent was evaporated using a rotary evaporator (Rotavapor® - Fisher
140 UK Extranet, R 210/215) under vacuum at 35°C. Then, the firstly extracted powder was
141 extracted with EtOAc under the same conditions as with PEth. The same procedure was
142 applied for the following solvents. Extracts were kept in amber vials and stored at 4°C for
143 further analysis.

144

145 **2.4. Determination of total phenolics content (TPC)**

146 The TPC of the extracts were estimated using spectrophotometric methods such as Folin-
147 Ciocalteu procedure. Gallic acid was used as reference and results were expressed as mg of
148 gallic acid equivalent per g of extract dry residue (GAE mg/g dr).

149

150 **2.5. Chromatographic analysis**

151

152 **2.5.1. HPLC analysis**

153 The analysis of various *Eucalyptus* extracts was performed using analytical HPLC-DAD
154 (Rahmani et al., 2020). The separation was conducted at ambient temperature, using a
155 Phermerex RP-C18 column, 25 cm x 4.6 mm and particle size 5 μm , equipped with a 5 μm
156 C18 guard column. The mobile phases were as follow: (A) acidified water (acetic acid, pH =
157 2.65) and (B) water/ACN (20:80, v/v, pH = 2.65). The gradient used was 0-35 min, 0.1-30%
158 B; 35-40 min, 30-50% B; 40-45 min, 50-99.9% B and 45-60 min, 99.9-0.1% B. Twenty μL
159 (20 μL) of the diluted samples were injected, and the detection was performed at 280 nm,
160 with a solvent flow rate 1.2 mL/min. Thereafter, the different samples were ultra-sonicated,
161 the samples were filtered through a Millex HA 0.25 μm filter before HPLC-DAD analysis.
162 The phenolic compounds were identified by comparing the retention time of the unknown
163 molecules with those of the standards.

164

165 **2.5.2. GC-MS analysis**

166 A GC coupled to MS (GCT 1er Waters), piloted by the software MassLynx was used to
167 identify the volatile compounds. One microliter (1 μL) of the diluted sample was injected in
168 the split mode (split ratio 1:10). Inlet pressure was 1 kPa and the carrier gas was helium at an
169 on-column flow of 1 mL/min. The gas chromatograph was programmed as follows:
170 isothermal at 60°C for 5 min, temperature gradient 60-270°C at 15°C/min, isothermal at
171 270°C for 6 min, then a second gradient was applied to 300°C at 50°C/min and finally
172 isothermal at 300°C for 4.5 min. Total analysis time was 30 min. High-resolution mass
173 spectrometer was adjusted for an emission current of 663 μA and electron multiplier voltage
174 70 eV. Trap temperature was 250°C and that of the transfer line was 300°C. Mass scanning
175 was performed from 40 to 650 amu. The volatile compounds were identified by the
176 determination of their formula using MassLynx and by comparison with those cited in NIST
177 11 spectrum library.

178

179 **2.5.2.1. Derivatization method**

180 In order to identify the non-volatile compounds, the extracts were undergone a derivatization
181 step, using silylation method. Briefly, 150 μL Pierce BSTFA + 1% TMCS was added to

182 extracts (1 mL, 5 mg/mL) in THF anhydrous, and mixed in a (2 mL) vial. Then, the mixture
183 was aerated with bubbling using nitrogen and shaken for 30 seconds. The reaction mixture
184 was maintained at 40°C for 15 min. One microliter (1 µL) of each derivatized solution was
185 finally injected into the GC-MS and analyzed as described in the previous section.

186

187 **2.6. Biological activities**

188

189 **2.6.1. Antioxidant activity (ABTS⁺)**

190 The antioxidant potential was evaluated by the radical cation ABTS (2,2'-azinobis-3-
191 ethylbenzothiazoline-6-sulphonate). 7 mM solution of ABTS at pH 7.4 (5 mM NaH₂PO₄, 5
192 mM Na₂HPO₄ and 154 mM NaCl) was mixed with 2.5 mM potassium persulfate (final
193 concentration) and stored in the dark at room temperature for 16 h before use. A dilution with
194 persulfate buffer of the mixture gave an absorbance of 0.70 ± 0.02 units at 734 nm using a
195 spectrophotometer (Helios, Unicam, Cambridge, UK). Then, 100 µL of each extract was
196 allowed to react with fresh ABTS solution (900 µL), and the absorbance was read 6 min after
197 initial mixing. Ascorbic acid was used as a standard. The capacity of free radical scavenging
198 was expressed by IC₅₀ (µg/mL) values which represents the concentration required to
199 scavenge 50% of ABTS radicals.

200

201 **2.6.2. Anti-15-lipoxygenase assay (15-LOX)**

202 The anti-inflammatory activity of various organic *Eucalyptus* extracts was performed. All
203 samples were tested at the concentration of 50 µg/mL in 96-well plates incubated at 25°C for
204 10 min. Each well contained a total volume of (250 µL) distributed as follows: 150 µL
205 phosphate buffer (pH 7.4), 20 µL sample, 60 µL of linoleic acid (3.5 mmol/L) and 20 µL 15-
206 LOX (soybean 500 U). The absorbance was measured at 234 nm in a microplate reader
207 (Multiskan Go, Finland). The nordihydroguaiaretic acid (NDGA) was used as standard. The
208 activity was defined as the percentage of inhibition of the 15-LOX enzyme, using the
209 following formula: % inhibition = 100 x (A blank - A sample) / A blank.

210 Where A sample was the absorbance of sample with the test material, and A blank was the
211 absorbance of sample without the enzyme.

212

213 **2.6.3. Anti-acetylcholinesterase assay (AChE)**

214 The anti-acetylcholinesterase activity of different extracts was determined by the detection of
215 the 5-thio-2-nitrobenzoic acid, yellow product, formed after the action of thiocholine on

216 DTNB. A volume (25 μ L) of each diluted extract (0.5 μ g/ μ L), 50 μ L of phosphate buffer (pH
217 8), 125 μ L of DTNB and 25 μ L of the AChE were added in 96-well plates. After 15 min pre-
218 incubation at 25°C, 25 μ L of acetylcholine iodide was added and the absorbance was
219 measured at 412 nm after 10 min incubation, using a microplate reader. Similarly, the control
220 was prepared using the same procedure replacing extracts with 5% dimethyl sulfoxide
221 (DMSO). Positive control was prepared using the same procedure replacing *Eucalyptus*
222 extracts by galanthamine. The percentage of inhibition of each sample was calculated using
223 the following formula: % inhibition = 100 x (A blank - A sample)/ A blank.

224 Where A sample was the absorbance of sample with the test material, and A blank was the
225 absorbance of sample without the enzyme.

226

227 **2.6.4. Anti-xanthine oxidase (XOD) assay**

228 Briefly, 60 μ L of sodium phosphate buffer (pH 7.5), 30 μ L of xanthine oxidase (0.1 μ mol/L),
229 and 50 μ L of extract (0.2 mg/mL) were placed in 96- well plates. After 15 min pre-incubation
230 at 25°C, 60 μ L of xanthine was added and the plate was incubated for 10 min. The control
231 was prepared using the same procedure replacing the extract with 5% DMSO solution. Then,
232 the absorbance was measured at 295 nm, using a microplate reader. Allopurinol, a potent anti-
233 xanthine oxidase was used as a positive control. The percentage of inhibition of each sample
234 was calculated using the following formula:

235 % inhibition = 100 x (A blank - A sample)/ A blank

236 Where A sample was the absorbance of the sample with the test material, and A blank was the
237 absorbance of the sample without the enzyme.

238

239 **2.6.5. Cytotoxic assay**

240 Extracts were tested for their cytotoxic activity against the following cells lines: human colon
241 cancer (HCT-116), human breast cancer (MCF-7) and human ovarian cancer (OVCAR).
242 MCF-7 cells line was maintained in DMEM growth medium and the two other cells lines
243 were maintained in RPMI growth medium. Cytotoxic effect was determined using a modified
244 MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) method. In 96-well
245 plates, 100 μ L of culture medium containing 10⁴ cells/well were placed. After 24 h incubation
246 at 37°C, 100 μ L of culture medium containing eucalyptus organic extracts were added. The
247 final sample concentration was 50 μ g/mL per well. Then, the mixture was incubated at 37°C
248 for 48 h in an humidified atmosphere, with 5% CO₂. The mixture was removed and replaced
249 with 50 μ L of 1 mg/mL MTT, after that incubated for 40 min at 37°C. The MTT solution was

250 then replaced with 50 μ L of DMSO to dissolve the MTT formazan precipitate and the
251 absorbance was read at 605 nm, using a microplate reader. The cytotoxic effect of different
252 extracts was estimated in terms of growth inhibition percentage. Tamoxifen (0.2 mg/L) was
253 used as reference.

254

255 **2.7. Statistical Analysis**

256 All measurements were carried out in quadruplicate. Two-way ANOVA was used for the
257 significance calculation using SPSS 20.1. Statistical differences between the solvents used in
258 the study were estimated using Tukey's test. The determination of the relationship between
259 TPC and biological activities was assessed by the coefficient of determination (R^2). Principal
260 component analysis (PCA) was done using XLSTAT (version 2014.5.03) for visualization of
261 discrimination between the different parameters.

262

263 3. Results and Discussion

264

265 3.1. Extraction Yield and total polyphenols content

266 According to the literature, no study has been previously reported on the effect of solvents on
267 the extraction yield and TPC of *E. stricklandii* and *E. brevifolia* leaves extracts. Both species
268 leaves powder were extracted using four organic solvents of increasing polarity (PEth,
269 EtOAc, MeOH and distilled water). The various extracts presented a remarkable difference in
270 terms of extraction yield. The highest extraction yields were observed for the MeOH and
271 aqueous extracts, for both species, with a superiority for the *E. brevifolia* (23 and 15%,
272 respectively) (Fig. 1). The polar extraction yield (MeOH) was about five-fold and two-fold
273 greater than the medium polar extracts yields (PEth and EtOAc), for *E. stricklandii* and *E.*
274 *brevifolia*, respectively. The variability in extraction yield might be explained by the
275 differences and the biochemical composition of the samples and the polarity of used solvents
276 (Ben Lataief et al., 2020).

277 Regarding the TPC, statistically, there was a significant difference between various solvents
278 used ($p \leq 0.05$). However, there was no significant difference between the two species ($p >$
279 0.05). While PEth and EtOAc extracts, for both species, exhibited a low TPC, which does not
280 exceed 100 mg GAE/g dr, the MeOH extracts showed a high level of phenolic compounds.
281 The *E. brevifolia* and *E. stricklandii* showed a TPC of 482 and 305 mg GAE/g dr, respectively
282 (Fig. 2). In addition, the aqueous extracts, showed a TPC of 59.8 and 216.4 mg GAE/g dr,
283 with superiority in favor of *E. stricklandii*. Quantitatively, TPC for the two *Eucalyptus* leaves
284 polar extracts (MeOH and aqueous) was greater than that reported in ethanolic extracts of *E.*
285 *globulus* Labill. and *Corymbia ficifolia* leaves (Dezsi et al., 2015). The authors found a TPC,
286 which not exceed the amount of 25 mg GAE/g dr. Furthermore, actual results were in the
287 range with previous work of Nile and Keum (2018) who illustrated that the TPC in *E.*
288 *globulus* Labill. was solvents dependent. They found that the aploar extract (CYHA)
289 exhibited a low content (100 mg GAE/g dr), while the polar extract (MeOH) reached the
290 amount of 240 mg GAE/g dr.

291

292 3.2. Chromatographic analysis

293

294 3.2.1. HPLC analysis

295 The phytochemical screening was established using HPLC-DAD. For each extract
296 chromatogram, the retention time of each peak was sequentially compared with those of
297 standards with known retention time. Overall, eight phenolic compounds were identified and
298 quantified, by means of their relative retention (Table 1). These compounds were divided into
299 three classes as follows: (i) phenolic acids (gallic acid, 7-hydroxycoumarin-3-carboxylic acid
300 and 2,4-dihydroxy-3,6-dimethylbenzoic acid), (ii) flavonoids (3',5'-dihydroxyflavone, icariin,
301 3,6,3'-trimethoxyflavone and 5-hydroxy-3'-methoxyflavone) and (iii) stilbenes (pinosylvin
302 monomethyl ether) (Table 1).

303 Actual results were consistent with those of Piug et al. (2018), reporting that the phenolic
304 profile of *E. globulus* leaves extracts revealed the presence of the same phenolic classes. In
305 addition, the contents of the identified compounds were ranged from 0.1 to 2.5 mg/g dr for *E.*
306 *brevifolia* and from 0.3 to 7.5 mg/g dr for *E. stricklandii*. When examining the
307 chromatograms of both species (Fig. 3; Fig. 4), it was observable that more than one
308 compound, with low intensity (0 to 75 mV), were detected in the PEth extracts (between 40
309 and 60 min).

310 HPLC-DAD analysis of EtOAc extracts showed that *E. brevifolia* and *E. stricklandii*
311 chromatograms were different, in terms of profile and intensity. While the first extract showed
312 an intensity between 0 and 100 mV, the second one showed a maximum intensity of 600 mV.
313 Despite the low peaks intensity, the *E. brevifolia* EtOAc extract exhibited the largest number
314 of peaks, compared to the *E. stricklandii* one and the other extracts. Overall, three compounds
315 were identified, in *E. brevifolia* EtOAc extract, and four compounds were identified in the *E.*
316 *stricklandii* one. Gallic acid and 3,6,3'-trimethoxyflavone were the common compounds
317 between the two species. The *E. brevifolia* EtOAc extract showed the concentrations of 0.1
318 and 0.2 mg/g dr, respectively, whereas *E. stricklandii* one showed the concentrations of 0.2
319 and 0.6 mg/g dr. The other uncommon compounds were pinosylvin monomethyl ether (0.3
320 mg/g dr) in the *E. brevifolia* EtOAc extract and 7-hydroxycoumarin-3-carboxylic acid (0.7
321 mg/g dr) and 5-hydroxy-3'-methoxyflavone (7.5 mg/g dr) in the *E. stricklandii* one (Fig. 3;
322 Fig. 4). The last mentioned compound displayed the highest concentration among all the
323 identified compounds in the different extracts of the two species. In addition, the highest
324 intensities (mV) were observed in the polar extracts (MeOH and aqueous), with an intensity,
325 which reached the 2500 mV. The total of six phenolic compounds were identified in the *E.*
326 *brevifolia* and *E. stricklandii* MeOH extracts, distributed as follows; four compounds in the *E.*
327 *brevifolia* (gallic acid: 0.1 mg/g dr; 7-hydroxycoumarin-3-carboxylic acid: 2.5 mg/g dr;
328 pinosylvin monomethyl ether: 0.2 mg/g dr and 3,6,3'-trimethoxyflavone: 0.1 mg/g dr). While,

329 only two compounds were identified in the *E. stricklandii* extract, which were icariin and 2,4-
330 dihydroxy-3,6-dimethylbenzoic acid with the contents of 1.1 and 0.4 mg/g dr, respectively
331 (Table 1; Fig. 3; Fig. 4). Concerning the aqueous extracts, phenolic compounds were
332 identified only the *E. brevifolia* species. The two compounds, were gallic acid (0.4 mg/g dr)
333 and 3',5'-dihydroxyflavone (0.3 mg/g dr). By comparison with the literature, all the identified
334 phenolic compounds were found for the first time in *E. brevifolia* and *E. stricklandii* leaves
335 extracts.

336

337 **3.2.2. GC-MS analysis**

338 The GC-MS analysis of *E. brevifolia* and *E. stricklandii* different organic extracts made it
339 possible to identify eleven and fifteen volatile compounds, respectively, before and after
340 derivatization (Table 2).

341 The volatile profile from the different extracts showed the presence of three organic
342 compound classes: terpenes, phenols and others. More than the 2/3 of the volatile compounds
343 was found in the PEth extract of *E. brevifolia*, whereas, no compounds were found in the
344 same extract of *E. stricklandii*, as well as, in the aqueous extracts, of both species (Table 2).
345 The rest of the compounds were distributed as follows: three compounds in the EtOAc
346 extracts and three in the MeOH ones. Since the majority of compounds were a sesquiterpene,
347 several studies mentioned their presence in the essential oil of *Eucalyptus* genus
348 (Benabdesslem et al., 2020). The most abundant compounds, such as, spathulenol and
349 eucalyptol, detected in the PEth extract of *E. brevifolia* were known by their antiproliferative
350 activity (Ivanov et al., 2021). In order to identify more volatile compounds in the different
351 extracts, a derivatization reaction was used to generate silylated products with
352 chromatographic properties and volatility. This step led to the identification of 15 compounds
353 in the different leaves extracts of both *Eucalyptus* species (Table 2). None of these
354 compounds was identified before now in the *Eucalyptus* genus. The volatile profile of the
355 different species extracts showed the presence of 5 classes of organic compounds: alcohols,
356 sugars, acids, ketones and fatty alcohols. The compounds were distributed as follows: twelve
357 and six compounds in the *E. brevifolia* and *E. stricklandii* extracts. The majority of the
358 identified compounds in the *E. brevifolia* species were found in the MeOH extract (twelve),
359 whereas the EtOAc one was the extract, which occupied the majority of molecules for the *E.*
360 *stricklandii* species (four). Only three compounds (ethylene glycol, α -D-xylose and glycerol)

361 were in common between the two species extracts. While ethylene glycol was very abundant
362 in the EtOAc extracts, glycerol was weakly abundant in the MeOH one (Table 2).

363 **3.3. Biological activities**

364 This is the first study that investigated, at the same time, the antioxidant, anti-15-LOX, anti-
365 AChE, anti-XOD, and cytotoxic activities of *E. stricklandii* and *E. brevifolia* leaves extracts.

366

367 **3.3.1. Antioxidant activity**

368 Figure 5 summarizes the antioxidant activity of the different *Eucalyptus* leaves extracts. This
369 activity was expressed in terms of inhibitory concentration (IC₅₀) in µg/mL.

370 It should be noted that the PEth extracts of *E. brevifolia* leaves have not been analyzed.
371 Regardless the eucalyptus species, MeOH extracts exhibited the best antiradical scavenging
372 activity. Aqueous extracts gained the second place, followed by EtOAc extracts, then PEth,
373 with respective IC₅₀ values (µg/mL) (3.0, 6.3, 23.3, and 97.5%) for *E. stricklandii*, and (5.4,
374 7.2, 46.4 and 99.2%) for and *E. brevifolia*. Vitamin C was used as standard and showed an
375 IC₅₀ value equal to 2 µg/mL (Fig. 5). Remarkably, the antioxidant power was improved with
376 the polarity of the extracts. Indeed, the aqueous and MeOH extracts (polar extracts) were
377 more antioxidant (active), than the PEth and EtOAc extracts (less polar). This variability of
378 antioxidant activity depending on the solvents polarity could be explained by the ability of the
379 solvent itself to dissolve the bioactive groups found in a mixture of compounds contained in a
380 natural raw material (Mendes et al., 2016). Statistically, the antioxidant activity of the
381 different extracts, for the two species, depends on the solvents polarity. Thus, there was a
382 significant difference ($p \leq 0.05$) between the PEth and EtOAc extracts of the two species,
383 compared to the other ones (PEth and EtOAc), in terms of antioxidant inhibition (Fig. 5). In
384 this context, it was noted that antioxidant activity of *E. blakelyi* leaves MeOH extract has a
385 higher value than that obtained with the aqueous extract, which is in accordance with the
386 current study (Boukhalfoun et al., 2020). Concerning aqueous extract, it was cited that *E.*
387 *robusta* extract presented an ABTS^{•+} value of 832.8 mg BHT/g which is lower than the IC₅₀
388 found in our study (Vuong et al., 2015). Interestingly, since the low IC₅₀ value, traduces the
389 power of antioxidant activity generated by the extract, there was a high negative correlation
390 between the TPC and their respective antioxidant activity (ABTS) ($R^2 = -0.64$). These
391 findings might be attributed to the richness of the extracts in phenolic acids, flavonoids,
392 tannins and the synergy between them.

393

394 **3.3.2. Anti-AChE activity**

395 From the results illustrated in Table 3, it was inferred that all *E. stricklandii* and *E. brevifolia*
396 leaves extracts exert an inhibitory AChE activity.

397 The inhibition percentage varies from 41.2 to 97.1%. Among them, MeOH one was the most
398 efficient. The following classification was provided by the increasing potency of AChE
399 inhibitory activity for *E. brevifolia*: Aqueous > MeOH > PEth > EtOAc. Contrariwise, the
400 potency of *E. stricklandii* extracts against AChE was as follow: MeOH > PEth > EtOAc >
401 Aqueous. Results depicted in Table 3 showed that *E. brevifolia* polar extracts (MeOH and
402 aqueous) exhibited the highest IC₅₀ value equal to 10 and 13 µg/mL, respectively. Mat-Ur-
403 Rasool et al. (2020) scrutinized the ability of *E. globulus* MeOH extract to inhibit AChE with
404 an IC₅₀ value equal to 1.1 mg/mL, while others demonstrated that *Eucalyptus galbie* MeOH
405 extract was exempt from cholinesterase activity (Gholam-Hoseinian et al., 2009). Some
406 reports, suggested a higher correlation between TPC and AChE inhibition activity (Amessis-
407 Ouchemoukh et al., 2014). Whereas, in this study there was a significant modest correlation
408 ($R^2 = 0.48$) between TPC and anti-AChE activity for both *Eucalyptus* species (Table 5).
409 Therefore, this anti-AChE activity may be related to polyphenolic compounds, which were
410 stipulated as neuroprotective agents (González-Burgos et al., 2018).

411

412 **3.3.3. Anti-15-LOX activity**

413 From table 3, it can be deduced that only the polar extracts (MeOH and aqueous), for both
414 species, showed an inhibitory activity against 15-LOX. Statistically, there was a significant
415 difference ($p \leq 0.05$) between the mentioned previous solvents. However, PEth and EtOAc
416 extracts does not show any inhibitory activity for both *Eucalyptus* species. The highest IC₅₀
417 values were obtained with the MeOH. As for *E. brevifolia*, the IC₅₀ value was 21.3 µg/mL,
418 while for *E. stricklandii* was about 9.7 µg/mL (Table 3). The current results were higher than
419 the inhibition activity highlighted by the aqueous extract of *E. citriodora* Kino species, which
420 showed an IC₅₀ equal to 103.56 µg/mL. It was reported that the anti-15-LOX activity was
421 rather caused by the EtOAc extract of *E. citriodora* Kino (Hung et al., 2018). Elgorashi and
422 McGaw (2019) indicated that the polar extracts (EtOH 50% and MeOH) of *E. camaladulensis*
423 Dehnh. were studied for their *in-vivo* pharmacological activities. Their findings elucidated
424 that *E. camaladulensis* Dehnh. extract can be considered non-lethal up to a dose of 2000
425 mg/kg, and these *Eucalyptus* leaf extract could be safe for use. The question is, what is behind

426 this anti-inflammatory power? The high correlation found between TPC and anti-15-LOX
427 activity ($R^2 = 0.74$), suggested the presence of other powerful phenolic compounds able to
428 inhibit 15-LOX enzyme. Due to their richness in polyphenols, *Eucalyptus* trees are known to
429 have a good anti-15-LOX activity by chelating the iron of the active site of the lipoxygenase
430 and/or by reducing the ferric form of the enzyme to an inactive ferrous form (Hartung et al.,
431 2019).

432

433 **3.3.4. Anti-XOD activity**

434 None of previous researches on *E. stricklandii* and *E. brevifolia* has investigated its inhibitory
435 capacity towards the XOD, an enzyme implicated in gout disease, catalyzes the oxidation of
436 hypoxanthine and xanthine to uric acid (Sung et al., 2021). Statistically, there was a
437 significant difference ($p \leq 0.05$) between MeOH extract, compared to the other extracts (PEth,
438 EtOAc and aqueous extracts). The percentage of inhibition depicted in Table 3, ranged from
439 6.9 to 51.1% (Table 3). It was demonstrated that all tested extracts exert inhibition potency
440 toward XOD, except *E. brevifolia* PEth and aqueous extracts, in addition to *E. stricklandii*
441 EtOAc extract. For both species, MeOH extracts gained the first place with an inhibition
442 percentage equal to 51.1 and 21.9%, respectively, for *E. stricklandii* and *E. brevifolia* (Table
443 3). It was reported that *E. deglupta* in Puerto Rico showed a high inhibition percentage of
444 XOD 51%, with an IC_{50} equal to 44.5 $\mu\text{g/mL}$ (Mehta and Nayeem, 2014), since there was a
445 high correlation between TPC and XOD ($R^2 = 0.66$). This considerable XOD activity might
446 be due to the presence of the phenolic compounds. It was also elucidated that *E. gunii* Hook.
447 F. MeOH extract exhibited higher antioxidant activity using XOD test compared to the
448 essential oil (Mimica-Dukić et al., 2012).

449

450 **3.3.5. Cytotoxic activity**

451 From Table 4, it was depicted that all cancer cell lines were highly inhibited by PEth followed
452 by EtOAc, aqueous then MeOH extracts.

453 The highest percentage of inhibition (96.2%), was assigned to *E. stricklandii* PEth extract
454 against HCT-116 followed by the same solvent used for *E. brevifolia*, with an inhibition
455 percentage equal to 92.5%, which is too close to the tamoxifen standard (99.5%) (Table 4).
456 HCT-116 was also strongly sensitive to EtOAc of both *Eucalyptus* species with an inhibition
457 percentage equal to 89.2 and 75.5%, respectively, for *E. stricklandii* and *E. brevifolia*. Among
458 the eight tested extracts, only the *E. stricklandii* MeOH was the less powerful against HCT-
459 116 (2.3%). The cytotoxicity of the different extracts depends on the solvents polarity.

460 Statistically, there was a significant difference ($p \leq 0.05$) between the four solvents.
461 Regarding MCF-7 and OVCAR cell lines, they were, also, affected by PEth and EtOAc
462 extracts of both *Eucalyptus* species, with an inhibition percentage comprised between 41.2
463 and 65.1% (Table 4). The previous study showed that flavonoids displayed an anticancer
464 effect against the OVCAR cells (Tavsan and Kayali, 2019). Moreover, it was cited that the
465 phenolic compounds identified in *E. globulus* bark EtOH extracts were responsible for the
466 antiproliferative potency of the latter plant material against breast cancer cell line MDAMB-
467 231 (Bhuyan, 2018). These precedent findings are proved by the current work, where there
468 was a high correlation between the different cell lines (MCF-7, HCT-116, and OVCAR), and
469 TPC, with R^2 of 0.87, 0.73, and 0.77, respectively.

470

471 3.4. PCA analysis

472 To better understand the relationship between TPC and the different phenolic compounds
473 with the biological activities of the two *Eucalyptus* species (*E. brevifolia* and *E. stricklandii*),
474 PCA analysis was done. The structuring of accessions showed 62.52% of the total variation
475 (Fig. 6). Axes were retained because they expressed 43.76% (PC1) and 18.77% (PC2).
476 Simultaneously, the loadings in the PCA loading plot express showed well that the principal
477 components correlate with the original variables, and also the correlations between the
478 different activities and TPC or the different phenolic compounds. As shown in Fig. 6, PC1
479 axis has a strong positive correlation with 15-LOX, XOD, and TPC for a loading of 0.96, 0.87
480 and 0.84, respectively. This being less pronounced for the compounds, 2,4-dihydroxy-3,6-
481 dimethylbenzoic acid and icariin ($r = 0.75$), and for the AChE activity ($r = 0.60$). Besides,
482 PC1 axis showed, also, a good correlation, in the negative side, with more than activity. PC1
483 was well negatively correlated with the level of cytotoxic activities (OVCAR, HCT-116 and
484 MCF-7 cell lines) with $r = -0.94$, $r = -0.89$ and $r = -0.82$, respectively (Table 5). However, it
485 was less pronounced for the variable, antioxidant activity ($r = -0.73$). Through this
486 distribution on PC1, several explanations can be deduced. On the one hand, TPC was closer to
487 the 15-LOX, XOD and AChE activities, and in a symmetrical position to the antioxidant
488 activity, expressed in IC_{50} , which suggest that the inhibition of this activity was greatly
489 enhanced by these compounds. Furthermore, icariin and 2,4-dihydroxy-3,6-dimethylbenzoic
490 acid were, probably, the most efficient molecules against XOD enzyme. On the other hand,
491 MCF-7, HCT-116, and OVCAR, were placed in a symmetrical position compared to the TPC,
492 which explain that these last, were not the responsible molecules for the mentioned activities.
493 For the PC2, there was a good positive correlation with the two phenolic compounds gallic

494 acid, 3,6,3'-trimethoxyflavone, and 7-hydroxycoumarin-3-carboxylic acid with loading of
495 0.82, 0.59 and 0.58, respectively. The two other axis (F3 and F4) showed a high positive
496 correlation with 3',5'-dihydroxyflavone ($r = 0.91$) and 5-hydroxy-3'-methoxyflavone ($r =$
497 0.71) (Table 5). Oval forms shown on Figure 7 grouped the different organic extracts in four
498 classes: C1 (*E. stricklandii* EtOAc), C2 (*E. brevifolia* MeOH and *E. brevifolia* aqueous), C3
499 (*E. stricklandii* PEth, *E. brevifolia* PEth, and *E. brevifolia* EtOAc), while C4 contains (*E.*
500 *stricklandii*- aqueous, *E. stricklandii* MeOH).

501 If the two plots (biplot) (Fig. 8) were gathering, many suggestions could be made. It was seen
502 that the high TPC level was related to the *E. brevifolia* MeOH extract, which exhibited the
503 highest anti-AChE activity. In addition, the richness of *E. stricklandii* MeOH extract with
504 icariin and 2,4-dihydroxy-3,6-dimethylbenzoic acid compounds, could explain its high XOD
505 activity.

506 Not only PCA showed the relationship between chemical composition and biological
507 activities. It was demonstrated that gallic acid plays a role as a strong antioxidant and free
508 radical scavenging activities, anti-inflammatory power, protects against degenerative disease
509 (Gao et al., 2019). This interesting chemical compound was present in EtOAc extract of both
510 eucalyptus species, as well as MeOH and aqueous *E. brevifolia* extracts. 7-Hydroxycoumarin-
511 3-carboxylic acid, found in *E. brevifolia* MeOH extract ($IC_{50} = 13 \mu\text{g/mL}$) was demonstrated
512 to be as cholinesterase inhibitors (Alipour et al., 2014).

513 It was reported, that icariin, which was present in the MeOH *E. stricklandii* extract, is a
514 molecule endowed with biological effects to counter many pathophysiological processes of
515 Alzheimer disease, and possess an impact on oxidative stress and inflammatory cascade
516 (Angeloni et al., 2019). 2,6-Dihydroxyacetophenone also known as 2-acetylresorcinol
517 observed after derivatization in PEth and EtOAc *E. stricklandii* extracts was reported to be
518 effective against severe diseases such as cancer, osteoporosis, and gastric ulcers (Durairaj,
519 2005). This is in agreement with our findings since the inhibitory percentage against
520 HCT116, MCF7 and OVCAR ranges from 96 to 41%.

521

522 **4. Conclusion**

523 Interestingly, based on the findings discussed for the first time in this report, *E. brevifolia* and
524 *E. stricklandii* various extracts could be used as a readily available source of natural bioactive
525 molecules. They have displayed an antioxidant, anti-inflammatory, anti-Alzheimer, anti-
526 xanthine oxidase, and antiproliferative properties. It was demonstrated that each *Eucalyptus*
527 extract species was selective for one or different bioactivities, as summarized in the principal

528 component analysis biplot. Nevertheless, it was shown that the polarity of solvent extracts has
529 been involved in the wide range of the biological activities of both *E. brevifolia* and *E.*
530 *stricklandii*. Moreover, the chemical composition analysis, using HPLC-DAD allowed the
531 identification of eight phenolic compounds belonging to the phenolic acids, flavonoids and
532 stilbenes chemical classes. GC-MS analysis provided eleven volatile molecules before
533 derivatization and fifteen ones after derivatization. The abundancy of these molecules was
534 solvent-extract dependent. We suggested that the *Eucalyptus* extracts mode of action was
535 related to the combination of the existing mixture of biomolecules, and not only to a single
536 one as, icariin or gallic acid, etc. Therefore, further studies are required to establish the
537 suitability of the investigated *Eucalyptus* extracts, connected to health benefits, for
538 incorporation either as a dietary supplement or as medicine.

539

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546

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553

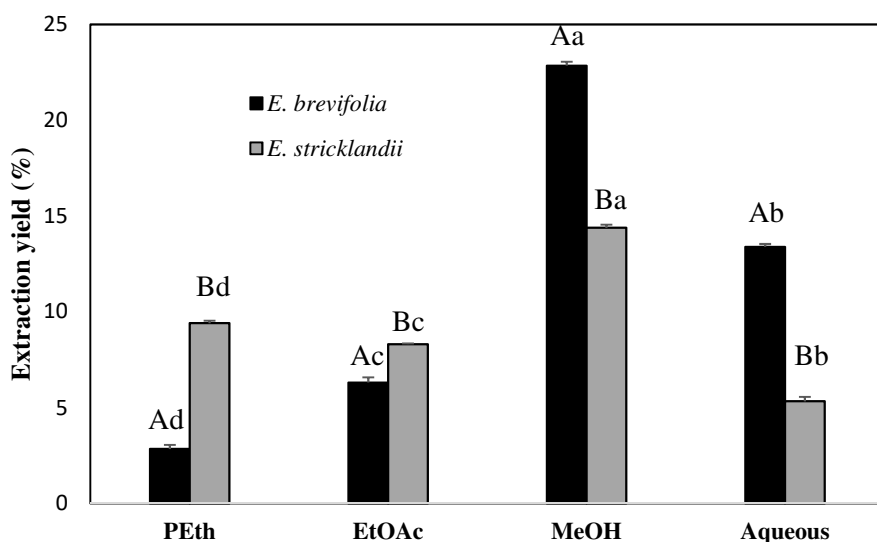
554 **Author Contribution Statement**

555 D. B. H conceived, performed the experiments, analyzed the data, and wrote the article. R. R.
556 performed the HPLC section, statistical analysis and contributed to the redaction of the
557 manuscript. J. P. S, M. A and J. B contributed samples/reagents/materials/analysis tools,
558 conceived experiments and validated results to be ready for publication.

559

560 **Conflict of interest**

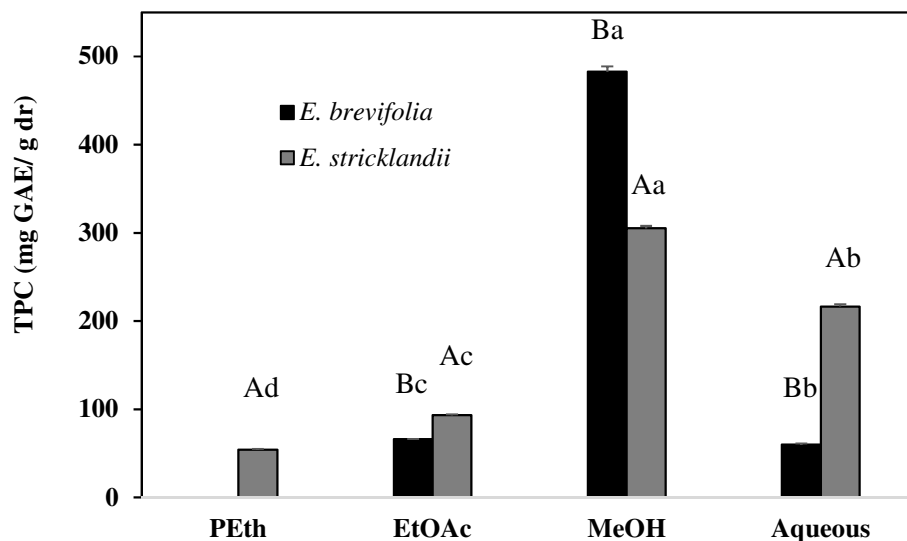
561 The authors declare that they have no conflict of interest.



562

563 **Figure 1:** Extraction yield of various *E. brevifolia* and *E. stricklandii* organic extracts. Capital
 564 letter means the difference between species; Lowercase letter means difference between
 565 solvents; (PEth: petroleum ether; EtOAc: ethyl acetate; MeOH: methanol; Aqueous: distilled
 566 water).

567



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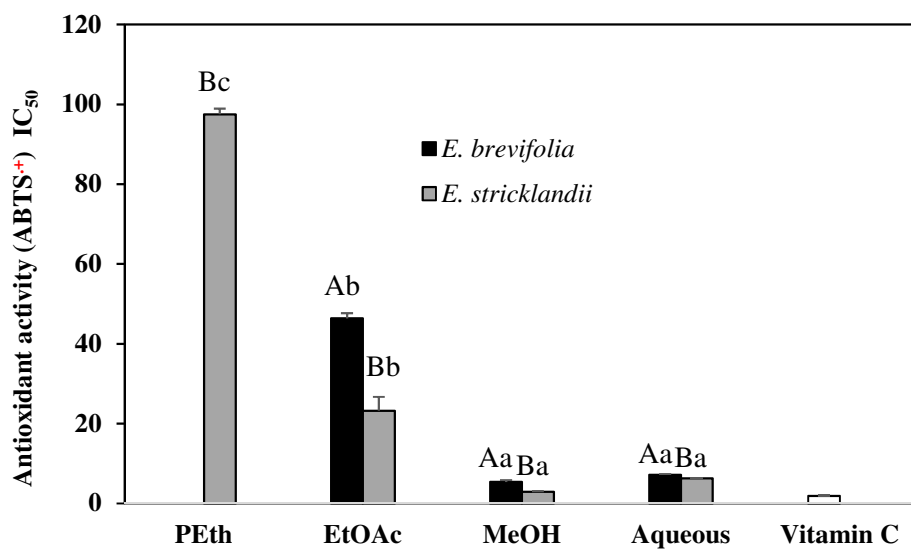
569 **Figure 2:** Total phenolics content (TPC) of different leaves extracts of *E. brevifolia* and *E.*

570 *stricklandii*. Capital letter means the difference between species; Lowercase letter means

571 difference between solvents; (PEth: petroleum ether; EtOAc: ethyl acetate; MeOH: methanol;

572 Aqueous: distilled water).

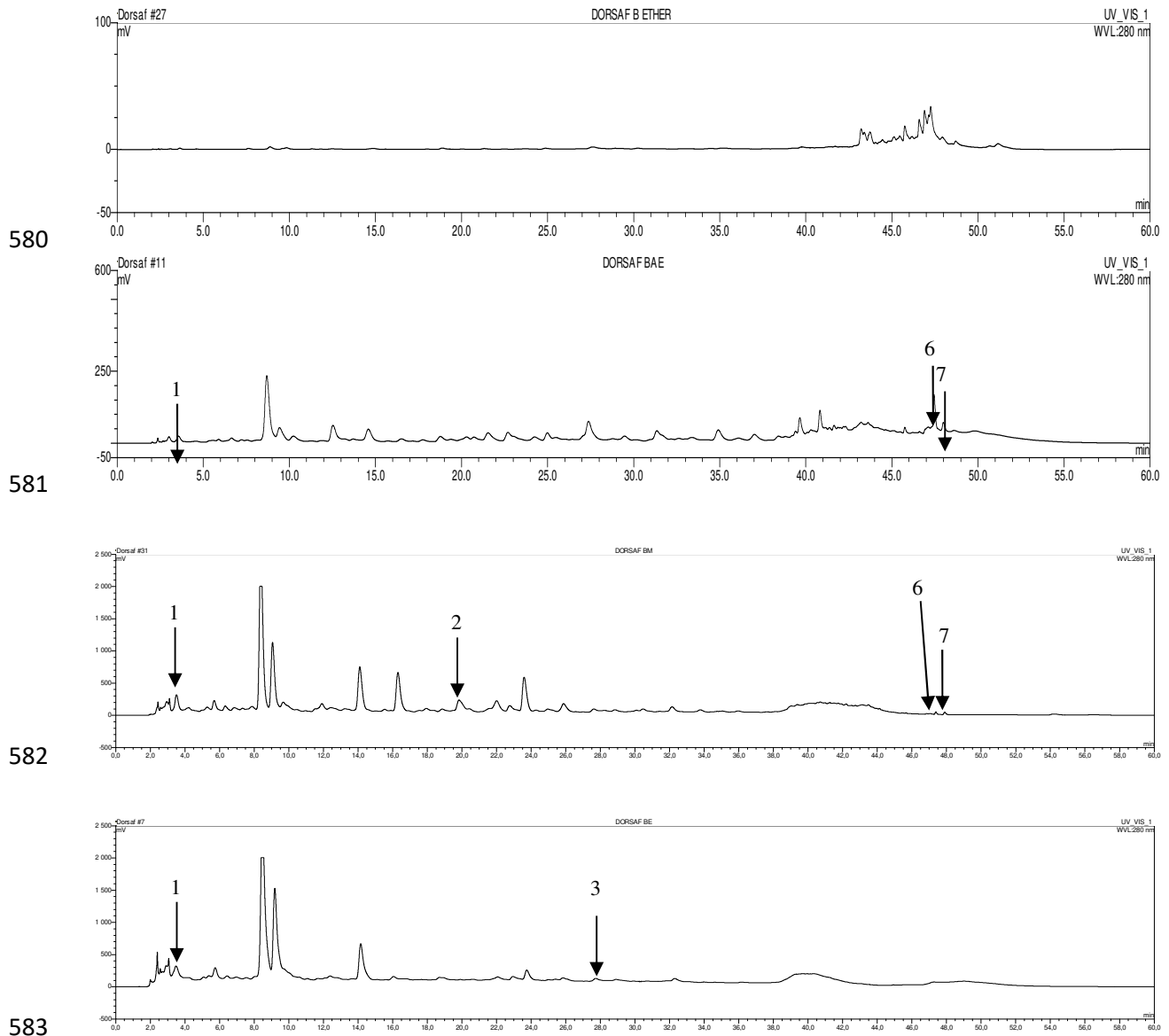
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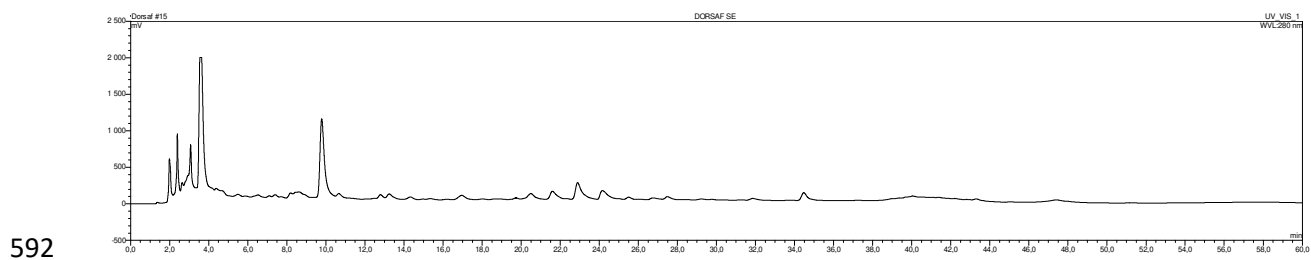
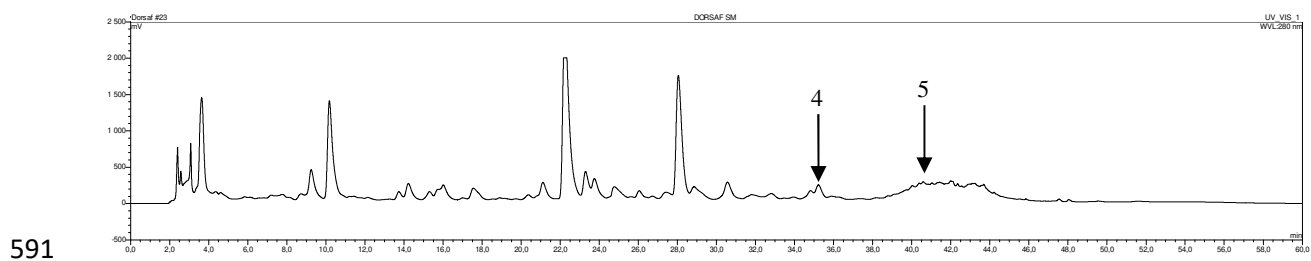
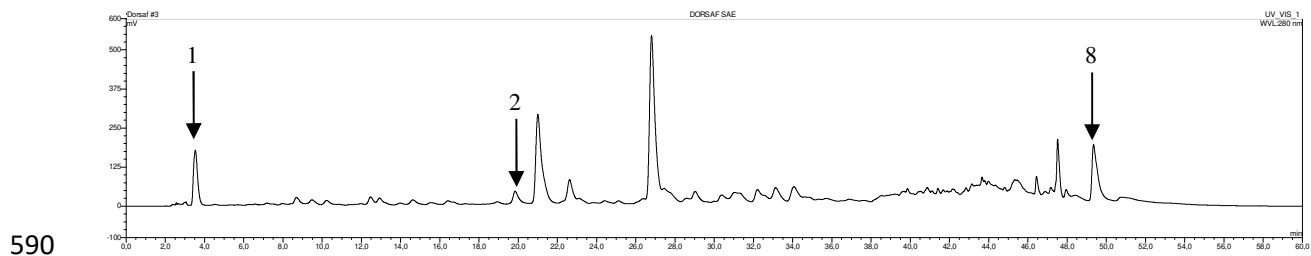
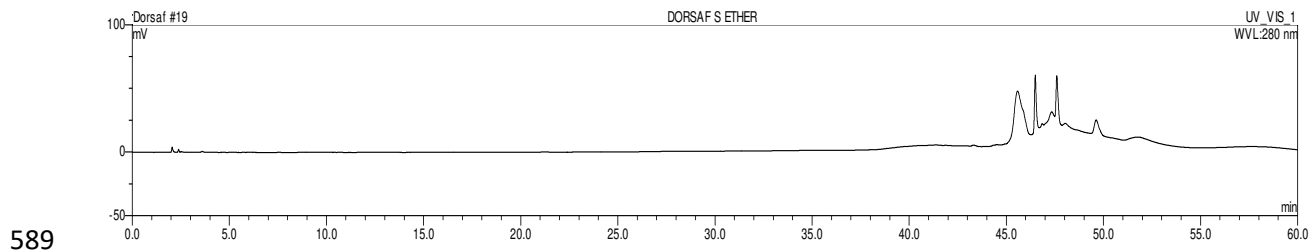
575 **Figure 3:** Antioxidant activity (anti-ABTS⁺) of the two *Eucalyptus* species (*E. brevifolia* and
 576 *E. stricklandii*). Capital letter means the difference between species; Lowercase letter means
 577 difference between solvents; (PEth: petroleum ether; EtOAc: ethyl acetate; MeOH: methanol;
 578 Aqueous: distilled water).

579



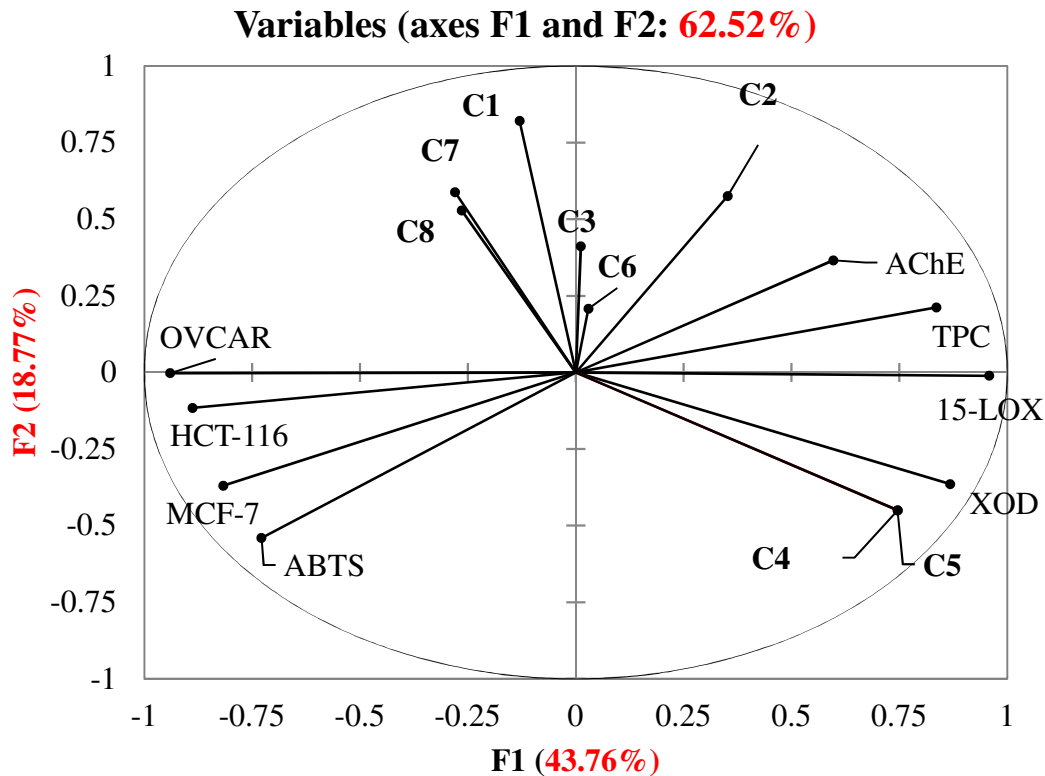
584 **Figure 4:** HPLC chromatograms of *E. brevifolia* leaves extracts. (PEth: petroleum ether;
 585 EtOAc: ethyl acetate; MeOH: methanol; Aqueous: distilled water). Peaks: **(1)** gallic acid; **(2)**
 586 7-hydroxycoumarin-3-carboxylic acid; **(3)** 3'.5'-dihydroxyflavone; **(6)** pinosylvin monomethyl
 587 ether **(7)** 3.6.3'-trimethoxyflavone.

588



593 **Figure 5:** HPLC chromatograms of *E. stricklandii* leaves extracts. (PEth: petroleum ether;
 594 EtOAc: ethyl acetate; MeOH: methanol; Aqueous: distilled water). Peaks: **(1)** gallic acid; **(2)**
 595 7-hydroxycoumarin-3-carboxylic acid; **(4)** 2,4-dihydroxy-3,6-dimethylbenzoic acid; **(5)**
 596 icariin; **(8)** 5-hydroxy-3'-methoxyflavone.

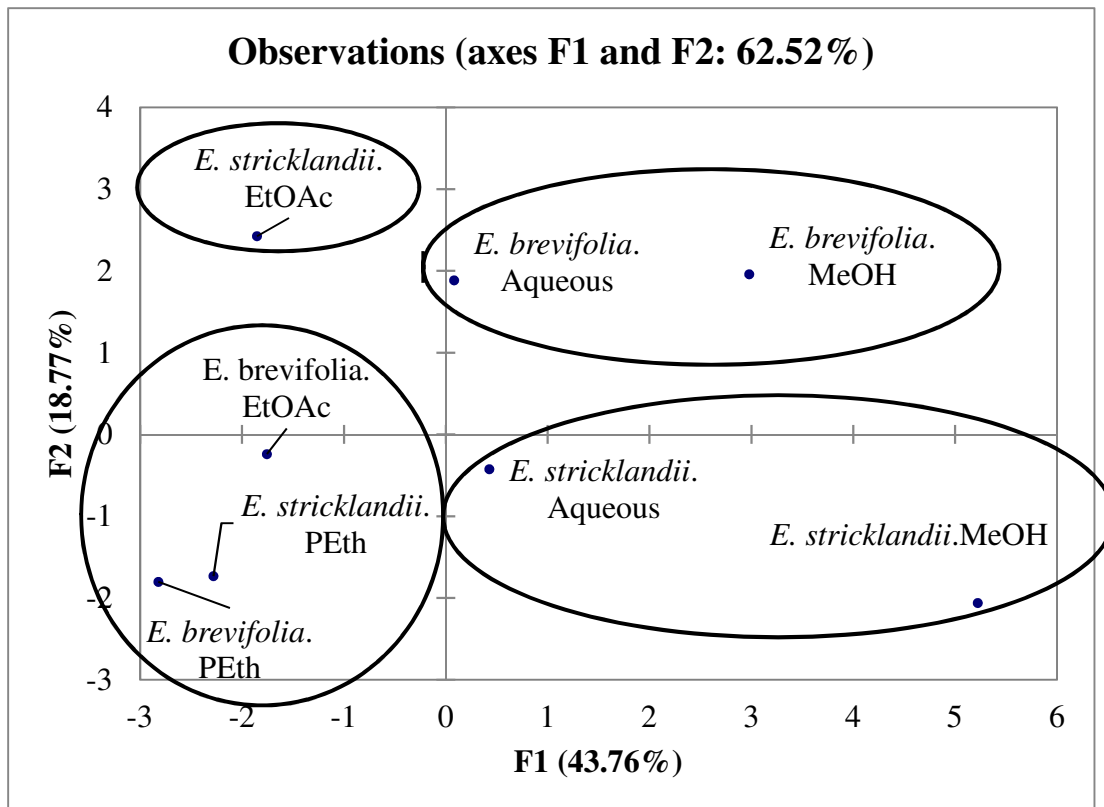
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598

599 **Figure 6:** Principal components analysis “loading plot” of chemical composition (TPC: total
 600 phenolics content) and biological properties; ABTS: antioxidant activity; XOD: anti-xanthine
 601 oxidase activity; 15-LOX: anti-15-lipoxygenase activity; AChE: anti-acetylcholinesterase
 602 activity; MCF-7, HCT-116 and OVCAR: cytotoxic activity) of *E. brevifolia* and *E.*
 603 *stricklandii* leaves extracts. **C1:** gallic acid; **C2:** 7-hydroxycoumarin-3-carboxylic acid; **C3:**
 604 3',5'-dihydroxyflavone; **C4:** 2,4-dihydroxy-3,6-dimethylbenzoic acid; **C5:** icariin; **C6:**
 605 pinosylvin monomethyl ether; **C7:** 3,6,3'-trimethoxyflavone; **C8:** 5-hydroxy-3'-
 606 methoxyflavone.

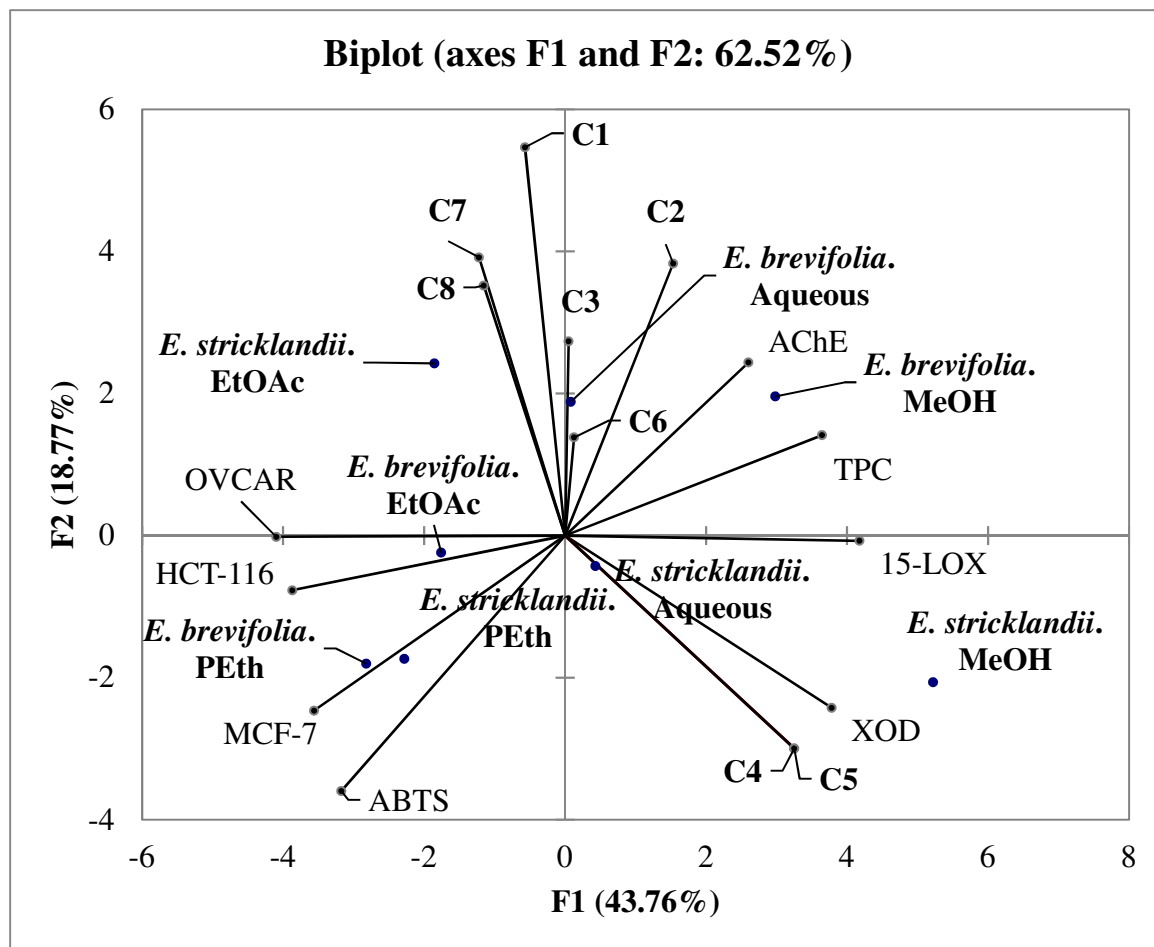
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608

609 **Figure 7:** Principal components analyses “score plot” of different *Eucalyptus* species extracts
 610 (PEth: petroleum ether; EtOAc: ethyl acetate; MeOH: methanol; Aqueous: distilled water).

611

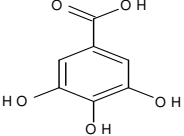
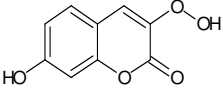
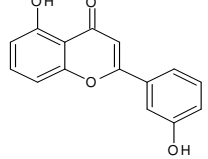
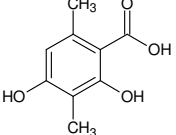
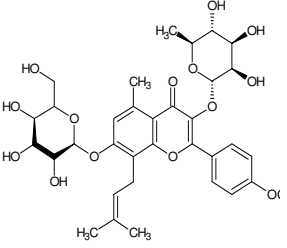
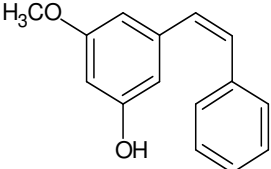


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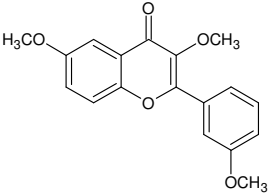
613 **Figure 8:** Principal components analysis biplot of biological activities of different *Eucalyptus*
 614 species extracts. **C1:** gallic acid; **C2:** 7-hydroxycoumarin-3-carboxylic acid; **C3:** 3'5'-
 615 dihydroxyflavone; **C4:** 2,4-dihydroxy-3,6-dimethylbenzoic acid; **C5:** icariin; **C6:** pinosylvin
 616 monomethyl ether; **C7:** 3,6,3'-trimethoxyflavone; **C8:** 5-hydroxy-3'-methoxyflavone.

617

618 **Table 1:** Identified and quantified phenolic compounds in *E. brevifolia* and *E. stricklandii*
 619 extracts using HPLC-DAD.

N°	Compounds and chemical structure	Rt (min)	Concentration (mg/g dr)								References
			<i>E. brevifolia</i>				<i>E. stricklandii</i>				
			PEth	EtOAc	MeOH	Aqueous	PEth	EtOAc	MeOH	Aqueous	
1	 Gallic acid	3.48	-	0.1	0.1	0.4	-	0.3	-	-	(Singh et al., 2017)
2	 7-hydroxycoumarin-3-carboxylic acid	19.83	-	-	2.5	-	-	0.7	-	-	(Wahdan et al., 2019)
3	 3',5'-dihydroxyflavone	27.73	-	-	-	0.3	-	-	-	-	(Mnif and Aifa, 2015)
4	 2,4-dihydroxy-3,6-dimethylbenzoic acid	35.21	-	-	-	-	-	-	0.4	-	(Filho et al., 2014)
5	 icariin	41.99	-	-	-	-	-	-	1.1	-	(Yang et al., 2020)
6	 pinosylvin monomethyl	47.05	-	0.3	0.2	-	-	-	-	-	(Vek et al., 2020)

ether

7		47.89	-	0.2	0.1	-	-	0.6	-	-	(Erdenetsog t et al., 2020)
---	---	-------	---	-----	-----	---	---	-----	---	---	-----------------------------------

3,6,3'-

trimethoxyflavone

8		49.35	-	-	-	-	-	7.5	-	-	(Voirin, 1983)
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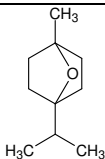
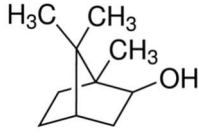
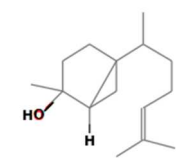
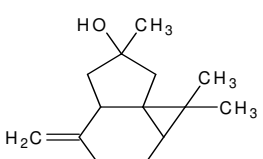
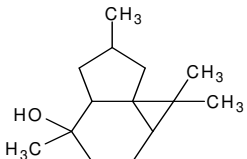
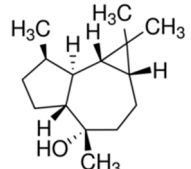
5-hydroxy-3'-

methoxyflavone

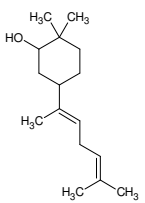
620 **Rt:** retention time; **PEth:** petroleum ether; **EtOAc:** ethyl acetate; **MeOH:** methanol; **Aqueous:** distilled water.

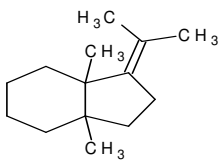
621

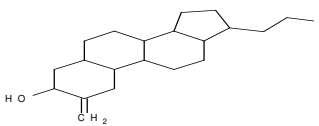
622 **Table 2:** Volatile compounds identified in *E. brevifolia* and *E. stricklandii* extracts before and after
 623 derivatization.

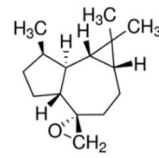
N°	Compounds and chemical structure	Rt (min)	<i>E. brevifolia</i>				<i>E. stricklandii</i>			
			PEth	EtOAc	MeOH	Aqueous	PEth	EtOAc	MeOH	Aqueous
Before derivatization										
1	 Eucalyptol	8.31	++	-	++	-	-	-	+	-
2	 Isoborneol	10.32	+	-	-	-	-	-	-	-
3	 <i>Trans</i> -sesquisabinene hydrate	13.28	+	-	-	-	-	-	-	-
4	 Spathulenol	14.29	+++	-	-	-	-	-	-	-
5	 Globulol	14.34	+++	-	-	-	-	++	-	-
6		14.43	+++	-	-	-	-	-	-	-

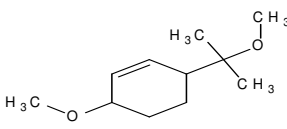
Epiglobulol

7		14.46	-	+++	+++	-	-	-	-	-
	<i>Trans-Z-α-bisabolene-epoxide</i>									

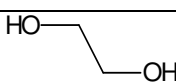
8		14.66	++	-	-	-	-	-	-	-
	1H-indene.1-ethylidene octahydro-7a-methy-cis									

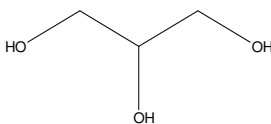
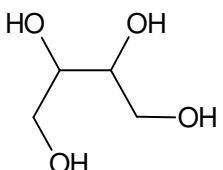
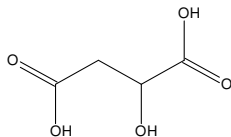
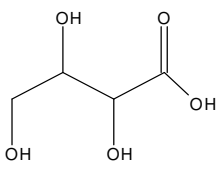
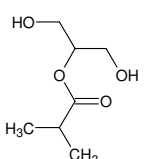
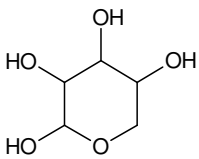
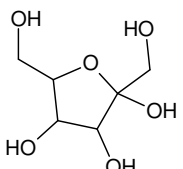
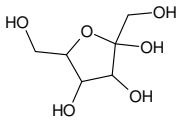
9		15.09	+	-	-	-	-	-	-	-
	2-methylene-5α-cholestan-3β-ol									

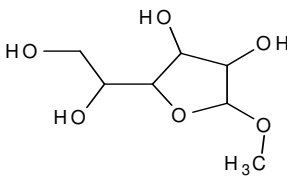
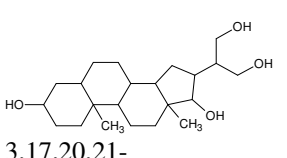
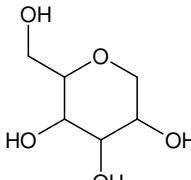
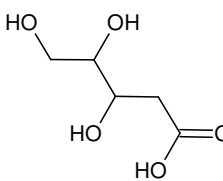
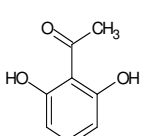
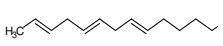
10		15.49	+	-	-	-	-	-	-	-
	Isoaromadendren-epoxide									

11		15.84	+	-	-	-	-	-	-	-
	Cyclohexene.4-isopropenyl.1-methoxy-methoxymethyl									

After derivatization

1		7.56	-	+++	++	-	-	+++	-	-
	Ethylene glycol									

2		11.30	-	-	+	-	-	-	+	-
	Glycerol									
3		13.45	-	-	-	-	-	-	+	-
	<i>meso</i> -Erythriol									
4		14.11	-	-	++	-	-	-	-	-
	Malic acid									
5		15.35	-	-	+	-	-	-	-	-
	2,3,4-Trihydroxybutyric acid									
6		15.58	-	-	+	-	-	-	-	-
	2-Hydroxy-1-(hydroxymethyl)ethyl 2-methylpropanoate									
7		15.64	-	-	+	-	-	+	-	-
	α -L-Xylose									
8		15.78	-	-	++	-	-	-	-	-
	α -D-tagatofuranose									
9		15.84	-	-	+++	-	-	-	-	-
	D-fructofuranose									

10		16.10	-	-	++	-	-	-	-	-
	Methyl .alpha.-D-glucofuranoside									
11		16.18	-	-	++	-	-	-	-	-
	3,17,20,21-Allopregnanetretol									
12		16.27	-	-	+	-	-	-	-	-
	1,5-anhydroglucitol (1,5-AG)									
13		16.38	-	-	+	-	-	-	-	-
	2-Desoxypentonic acid									
14		16.87	-	-	-	-	++	++	-	-
	2,6-Dihydroxyacetophenone (2-Acetylresorcinol)									
15		20.83	-	-	-	-	-	+	-	-
	Monolinolenin									

624 **Rt:** retention time; **PEth:** petroleum ether; **EtOAc:** ethyl acetate; **MeOH:** methanol; **Aqueous:** distilled water;

625 -: not detected; +++: very abundant; +: weakly abundant

626

627 **Table 3:** Biological activities of *E. brevifolia* and *E. stricklandii* extracts.

Species	Solvents	Anti-AChE		Anti-15-LOX		Anti-XOD
		IP (%) at 50 µg/mL	IC ₅₀ (µg/mL)	IP (%) at 50 µg/mL	IC ₅₀ (µg/mL)	IP (%) at 50 µg/mL
<i>E. brevifolia</i>	PEth	57.5±1.6	-	na	-	Na
	EtOAc	56.3±3.1	-	na	-	11.0±3.6
	MeOH	96.8±0.0	13.0±1.7	75.6±0.1	21.3±1.5	21.9±7.4
	Aqueous	97.1±0.0	10.0±0.0	45.2±0.1	59.3±1.1	na
<i>E. stricklandii</i>	PEth	61.5±3.3	-	na	-	7.9±1.0
	EtOAc	58.1±2.3	-	na	-	na
	MeOH	84.1±0.0	48.7±0.6	100.9±8.5	9.7±0.6	51.1±2.3
	Aqueous	41.2±0.1	-	10.4±0.0	-	6.9±0.0
Standards	Galanthamine	-	1.0±0.6	-	-	-
	NDGA	-	-	-	2.2±0.1	-
	Allopurinol	-	-	-	-	60.0±1.3

628 **PEth:** petroleum ether; **EtOAc:** ethyl acetate; **MeOH:** methanol; **Aqueous:** distilled water; **na:** not
629 active.

630

631 **Table 4:** Cytotoxic activities of *E. brevifolia* and *E. stricklandii* organic extracts against human cancer cell lines
 632 (HCT-116, MCF-7 and OVCAR) expressed as percentage inhibition (at 50 µg/mL) means.

Species	Solvents	HCT-116 (%)	MCF-7 (%)	OVCAR (%)
<i>E. brevifolia</i>	PEth	92.5±0.6	65.1±1.7	63.5±6.2
	EtOAc	75.5±5.5	48.4±0.9	62.6±15.9
	MeOH	17.8±0.3	15.6±2.7	35.4±3.4
	Aqueous	26.1±1.5	39.2±3.9	49.2±6.5
<i>E. stricklandii</i>	PEth	96.2±0.4	59.1±1.3	56.9±3.8
	EtOAc	89.2±0.6	41.2±2.6	47.0±1.6
	MeOH	18.0±0.2	24.7±3.1	16.22±1.3
	Aqueous	13.7±4.2	20.1±3.9	39.3±6.9
Tamoxifen		99.5±0.0	47.2±4.3	60.5±4.3

633 **PEth:** petroleum ether; **EtOAc:** ethyl acetate; **MeOH:** methanol; **Aqueous:** distilled water.

634 Tamoxifen (a standard) was tested at 0.2 mg/L.

635

Table 5: Correlations between variables and factors.

	F1	F2	F3	F4
TPC	0.84	0.21	-0.38	-0.29
AChE	0.60	0.37	0.42	-0.20
XOD	0.87	-0.36	-0.18	0.10
15-LOX	0.96	-0.01	0.14	0.01
MCF-7	-0.82	-0.37	0.18	0.09
HCT-116	-0.89	-0.12	-0.21	0.15
OVCAR	-0.94	-0.00	0.08	-0.27
ABTS⁺	-0.73	-0.54	-0.05	-0.13
C1	-0.13	0.82	0.45	0.26
C2	0.35	0.58	-0.46	-0.40
C3	0.01	0.41	0.91	-0.03
C4	0.75	-0.45	0.04	0.43
C5	0.75	-0.45	0.04	0.43
C6	0.03	0.21	-0.40	-0.58
C7	-0.28	0.59	-0.50	0.53
C8	-0.27	0.53	-0.36	0.71

637 **C1:** gallic acid; **C2:**7-hydroxycoumarin-3-carboxylic acid; **C3:** 3',5'-dihydroxyflavone; **C4:** 2,4-
638 dihydroxy-3,6-dimethylbenzoic acid; **C5:** icariin; **C6:** pinosylvin monomethyl ether; **C7:** 3,6,3'-
639 trimethoxyflavone; **C8:** 5-hydroxy-3'-methoxyflavone.

641 **References**

- 642 • Alipour, M., Khoobi, M., Moradi, A., Nadri, H., Moghadam, F. H., Emami, S.,
643 Hasanpour, Z., Foroumadi, A., Shafiee, A., 2014. Synthesis and anti-cholinesterase
644 activity of new 7-hydroxycoumarin derivatives. *European Journal of Medicinal*
645 *Chemistry* 82, 536-544.
- 646 • Alsharif, W., Saad, M. M., Hirt, H., 2020. Desert microbes for boosting sustainable
647 agriculture in extreme environments. *Frontiers in Microbiology* 11, 1666.
- 648 • Amat-Ur-Rasool, H., Symes, F., Tooth, D., Schaffert, L. N., Elmorsy, E., Ahmed, M.,
649 Hasnain, S., Carter, W. G., 2020. Potential nutraceutical properties of leaves from
650 several commonly cultivated plants. *Biomolecules* 10, 1-22.
- 651 • Amessis-Ouchemoukh, N., Madani, K., Falé, P. L. V., Serralheiro, M. L., Eduarda,
652 M., Araújo, M., 2014. Antioxidant capacity and phenolic contents of some
653 Mediterranean medicinal plants and their potential role in the inhibition of
654 cyclooxygenase-1 and acetylcholinesterase activities. *Industrial Crops and Products*
655 53, 6-15.
- 656 • Angeloni, C., Barbalace M. C., Hrelia, S., 2019. Icariin and its metabolites as potential
657 protective phytochemicals against Alzheimer's disease. *Frontiers in Pharmacology* 10,
658 271.
- 659 • Ben Hassine, D., Abderrabba, M., Cazaux, S., Bouajila, J., 2013. Contribution to the
660 chemical and biological study of *Eucalyptus* leaves extracts: Effect on frying oil.
661 *Journal of Medical and Bioengineering* 2, 242-247.
- 662 • Ben Lataief, S., Zourgui, M. N., Rahmani, R., Najjaa, H., Gharsallah, N., Zourgui, L.,
663 2020. Chemical composition, antioxidant, antimicrobial and cytotoxic activities of
664 bioactive compounds extracted from *Opuntia dillenii* cladodes. *Journal of Food*
665 *Measurement and Characterization* 15, 782-794.
- 666 • Benabdesslem, Y., Hachem, K., Mébarki, M., 2020. Chemical composition of the
667 essential oil from the leaves of *Eucalyptus globulus* Labill. growing in southwest
668 Algeria. *Journal of Essential Oil Bearing Plants* 23, 1154-1160.
- 669 • Bhuyan, D. J., 2018. Phytochemicals derived from Australian *Eucalypts* as anticancer
670 agents for pancreatic malignancies. Thesis 356 pp. University of Newcastle, Australia.
- 671 • Boukhalafoun, L., Kirouani, A., Behidj, N., Gana, S., 2020. Assessment of some
672 biological activities of *Eucalyptus blakelyi* Maiden using the essential oil, methanolic
673 and aqueous extracts. *Journal of Essential Oil Bearing Plants* 23, 266-275.

- 674 • Dezsi, Ş., Bădărău, A. S., Bischin, C., Vodnar, D. C., Silaghi-Dumitrescu, R., Gheldiu,
675 A. M., Mocan, A., Vlase, L., 2015. Antimicrobial and antioxidant activities and
676 phenolic profile of *Eucalyptus globulus* Labill. and *Corymbia ficifolia* (F. Muell.)
677 K.D. Hill & L.A.S. Johnson leaves. *Molecules* 20, 4720-4734.
- 678 • Durairaj, R. B., 2005. Resorcinol chemistry in pharmaceuticals applications. In:
679 Resorcinol. Springer, Berlin, Heidelberg. https://doi.org/10.1007/3-540-28090-1_9
- 680 • Elaieb, M. T., Ayed, B. S., Dumarçay, S., De-Faria, B. F. H., Thévenon, M. F.,
681 Gérardin, P., Candelier, K., 2020. Natural durability of four Tunisian *Eucalyptus* spp.
682 and their respective compositions in extractives. *Holzforschung* 74, 260-274.
- 683 • Elansary, H. O., Salem, M. Z. M., Ashmawy, N. A., Yessoufou, K., El-Settawy, A. A.
684 A., 2017. *In vitro* antibacterial, antifungal and antioxidant activities of *Eucalyptus* spp.
685 leaf extracts related to phenolic composition. *Natural Product Research* 31, 2927-
686 2930.
- 687 • Elgorashi, E. E., McGaw, L. J., 2019. African plants with *in vitro* anti-inflammatory
688 activities: A review. *South African Journal of Botany* 126, 142-169.
- 689 • Erdenetsogt, U., Nadmid, S., Paulus, C., Chanagsuren, G., Dolgor, E., Gotov, C.,
690 Dahse, H.M., Luzhetskyy, A., Dagvadorj, E., 2020. Bioactive flavonoids from plant
691 extract of *Pyrethrum pulchrum* and its acute toxicity. *Natural Product Research*.
692 DOI: 10.1080/14786419.2020.1806271
- 693 • Filho, M. B., Caputo, D. C. S., Marin, P. J., Chang, S., 2014. The mechanism of
694 auriculotherapy: A case report based on the fractal structure of meridian system.
695 *African Journal of Traditional, Complementary and Alternative Medicines* 11, 30-37.
- 696 • Gao, J., Hu, J., Hu, D., Yang, X., 2019. A role of gallic acid in oxidative damage
697 diseases: A comprehensive review. *Natural Product Communications* 1-9.
- 698 • Gholam-Hoseinian, A., Moradi, N. M., Sharifi-Far, F., 2009. Screening the methanol
699 extracts of some Iranian plants for acetylcholinesterase inhibitory activity.
700 *Research in Pharmaceutical Sciences* 4, 105-112.
- 701 • González-Burgos, E., Liaudanskas, M., Viskelis, J., Zvikas, V., Janulis, V., Gomez-
702 Serranillos, M. P., 2018. Antioxidant activity, neuroprotective properties and bioactive
703 constituents' analysis of varying polarity extracts from *Eucalyptus globulus* leaves.
704 *Journal of Food and Drug Analysis* 26, 1293-1302.
- 705 • Hartung, N. M., Fischer, J., Ostermann, A. I., Willenberg, I., Rund, K. M., Schebb, N.
706 H., Garscha, U., 2019. Impact of food polyphenols on oxylipin biosynthesis in human

- 707 neutrophils. *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids*
708 1864, 1536-1544.
- 709 • Hung, W. J., Chen, Z. T., Lee, S. W., 2018. Antioxidant and lipoxygenase inhibitory
710 activity of the kino of *Eucalyptus citriodora*. *Indian Journal of Pharmaceutical*
711 *Sciences* 80, 955-959.
- 712 • Ivanov, M., Kannan, A., Stojkovic, D. S., Glamoclija, J., Calhelha, R. C., Ferreira, I.
713 C. F. R., Sanglard, D., Sokovic, M., 2021. Camphor and eucalyptol-anticandidal
714 spectrum, antivirulence effect, efflux pumps interference and cytotoxicity.
715 *International Journal of Molecular Sciences* 22, 1-14.
- 716 • Maiden, J. H., 1914. Critical revision of the genus *Eucalyptus*.
717 <http://adc.library.usyd.edu.au/view?docId=ozlit/xml-main-texts/p00109v2.xml>
- 718 • Mehta, S. K., Nayeem, N., 2014. Natural xanthine oxidase inhibitors for management
719 of gout: A Review. *Journal of Medical and Health Sciences* 3, 4-13.
- 720 • Mendes, M., Carvalho, A. P., Magallanes, J. M. C. S., Moreira, M., Guido, L., Gomes,
721 A. M., Delerue-Matos, C., 2016. Response surface evaluation of microwave-assisted
722 extraction conditions for *Lycium barbarum* bioactive compounds. *Innovative Food*
723 *Science and Emerging Technologies* 33, 319-326.
- 724 • Mimica-Dukić, N., Jovin, E., Grbović, S., Knežević-Vukčević, J., Mitić-Ćulafić, D.,
725 Orčić, D., 2012. *Eucalyptus gunii* Hook. f. New source of valuable bioactive
726 compounds. *Planta Medica* 78, PI206.
- 727 • Mnif, S., Aifa, S., 2015. Cumin (*Cuminum cyminum* L.) from traditional uses to
728 potential biomedical applications. *Chemistry and Biodiversity* 12, 733-742.
- 729 • Nile, S. H., Keum, Y. S., 2018. Chemical composition, antioxidant, anti-inflammatory
730 and antitumor activities of *Eucalyptus globulus* Labill. *Indian Journal of Experimental*
731 *Biology* 56, 734-742.
- 732 • Ossipov, V., Koivuniemi, A., Mizina, P., Salminen, J. P., 2020. UPLC-PDA-Q
733 extractive orbitrap-MS profiling of the lipophilic compounds product isolated from
734 *Eucalyptus viminalis* plants. *Heliyon* 6, e05768.
- 735 • Puig, C. G., Reigosa, M. J., Valentão, P., Andrade, P. B., Pedrol, N., 2018.
736 Unravelling the bioherbicide potential of *Eucalyptus globulus* Labill: Biochemistry
737 and effects of its aqueous extract. *PLOS One* 13, 1-16.

- 738 • Rahmani, R., Jouaidi, M., Bouajila, J., Debouba, M., 2020. African mustard (*Brassica*
739 *tournefortii*) as source of nutrients and nutraceuticals properties. Journal of Food
740 Science 85, 1856-1871.
- 741 • Sabo, A.V., Knezevic, P., 2019. Antimicrobial activity of *Eucalyptus camaldulensis*
742 Dehn. plant extracts and essential oils: A review. Industrial Crops and Products 132,
743 413-429.
- 744 • Sharifi-Rad, J., Sureda, A., Tenore, G.C., Daglia, M., Sharifi-Rad, M., Valussi, M.,
745 Tundis, R., Sharifi-Rad, M., Loizzo, M. R., Ademiluyi, A. O., Sharifi-Rad, R.,
746 Ayatollahi, S. A., Iriti, M., 2017. Biological activities of essential oils: From plant
747 chemoecology to traditional healing systems. Molecules 22,70.
- 748 • Sharmeen, J. B., Mahomoodally, F. M., Zengin, G., Maggi, F., 2021. Essential oils as
749 natural sources of fragrance compounds for cosmetics and cosmeceuticals. Molecules
750 26, 666.
- 751 • Singh, A., Gupta, R., Pandey, R., 2017. Exogenous application of rutin and gallic acid
752 regulate antioxidants and alleviate reactive oxygen generation in *Oryza sativa* L.
753 Physiology and Molecular Biology of Plants 23, 301-309.
- 754 • Sung, Y. Y., Yuk, H. J., Kim, D. S., 2021. Saengmaeksan, a traditional herbal
755 formulation consisting of *Panax ginseng*, ameliorates hyperuricemia by inhibiting
756 xanthine oxidase activity and enhancing urate excretion in rats. Journal
757 of Ginseng Research 565-574.
- 758 • Tavsan, Z., Kayali, H. A., 2019. Flavonoids showed anticancer effects on the ovarian
759 cancer cells: Involvement of reactive oxygen species, apoptosis, cell cycle and
760 invasion. Biomedicine and Pharmacotherapy 116, 109004.
- 761 • Vek, V., Poljanšek, I., Humar, M., Willför, S., Oven, P., 2020. *In vitro* inhibition of
762 extractives from knot wood of Scots pine (*Pinus sylvestris*) and black pine (*Pinus*
763 *nigra*) on growth of *Schizophyllum commune*, *Trametes versicolor*, *Gloeophyllum*
764 *trabeum* and *Fibroporia vaillantii*. Wood Science and Technology 54, 1645-1662.
- 765 • Voirin, B., 1983. UV spectral differentiation of 5-hydroxy- and 5-hydroxy-3-
766 methoxyflavones with mono-(4'), di-(3',4') or tri-(3',4',5')-substituted B rings.
767 Phytochemistry 22, 2107-2145.
- 768 • Von Mueller, F., 1859. Monograph of the Eucalypti of tropical Australia. Journal of
769 the Proceedings of the Linnean Society. Botany 3, 84.

- 770 • Vuong, Q. V., Hiruna, S., Chuena, T. L. K., Goldsmitha, C. D., Munro, B., Bowyera,
771 M. C., Chalmers, A. C., Sakoff, J. A., Phillips, P. A., Scarlett, C. J., 2015.
772 Physicochemical, antioxidant and anticancer activity of a *Eucalyptus robusta* (Sm.)
773 leaf aqueous extract. *Industrial Crops and Products* 64, 167-174.
- 774 • Wahdan, O. A., Aly-Badr, S. E. R., Abdelfattah, M. S., 2019. Phytochemical analysis,
775 antibacterial and anticancer activities of the *Physalis peruviana* calyces growing in
776 Egypt. *Food and Nutrition Journal* 4, 197-203.
- 777 • Yang, X., Chen, J., Huang, W., Zhang, Y., Yan, X., Zhou, Z., Wang, Y., 2020.
778 Synthesis of icariin in tobacco leaf by over expression of a glucosyl transferase gene
779 from *Epimedium sagittatum*. *Industrial Crops and Products* 156, 112841.
780