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1 **Investigation of antiparasitic activity of ten European tree bark extracts on *Toxoplasma***
2 ***gondii* and bioguided identification of triterpenes in *Alnus glutinosa* barks**

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18

19 Running Head: Anti-*toxoplasma* activity of ten European tree barks extracts

20

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22

23 **Abstract:**

24 Toxoplasmosis is a worldwide parasitosis that affects one-third of the population. People at risk,
25 such as immunocompromised patients (AIDS, chemotherapy treatment) or fetuses (maternal-fetal
26 transmission) can develop severe forms of the disease. The antiparasitic activity of extracts of

27 different polarities (*n*-heptane, MeOH, MeOH/H₂O) of ten tree species endemics to temperate
28 regions was investigated against *Toxoplasma gondii* infection *in vitro*. Our results showed that
29 the *n*-heptane extract of the black alder (*Alnus glutinosa*) exhibited a significant antiparasitic
30 activity without any cytotoxicity at the tested concentrations, with an IC₅₀ of up to 25.08 µg/mL
31 and a selectivity index higher than 3.99. The chemical profiling of this extract revealed
32 triterpenes as major constituents. The ability of commercially available triterpene (betulin,
33 betulinic acid, and betulone) to inhibit the growth of *T. gondii* was evaluated and showed growth
34 inhibition rates of 44%, 49%, and 99% at 10 µM, respectively.

35

36 **Keywords:** *Toxoplasma gondii*, *Alnus glutinosa*, Betulaceae, triterpene, antiparasitic,
37 apicomplexa, bark

38

39 **Introduction:**

40 Toxoplasmosis is a worldwide disease caused by the protozoan parasite *Toxoplasma gondii*
41 which belongs to the Apicomplexa phylum.

42 Toxoplasmosis is one of the most common parasitic infections, generally benign in
43 immunocompetent individuals. However severe effects can be observed in the case of mother-to-
44 child transmission of the parasite during pregnancy of primo-infected women (congenital
45 toxoplasmosis)(1) or in the case of reactivation of a previous infection triggering cerebral or
46 global toxoplasmosis in immunocompromised patients(2).

47 The therapeutic armamentarium against *Toxoplasma gondii* is relatively poor, old, and presents
48 side effects, especially the pyrimethamine and sulfadiazine combination. Moreover, parasites can
49 develop a resistance against drugs, as demonstrated *in vitro* for *T. gondii* (3–5). Some *T. gondii*
50 strains can also naturally present different susceptibility toward drugs (6, 7). Due to the lack of

51 specificity (available drugs are limited and none of them are specific of toxoplasmosis) and
52 limited efficacy of the current treatments, new active compounds are needed to treat
53 toxoplasmosis.

54 The use of plants for therapeutic purposes has always existed in human and animal behaviors.
55 Newman and Cragg reported that between 1981 and 2019, 20 new antiparasitic drugs were
56 discovered of which two are natural products, seven are compounds derived from natural
57 products and three are synthetic drugs with a natural pharmacophore (8). Traditional medicine is
58 also still very present in some countries, especially to treat parasitic diseases such as
59 trypanosomiases (9) or malaria (10). Many treatments currently used against diverse pathologies
60 have a natural origin, such as artemisinin from *Artemisia annua* (11) and quinine from *Cinchona*
61 *officinalis* (12) against malaria, or paclitaxel from *Taxus baccata* against cancer (13). For twenty
62 years, one can note a significant increase in the number of publications relating to the search for
63 new compounds to fight against *T. gondii*. In this field of research, works on new anti-
64 *toxoplasma* molecules derived from terrestrial plants, marine organisms, microorganisms, and
65 even animals have intensified since 10 years, some of them being based on ethnopharmacological
66 considerations(14, 15). To our knowledge, no natural product currently exists that was patented
67 as an anti-*toxoplasma* active substance.

68
69 In the present study, ten tree species with high occurrence in European forests were investigated
70 for their anti-*T. gondii* activities: *Fagus sylvatica* L. (common beech, Fagaceae), *Quercus robur*
71 L. (common oak, Fagaceae), *Alnus glutinosa* (L.) Gaertn. (black alder, Betulaceae), *Prunus*
72 *avium* (L.) L. (wild cherry, Rosaceae), *Acer pseudoplatanus* L., (sycamore maple, Sapindaceae),
73 *Fraxinus excelsior* L. (common ash, Oleaceae), *Populus tremula* L. (common aspen, Salicaceae),

74 *Populus x canescens* Aiton (robusta poplar, Salicaceae), *Larix decidua* Mill. (European larch,
75 Pinaceae), and *Picea abies* (L.) H.Karst (Norway spruce, Pinaceae).

76 Since barks constitute an interface between the tree heart and its environment, metabolites
77 extracted from barks can lead to the discovery of protective agents against diverse pathogens or
78 aggressions(16). Therefore, several extracts were produced from the barks of the ten European
79 tree species and tested *in vitro* to evaluate their activity against *T. gondii* growth inhibition and
80 cytotoxicity on non-infected Vero cells.

81 Then a ¹³C NMR-based (Nuclear Magnetic Resonance) dereplication workflow combined with a
82 bioactivity-guided fractionation process was applied on the most active extract to identify the
83 compounds being involved in the biological effect against *T. gondii*. In addition, the screening of
84 four commercially available identified compounds was performed at 1 and 10 μM.

85

86 **Material & methods:**

87 **Plant materials.** France signed the Nagoya Protocol in 2011 and is a party since 2014. We
88 received authorization from the French “Office National des Forêts” (ONF) to collect samples
89 and use bark extracts at the University of Reims Champagne-Ardenne. Ten tree species
90 abundantly represented in temperate forests were selected: *Fagus sylvatica* L., *Quercus robur* L.,
91 *Alnus glutinosa* (L.) Gaertn., *Prunus avium* (L.) L., *Acer pseudoplatanus* L., *Fraxinus excelsior*
92 L, *Populus tremula* L, *Populus x canescens* Aiton, *Larix decidua* Mill., and *Picea abies* (L.)
93 H.Karst. All bark samples were collected in the Champagne-Ardenne territory, northeast of
94 France, in 2014. Approximately two kilograms were manually harvested from trunks two months
95 after cutting over professional forestry activities. Samples were dried for three days at 30°C
96 before being crushed by a hammer mill (VEM Motors GmbH, Germany) to a thin powder.
97 Botanical identification was made according to the phenotypic characteristics of the trees, such as

98 the shape, arrangement, and contours of leaves. Voucher specimens were deposited in the
99 Herbarium of the Botanical laboratory at the faculty of Pharmacy of Reims (University of Reims
100 Champagne-Ardenne, Reims, France) : *F. sylvatica* (JH-2014-1), *Q. robur* (JH-2014-2), *A.*
101 *glutinosa* (JH-2014-3), *P. avium* (JH-2014-4), *A. pseudoplatanus* (JH-2014-5), *F. excelsior* (JH-
102 2014-6), *P. x canescens* (JH-2014-7), *L. decidua* (JH-2014-8), *P. abies* (JH-2014-9), and *P.*
103 *tremula* (JH-2014-10).

104

105 **Chemicals.** Acetonitrile (CH₃CN), methanol (MeOH), methyl-*tert*-butyl ether (MtBE) and *n*-
106 heptane were purchased from Carlo Erba Reactifs SDS (Val de Reuil, France). Deuterated
107 chloroform (chloroform-d), deuterated methanol (methanol-d₄), lupenone (CAS: 1617-70-5),
108 betulin (CAS: 473-98-3), and betulinic acid (CAS: 472-15-1) were purchased from Sigma-
109 Aldrich (Saint-Quentin-Fallavier, France). Betulone (CAS: 7020-34-0) was purchased from
110 Clickbetulin (Riga, Latvia). Deionized water was used to prepare aqueous solutions.

111

112 **Preparation of bark extracts.** Three consecutive solid-liquid extractions were performed on
113 powdered bark with solvents of increasing polarity to cover a large chemical space. The first
114 extraction was performed on 100 g of bark powder with 1.5 L of *n*-heptane for 18 hours at room
115 temperature under magnetic stirring. After filtration under vacuum on a sintered-glass filter of 0.4
116 μm, the solvent was evaporated under vacuum to give a dry *n*-heptane extract containing the less
117 polar compounds (like fatty acids, sterols, triterpenes...). The bark residue exhausted by *n*-
118 heptane was further dried and submitted to another solid-liquid extraction in 1.5 L of methanol
119 for 18 hours at room temperature under magnetic stirring. Methanol was evaporated under
120 vacuum, resulting in a dry MeOH extract. The bark residue exhausted by methanol was finally
121 submitted to a third extraction process in 1.5 L of methanol/water 1/1 (v/v) under the same

122 conditions. In total, thirty extracts (three extracts for ten trees) were obtained and were weighed
123 after evaporation.

124 **Centrifugal Partition Chromatography (CPC).** The *n*-heptane extract of *A. glutinosa* bark was
125 fractionated by CPC on a column of 303.5 ml capacity (FCPE300®, Rousselet-Robatel-
126 Kromaton, Annonay, France) containing seven partition disks engraved with 231 twin partition
127 cells. A two-phase solvent system composed of *n*-heptane / ethyl acetate / methanol / water
128 (9/1/9/1, v/v/v/v) was prepared in a separatory funnel. The lower phase was used as the stationary
129 phase and pumped into the column (50 ml/min and 27 g) in the ascending mode. The rotation
130 speed was then set at 158 g. The sample (1 g) was solubilized in 15 ml of a mixture of lower
131 phase / upper phase in the proportions (80/20, v/v) and loaded into the CPC column through a
132 3725 Rheodyne injector valve equipped with a 15 mL sample loop. The UP was used as the
133 mobile phase and pumped at a flow rate of 20 mL/min for 70 min. Then, the most polar
134 compounds retained inside the column were recovered by extrusion of the stationary phase for 10
135 min. Fractions of 20 mL were collected over the whole experiment and combined according to
136 their Thin Layer Chromatography profile similarities (data not shown), resulting in a final series
137 of twenty fractions.

138
139 **Chemical profiling of the CPC fractions.** Aliquots (up to \approx 15 mg when possible) of CPC
140 fractions (n=20) obtained from the *n*-heptane extract of *A. glutinosa* bark were dissolved in
141 600 μ L of CDCl₃ and analyzed by Nuclear Magnetic Resonance (¹H, ¹³C, HSQC, HMBC, and
142 COSY) at 298 K on a Bruker Avance AVIII-600 spectrometer (Karlsruhe, Germany) equipped
143 with a TXI cryoprobe. ¹³C NMR spectra were acquired at 150.91 MHz using a standard zgpg
144 pulse sequence with an acquisition time of 0.9 s, a relaxation delay of 3 s, and a total of 1024
145 scans. After spectra processing using the TOPSPIN 3.5 software (Bruker), the absolute intensities

146 of all ^{13}C NMR signals detected in all spectra were collected by automatic peak picking. Then the
147 ^{13}C NMR spectral width (from 0 to 240 ppm) was divided into chemical shift buckets of 0.2 ppm
148 and the absolute intensity of the NMR peaks detected in all spectra was associated to the
149 corresponding bucket. This step was performed using a locally developed computer script written
150 in Python, resulting in a table with 20 columns corresponding to the CPC fractions, and 295 rows
151 corresponding to the NMR spectral buckets for which at least one ^{13}C NMR peak was detected in
152 at least one spectrum. Hierarchical Clustering Analysis (HCA) was performed on the rows for
153 data visualization of signals corresponding to major compounds contained in the *n*-heptane
154 extract of *Alnus glutinosa* bark. The higher the intensity of ^{13}C NMR peaks, the brighter the
155 yellow color in the map. The proximity between samples was measured with the Euclidian
156 distance and data agglomeration was performed with Ward's method. The resulting clusters of
157 ^{13}C NMR chemical shifts were visualized as dendrograms on a heat map (Figure 2). The ^{13}C
158 NMR chemical shifts regrouped with the HCA were submitted to a local database containing the
159 structures and predicted NMR chemical shifts (ACD/NMR Workbook Suite 2012 software,
160 ACD/Labs, Ontario, Canada) of around 3000 natural metabolites (March 2020), to identify the
161 corresponding chemical structures. This dereplication procedure is described in a previous
162 publication (17). A tolerated ^{13}C NMR chemical shift difference between the predicted database
163 spectrum and the real spectrum was established at 2 ppm. Finally, each proposition given by the
164 database was confirmed by interpretation of 1D and 2D NMR data (^1H NMR, HSQC, HMBC,
165 COSY).

166

167 ***T. gondii* strain.** RH (genotype I) strain of *T. gondii* was provided by the French Biological
168 *Toxoplasma* Resource Centre (BRC *Toxoplasma*, France).

169

170 **Solubilization of extracts and fractions.** All solid-liquid extracts and CPC fractions were
171 solubilized in DMSO. The final DMSO concentration was of 1/4000 (DMSO/medium culture,
172 v/v). It has also been demonstrated that DMSO was a good lipophilic vehicle, commonly used for
173 *in vitro* and *in vivo* experiments (18).

174

175 **Parasite growth.** *T. gondii* RH strain tachyzoites were cultured on Vero cell monolayers (ATCC,
176 CCL-81) at 37°C, 5% CO₂ in a humidified incubator. Both cells and parasites were grown in
177 complete medium Iscove's Modified Dulbecco's Medium/Glutamax (IMDM) (Invitrogen,
178 France) supplemented with 2% (v/v) fetal calf serum (Biowest, France) and antibiotics (100
179 IU/mL penicillin and 0.1 mg/mL streptomycin) (GIBCO). Host cells were infected at a 1:2
180 parasite to cell ratio.

181

182 **Screening of bark extracts on *T. gondii*.** *n*-heptane, methanol, and methanol/water 1:1 (v/v)
183 bark extracts were tested on *T. gondii* (Figure 1). Vero cells were seeded in a 96-wells plate, each
184 well containing 200 µL of cell suspension with 20,000 cells. Plates were then incubated for four
185 hours at 37°C and 5% CO₂. Tachyzoites grown on Vero cells were counted using a Kova Slide
186 counting chamber as described above. Each well was inoculated with 50 µL of parasite
187 suspension containing 10,000 *T. gondii* tachyzoites (in IMDM supplemented with 2% fetal calf
188 serum). Four wells were not inoculated and served as reference using Vero cells. These wells
189 only contained host cells, and 50 µL of IMDM supplemented with 2% fetal calf serum were
190 added. Four supplementary wells were inoculated with *T. gondii* as parasitic growth control in
191 untreated condition and were also containing 50 µL of IMDM supplemented with 2% fetal calf
192 serum. Four last wells were inoculated in the same conditions as described above with
193 pyrimethamine at 1 µM as control. Plates were incubated for three hours at 37°C and 5% CO₂.

194 Then 25 μL of bark extract solubilized in DMSO were added to each well and tested at 100
195 $\mu\text{g}/\text{mL}$. Finally, plates were incubated 72 hours at 37°C and 5% CO_2 before being fixed with cold
196 methanol.

197

198 **Cytotoxicity evaluation of CPC fractions of *n*-heptane extract of *Alnus glutinosa*.** The *in*
199 *vitro* cytotoxicity of 20 CPC fractions was evaluated on Vero cell cultures using the UptiBlue®
200 viable cell-counting assay (Interchim, France) (Figure 3). A suspension of IMDM®
201 supplemented with 2% (v/v) fetal calf serum containing 20 000 cells was used for each
202 concentration. After 4 hours of incubation, substances to test were deposited in wells. After 72h
203 of incubation at 37°C and 5% CO_2 , wells were emptied and washed with cold Phosphate
204 Buffered Saline (Sigma-Aldrich, France). Then, 100 μL of IMDM® supplemented with 2% (v/v)
205 fetal calf serum and 10% (v/v) UptiBlue® were added in each well. Afterwards, plates were
206 incubated at 37°C for 3 hours. The protocol was the same as described in the “Screening of bark
207 extracts on *T. gondii*” section, except that no parasite was inoculated in wells.
208 Spectrophotometric readings (FLUOstar Omega microplate reader, BMG Labtech, France) were
209 made at 570 nm, corrected at 600 nm. Visual control was made as described above. A
210 cytotoxicity threshold was arbitrarily defined at cell viability of 80%.

211

212 **Screening of active CPC fractions of *A. glutinosa* on *T. gondii*.** The 15 non cytotoxic CPC
213 fractions from the *n*-heptane extract of black alder bark were tested at 25 $\mu\text{g}/\text{mL}$ to avoid any
214 cytotoxic activity on Vero cells (Figure 4). All measures were performed in triplicate, as
215 described in the half maximal inhibitory concentration.

216

217 **Half maximal inhibitory concentration (IC₅₀).** The *in vitro* IC₅₀ of the active CPC fractions
218 against *T. gondii* was assessed using 96-wells plates for each fraction inhibiting at least 50% of
219 parasite growth at 25 µg/mL within 72 hours. Briefly, 200 µL aliquots of cell suspension
220 containing 20,000 Vero cells were placed into each well and incubated at 37°C and 5% CO₂ for
221 four hours to adhere. Each well, except the eight negative-control wells, was filled with 50 µL of
222 a parasite suspension containing 10,000 *T. gondii* tachyzoites. The plates were incubated at 37°C
223 and 5% CO₂ for three hours. Each well was filled with 25 µL of each active fraction at eight
224 concentrations obtained by serial dilutions in the culture medium (from 25 to 0.20 µg/mL).
225 Finally, plates were incubated at 37°C and 5% CO₂ for 72 hours before being fixed with cold
226 methanol. Parasite growth was determined by an enzyme immunoassay (3) on the fixed infected
227 cultures with an anti-*T. gondii* SAG-1-HRP conjugated monoclonal antibody (Argene Biosoft,
228 France) and a secondary antibody coupled with horseradish peroxidase. All plates were revealed
229 using O-Phenylenediamine Dihydrochloride (Sigma-Aldrich, France) end point by addition of
230 hydrochlorid acid. Spectrophotometric readings (FLUOstar Omega microplate reader, BMG
231 Labtech, France) were made at 450 nm, corrected at 630 nm. For visual control, the last well of
232 each condition was stained with kit RAL 555 (RAL Diagnostics, France) and examined
233 microscopically (AxioVert 200 M, Zeiss, France) at magnification 20x (data not shown).

234

235 **Chemosensitivity at 1 and 10 µM.** Pure commercially compounds were assessed in three wells.
236 In a 96 wells plate, 200 µL of IMDM® supplemented with 5% (v/v) fetal calf serum containing
237 20 000 Vero cells were set. After incubation for four hours at 37°C and 5% CO₂, 15 000 *T. gondii*
238 tachyzoites were added in a volume of 50 µL in each well, except three wells for reference. After
239 3 hours of incubation, the wells were emptied and 100 µL of drugs at a concentration of 1 µM or
240 10 µM in IMDM® supplemented with 5% (v/v) fetal calf serum were added. A final incubation

241 time of 72 hours was accomplished before the determination of the parasite growth as described
242 above (section “Half maximal inhibitory concentration (IC₅₀).”).

243

244 **Selectivity indexes.** A selectivity index (SI) was calculated for each sample, as the ratio between
245 cytotoxic and antiparasitic activities:

$$SI_{parasite} = \frac{CC_{50\ Vero}}{IC_{50\ parasite}}$$

246 The antiparasitic effect was considered selective when SI > 4.

247

248 **Statistical analysis.** Statistical analyses were performed using the R software (version
249 4.1.1) implemented in the RStudio IDE (version 1.4.1717). The global comparison of data
250 associated to screening and cytotoxicity of bark extracts was performed using a Kruskal-Wallis
251 rank sum test.

252 Before pairwise comparisons, data normality was assessed by a Shapiro test. Further,
253 variance homogeneity between samples were checked using a Fisher test. Pairwise comparisons
254 of extracts or products were run out by a classical Student t-test when both normality and
255 variance homogeneity null hypothesis were verified or a Welsh two samples t-test when only the
256 null hypothesis about data normality was verified. A Wilcoxon rank sum exact test was used for
257 all other cases.

258 Significance levels for p-values are: lower than 0.05 (*), lower than 0.01 (**), lower than 0.001
259 (***).

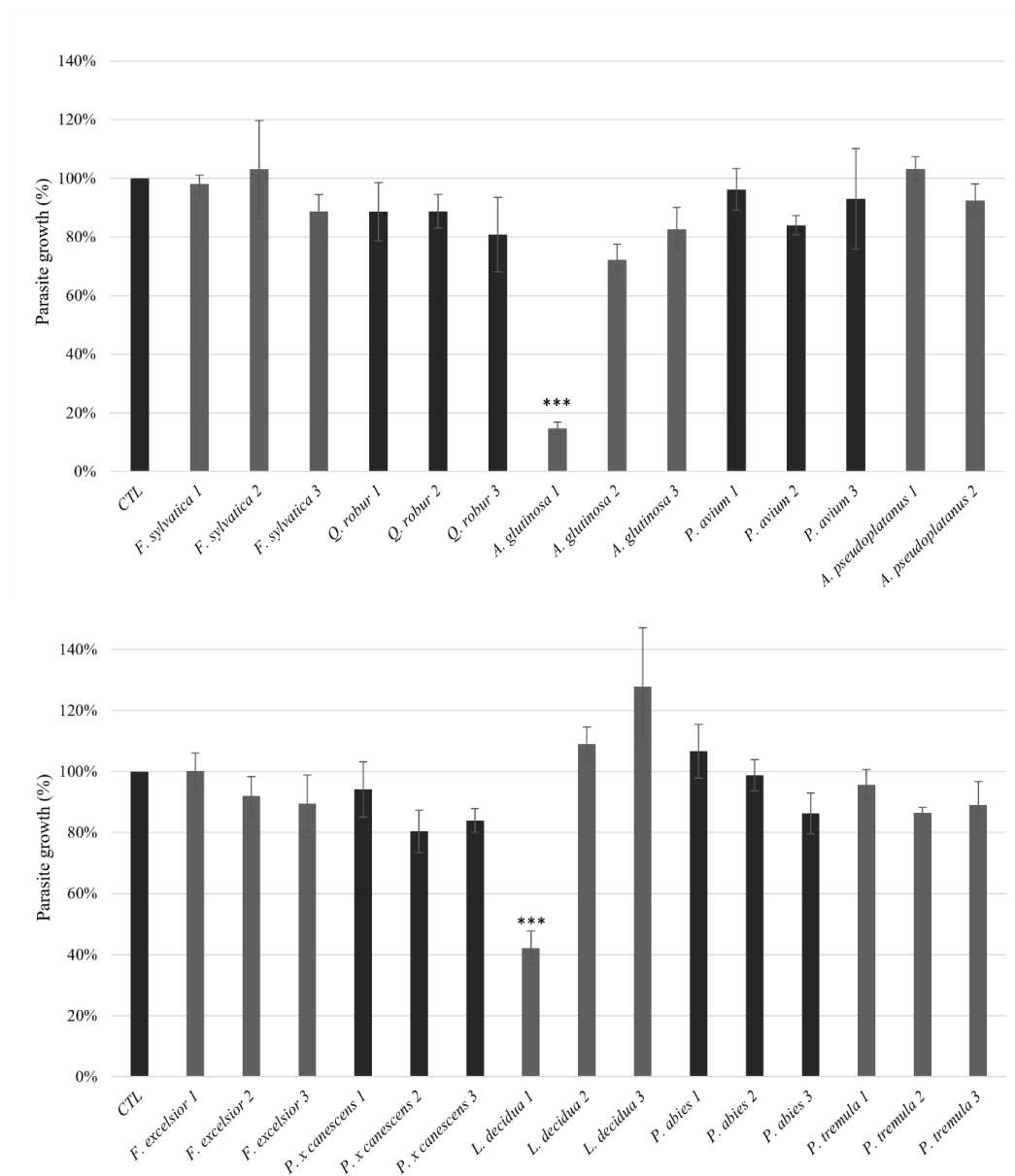
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261 **Results:**

262 **Solid-liquid extraction of European tree barks.** The ten different barks were macerated at
263 room temperature successively with *n*-heptane, MeOH, and MeOH-water 1:1 (v/v) (19) yielding
264 30 solid-liquid crude extracts. Yields are presented in supplementary table 1.

265

266 **Biological screening of the barks extracts on *T. gondii*.** *n*-heptane, MeOH and MeOH/water
267 1:1 (v/v) bark extracts were all tested on *T. gondii* (Figure 1) at 100 µg/mL, except the *A.*
268 *pseudoplatanus* methanol/water 1:1 (v/v) extract that was not soluble in DMSO.
269 Figure 1 shows that the *n*-heptane extract of *A. glutinosa* and *L. decidua* showed the highest
270 activity against *T. gondii* with 85 % and 58 % growth inhibition, respectively. These observations
271 were confirmed microscopically. On the contrary, MeOH and MeOH/H₂O extracts showed no
272 significant activity against *T. gondii* tachyzoites. The *n*-heptane extract of *A. glutinosa* which
273 presented the most promising activity was further fractionated by CPC and chemically profiled
274 based on the ¹³C NMR data (17) to identify the compounds responsible for the antiparasitic
275 activity.
276



277

278 **Figure 1.** *In vitro* screening of bark extracts on *T. gondii* tachyzoites (RH strain) growth after 72

279 hours of incubation. Each graph shows the tachyzoites growth compared to positive control and

280 determined by enzyme immunoassay with infected monolayers (y axis) versus bark extract (x

281 axis). Bark extracts were tested at 100 µg/mL. Number 1, 2 and 3 respectively stand for *n*-
282 heptane, MeOH and MeOH/H₂O extracts.

283

284 **Fractionation of the *n*-heptane extract of *A. glutinosa* by CPC.** The *n*-heptane extract of *A.*
285 *glutinosa* was fractionated by CPC, yielding 19 chemically simplified fractions, 16 corresponding
286 to the elution step and the last four being obtained after a stationary phase extrusion step (20).

287 The chemical profiling of the CPC fractions was achieved by a dereplication process based on
288 unsupervised clustering of recorded ¹³C NMR data (21, 22). The resulting heat map after
289 hierarchical clustering analysis on the ¹³C chemical shifts (*i.e* on the lines of the table) highlights
290 nine clusters (Figure 2). The chemical shifts corresponding to each cluster were one by one

291 submitted to an in-house database. This database contains more than 3000 compounds to date and
292 associates natural product structures to the predicted ¹H and ¹³C NMR chemical shifts calculated
293 by the ACD/Labs predictor. A literature survey was carried out on the plant *A. glutinosa*,

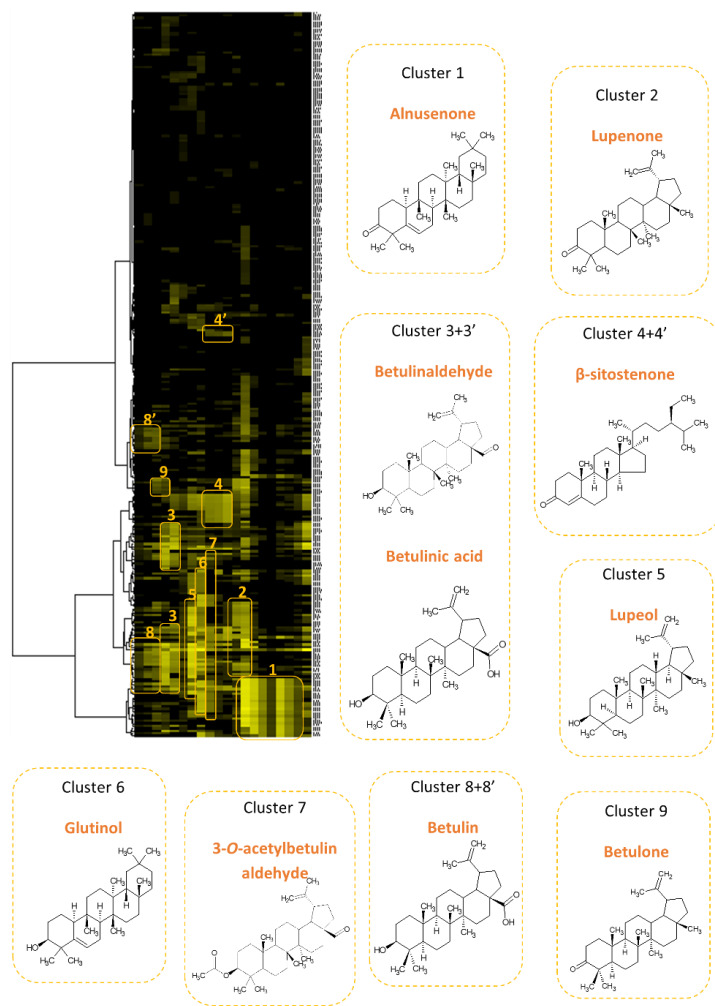
294 resulting in 68 metabolites stored in the database. As a result, ten triterpenes - major constituents
295 of the *n*-heptane bark extract - were identified and confirmed by interpretation of 1D and 2D
296 NMR data. Fractions 1-5 corresponded to mixtures of betulin (cluster 8 and 8'), betulone (cluster
297 9), betulinaldehyde and betulinic acid (cluster 3 and 3') at different ratios (Figure 2). The main
298 compounds in fraction 7 and fraction 8 are lupeol (cluster 5) and glutinol (cluster 6), respectively.

299 Fraction 9 mainly contains β-sitostenone (cluster 4 and 4') and 3-*O*-acetylbetulin-aldehyde
300 (cluster 7), whereas β-sitostenone is the major compound in fractions 10-11. Fraction 12 is

301 mainly composed of lupenone (cluster 2), the latter being in a mixture with alnusenone (cluster 1)
302 in fraction 13. Finally, alnusenone is the major compound in fractions 14-19. The composition of

303 fraction 20 corresponded almost to the composition of the initial extract, a phenomenon
304 frequently encountered and probably due to a dead volume in the line of fluid discharged during

305 the extrusion step. Fraction 20 was excluded from this study. Fraction compositions are
306 summarized in Table I.



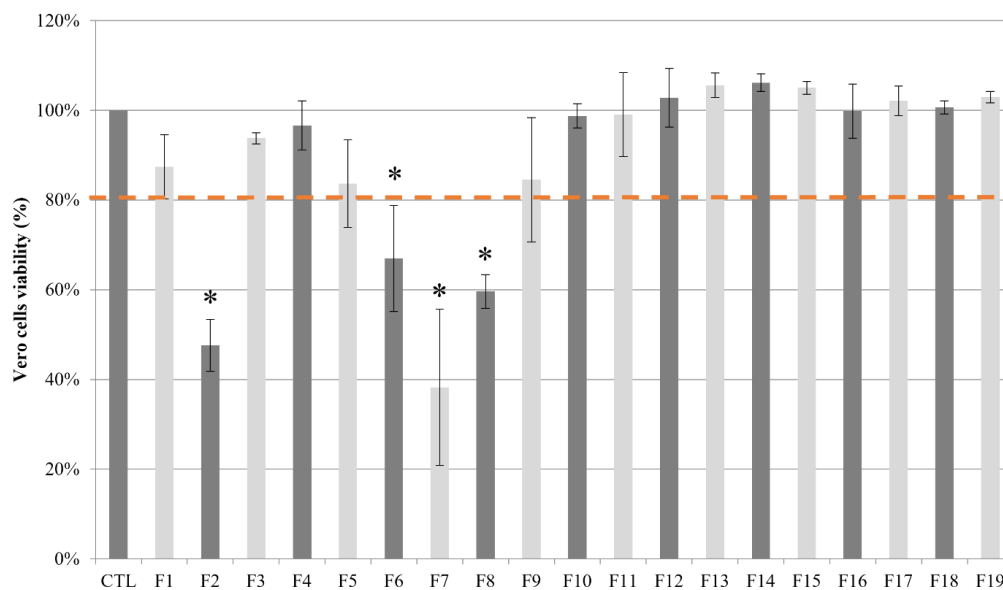
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308 **Figure 2.** Heatmap of peak intensities of ^{13}C NMR (rows) and fractions (columns) from *Alnus*
309 *glutinosa* n-heptane extract. This representation allows the visualization of the carbon skeleton of
310 the major compounds. The 9 clusters represent the 10 major compounds identified by the
311 CAMEL dereplication procedure. The molecules were designed with ChemDraw 18.0.

312

313

314 **Cytotoxicity assay.** Each fraction was screened on Vero cells at 25 $\mu\text{g/mL}$ to determine its
315 cytotoxicity (Figure 3).



316

317 **Figure 3.** *In vitro* screening of the 19 fractions obtained from the *n*-heptane bark extract of *A.*
318 glutinosa on Vero cells after 72 hours of incubation. Each graph shows cell viability growth
319 compared to positive control and determined by using the UptiBlue® viable cell counting assay.
320 Fractions were tested at 25 $\mu\text{g/mL}$. The dotted line indicates a 20% reduction in cell viability.

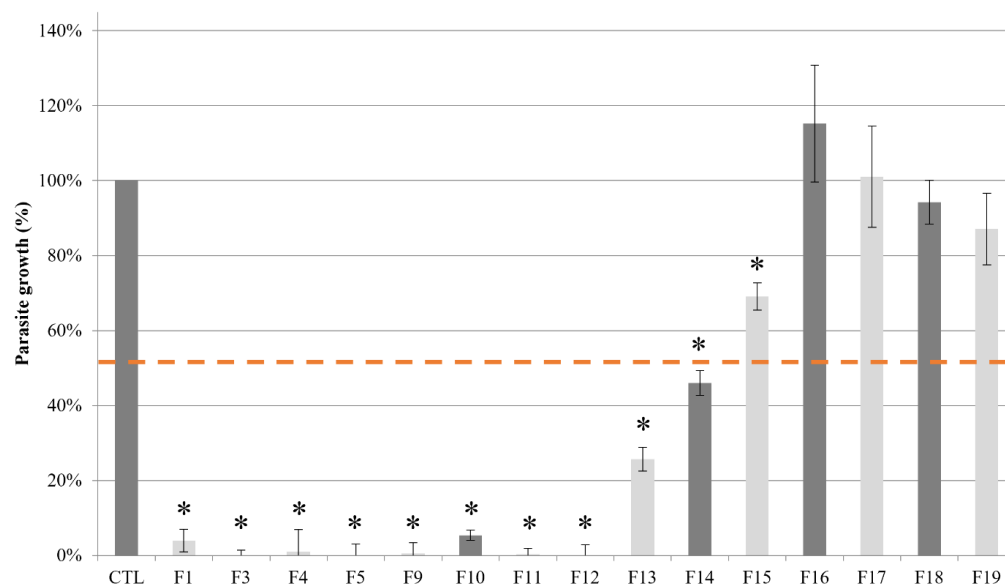
321

322 Four fractions showed a significant cytotoxic effect on Vero cells (cell viability under 80%): F2
323 and F6-F8. Therefore, they were excluded from this study. The other fractions were tested against

324 *T. gondii*.

325

326 **Screening of the 15 noncytotoxic fractions on *T. gondii*.** The 15 fractions of *A. glutinosa* *n*-
 327 heptane extract that showed no cytotoxicity on Vero cells were screened on *T. gondii* (Figure 3).
 328 Among them, 10 fractions showed a significant anti-*T. gondii* effect, with more than 50% parasite
 329 growth inhibition: F1, F3, F4, F5, F9, F10, F11, F12, F13, F14 (Figure 4). Eight fractions were
 330 inhibiting the parasite growth under 10% (F1, F3, F4, F5, F9, F10, F11, and F12).



331

332 **Figure 4.** *In vitro* screening of the 15 fractions on *T. gondii* tachyzoites (RH strain) growth after
 333 72 hours of incubation. The tachyzoites growth was compared to a positive control and
 334 determined by enzymatic immunoassay with infected monolayers (y axis) versus bark extract (x
 335 axis). Fractions were tested at 25 $\mu\text{g}/\text{mL}$. Values are expressed as mean \pm SD ($n = 3$). The dotted
 336 lines indicate a 50% reduction in parasite growth.

337

338 **CC₅₀, IC₅₀ and selectivity indexes determination.** The active CPC fractions on *T. gondii* were
 339 tested to determine their respective CC₅₀, IC₅₀, and selectivity indexes (Table I). The results were
 340 confirmed microscopically. All fractions considered as active according to the previous screening
 341 were selective ($SI > 4$) against *T. gondii*. Fractions F3, F4, and F5 showed the highest selectivity

342 indexes. Based on ^{13}C NMR dereplication analysis, these fractions contained four lupane type
343 triterpenes: betulin, betulinic acid, betulone, and betulinaldehyde.

344

345

346

347 **Table I.** Activity of the selected fractions (> 50% of parasite growth inhibition at 25 $\mu\text{g}/\text{mL}$)
348 fractions obtained from the *n*-heptane bark extract of *A. glutinosa* on Vero cells (CC_{50}), *T. gondii*
349 (IC_{50}), and their respective selectivity indexes (SI). Values are expressed as mean \pm SD.

350 Underlined values are theoretical based on the trend line since CC_{50} was not reached. The main
351 compounds contained in the *n*-heptane bark extract of *A. glutinosa* after its fractionation by CPC
352 were annotated based on the ^{13}C NMR dereplication workflow.

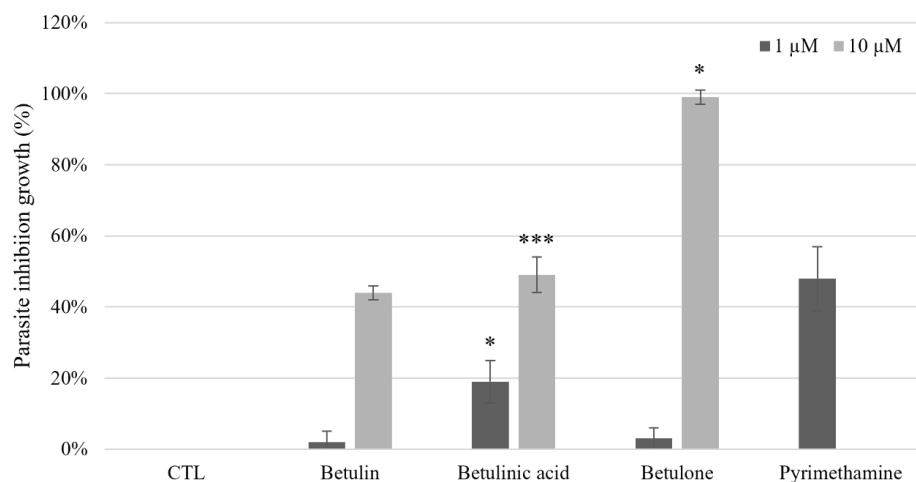
<i>A. glutinosa</i> active fractions (from <i>n</i> -heptane extract)	Main compounds	CC_{50} Vero cells ($\mu\text{g}/\text{mL}$)	IC_{50} <i>T. gondii</i> ($\mu\text{g}/\text{mL}$)	SI <i>T.</i> <i>gondii</i>
F1	betulin, betulinaldehyde, betulone and betulinic acid	62.03	7.24 ± 1.45	8.57
F3		58.23	3.31 ± 0.12	17.62
F4		42.93	2.64 ± 0.61	16.26
F5		44.76	3.85 ± 0.36	11.64
F9	β -sitostenone and 3- <i>O</i> -acetylbetulinaldehyde	54.41	6.25 ± 0.83	8.71
F10	β -sitostenone	102.01	7.11 ± 1.06	14.35
F11		148.48	6.21 ± 1.77	23.93
F12	lupenone	58.47	2.95 ± 1.42	19.85
F13	lupenone and alnusenone	> 100	13.51 ± 4.34	<u>> 7.40</u>
F14	alnusenone	> 100	21.50 ± 7.08	<u>> 4.65</u>
Extract	-	> 100	25.08 ± 4.63	> 3.99

353

354

355

356 **Chemosensitivity of *T. gondii* to lupane type triterpenes at 1, and 10 μ M.** The ability of
357 commercially available compounds (betulin, betulinic acid, betulone, and lupenone) to inhibit the
358 growth of *T. gondii* at a concentration of 1 and 10 μ M was evaluated. Results, shown in **Error!**
359 **Reference source not found.**, highlighted the anti-toxoplasmic potential of betulone. Results at 1
360 μ M did not show significant inhibition of parasite growth while chemosensitivity at 10 μ M
361 induced an inhibition growth of 44%, 49%, and 99% for betulin, betulinic acid, and betulone,
362 respectively. Betulone appears to be the most active triterpene. All CC_{50} associated to these
363 compounds were above 80 μ M (data not shown). Selectivity indexes were higher than for betulin,
364 betulinic acid, betulone and lupenone, respectively, at 10 μ M.



365

366 **Figure 5.** Chemosensitivity of *T.gondii* to commercially available lupane type triterpenes at 1 and
367 10 μ M identified in *Alnus glutinosa* n-heptane extract with positive control (pyrimethamine at 1
368 μ M) and negative control (no inhibition).

369

370 **Discussion:**

371 Barks are one of the most abundant biomasses and their valorization is almost totally neglected.
372 Currently, this low-value by-product is mainly burnt for combined heat and energy production.
373 Nevertheless, potential applications could be found in many high added-value fields, including
374 pharmaceutical and cosmetic sectors. This study aimed to investigate the potential antiparasitic
375 activity of several extracts from ten tree barks against *T. gondii*. Three solid-liquid extracts of
376 increasing polarity (*n*-heptane, MeOH, and MeOH/H₂O 1:1) were obtained (30 extracts) and all
377 of them were submitted to a preliminary screening against *T. gondii*. The latter showed that the *n*-
378 heptane extract of *A. glutinosa* was particularly active against *T. gondii*. This extract was then
379 fractionated by CPC by using the quaternary biphasic solvent system *n*-heptane / ethyl acetate /
380 methanol / water (9/1/9/1, v/v/v/v) and chemically profiled using a ¹³C NMR-based dereplication
381 workflow. Finally, the anti-toxoplasmic potential of the generated CPC fractions was evaluated
382 through *in vitro* bioassays to correlate their biological activity to specialized metabolite families
383 present in these extracts.

384 The fractions mainly containing oleanane or lupan triterpenes such as glutinol, betulin, and lupeol
385 (Fractions F2, F6-F8) have proven to be cytotoxic at 25 µg/mL (< 80% cell viability) and thus,
386 were excluded from the rest of the study. Recently, a cytotoxic effect on SK-MEL cancer cell line
387 was reported for glutinol (IC₅₀ of 45.3 µM) whereas the latter was mentioned as non-cytotoxic on
388 Vero cells(23). Cytotoxicity on Vero cells of betulin was previously reported with a CC₅₀ of 160
389 µM(24). Finally, lupeol has only been reported as a very weak cytotoxic compound on Vero
390 cells(25). To the best of our knowledge, no information exists concerning glutinol cytotoxicity on
391 Vero cells. Minor compounds present in F6-F8 could also be responsible for the observed
392 cytotoxicity, this phenomenon being today well described in the field of natural product
393 chemistry and called residual complexity(26).

394 A bioassay on tachyzoites was then performed on *T. gondii* to determine the activity of the other
395 nontoxic fractions. Fractions F1, F3-F5, and F9-F14 were active against *T. gondii*. Both their
396 CC_{50} and IC_{50} were determined and then their selectivity indexes were calculated. All tested
397 fractions were significantly selective against the parasite. The most promising activities were
398 obtained for fractions F3-F5 and F12, with IC_{50} between 2.64 $\mu\text{g/mL}$ and 3.85 $\mu\text{g/mL}$,
399 respectively. All triterpenes found in these fractions were pentacyclic lupane type (betulin,
400 betulinaldehyde, betulone, betulinic acid, and lupenone). Screening of commercially available
401 compounds (betulin, betulinic acid and betulone) at 1 μM and 10 μM was thus carried out.
402 Lupenone, insoluble in our bioassay conditions, was not tested. As highlighted by Figure 5, the
403 anti-toxoplasmosis activities observed with fractions F3-F5 and F7 were confirmed on pure
404 compounds with an inhibition activity of *T. gondii* growth between 44 % and 99 % at 10 μM . The
405 most active compound was betulone. Little information is reported in the literature concerning
406 the evaluation of triterpenes specifically as anti-toxoplasmosis agents. Nevertheless, a review
407 reports the effect of pentacyclic triterpenes against tropical parasitic disease (27). One can
408 mention for instance the action against *T. gondii* of maslinic acid, another pentacyclic triterpene
409 with a carboxylic function on its C-17 position such as betulinic acid (28). The authors mention
410 an interesting dual effect on *T. gondii* combining both the mobility inhibition of the parasite as well
411 as entrance inhibition into Vero cells. The same pentacyclic triterpene was also mentioned for its
412 parasitostatic activity toward some parasites belonging to the Apicomplexa phylum including *T.*
413 *gondii* (29). More recently, anti-*Toxoplasma* triterpenoids (29-norlupan-3,20-dione, oleanic acid
414 acetate, and ursolic acid acetate) were isolated from *Quercus crispula* (Blume) outer bark (30).
415 These three compounds possess also either a carboxylic substituent on C-17 or a ketone or
416 hydroxyl on C-3, as we observed for the two most active compounds in our study: betulone and

417 betulinic acid for which a parasite growth inhibition at 10 μM of 99 % and 49 % were found,
418 respectively. Endo *et al.* (30) also mentioned that betulin exhibited an interesting activity on
419 *Plasmodium* with IC_{50} of 18.3 μM , but cytotoxicity against human foreskin fibroblast led to a
420 very low selectivity ($\text{SI}=0.3$). More publications report the antimalarial activity of triterpenes,
421 *Plasmodium sp.* parasites belonging as *T. gondii* to the Apicomplexa phylum. For instance, *in*
422 *vitro* and *in vivo* inhibition of *Plasmodium falciparum* growth was reported for betulinic acid – an
423 intensively studied triterpene - and betulinic acid derivatives (31). By broadening the spectrum of
424 the tested parasites beyond the Apicomplexa phylum, one can mention for example the lupenone,
425 a pentacyclic triterpene with a ketone on C-3, that can induce a synergistic effect against
426 *Trypanosoma cruzi* when coupled with caryophyllene oxide (32).

427 Considering the screening results on pure compounds and the IC_{50} measured on the most
428 promising CPC fractions, the most interesting triterpenes possess a lupane skeleton. Oxidizing
429 the free hydroxyl group at C-3 increases the anti-*toxoplasma* activity as clearly seen by the *T.*
430 *gondii* inhibition growth level of betulin (or betulinaldehyde) and betulone. Moreover, the
431 presence of a C-17 highly oxidized substituent such as a carboxylic acid group seems to
432 counterbalance this phenomenon as highlighted by the high inhibition growth level of betulinic
433 acid. These observations are in good agreement with the structure-activity relationship proposed
434 by Endo *et al.*(30).

435 Finally, β -sitostenone (tetracyclic skeleton) with a ketone function in C-3, the main compound of
436 F10-F11, showed an anti-*toxoplasma* activity of about two times lower than that observed for
437 lupane triterpene containing fractions, but with lower cytotoxicity suggesting a different mode of
438 action.

439 In conclusion, several potent anti-*Toxoplasma* triterpenes were identified in the *n*-heptane extract
440 of *Alnus glutinosa*. Structure-activity relationships suggest that the lupane skeleton with a ketone
441 function on C-3 or a carboxylic substituent on C-17 provides structural elements important for
442 potent anti-*Toxoplasma* activity and selectivity. Similar results obtained in *Mycobacterium*
443 *tuberculosis* (a model that could be comparable to *T. gondii* since they share common
444 characteristics(33)) support this structure-activity relationship hypothesis(34). Betulone exhibits
445 the most interesting activity and should be a very promising candidate for the development of
446 new anti-*Toxoplasma* drugs with high efficacy and selectivity. Further synthesis of analogues or
447 derivatives as well as *in silico* and *in vitro* investigation of their mode of action will open the
448 door for the novel application of pentacyclic triterpenoids obtained from natural resources like
449 temperate northern hemisphere tree barks.

450

451 Conflict of interest:

452 The authors declare no conflict of interest associated with this publication.

453

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460

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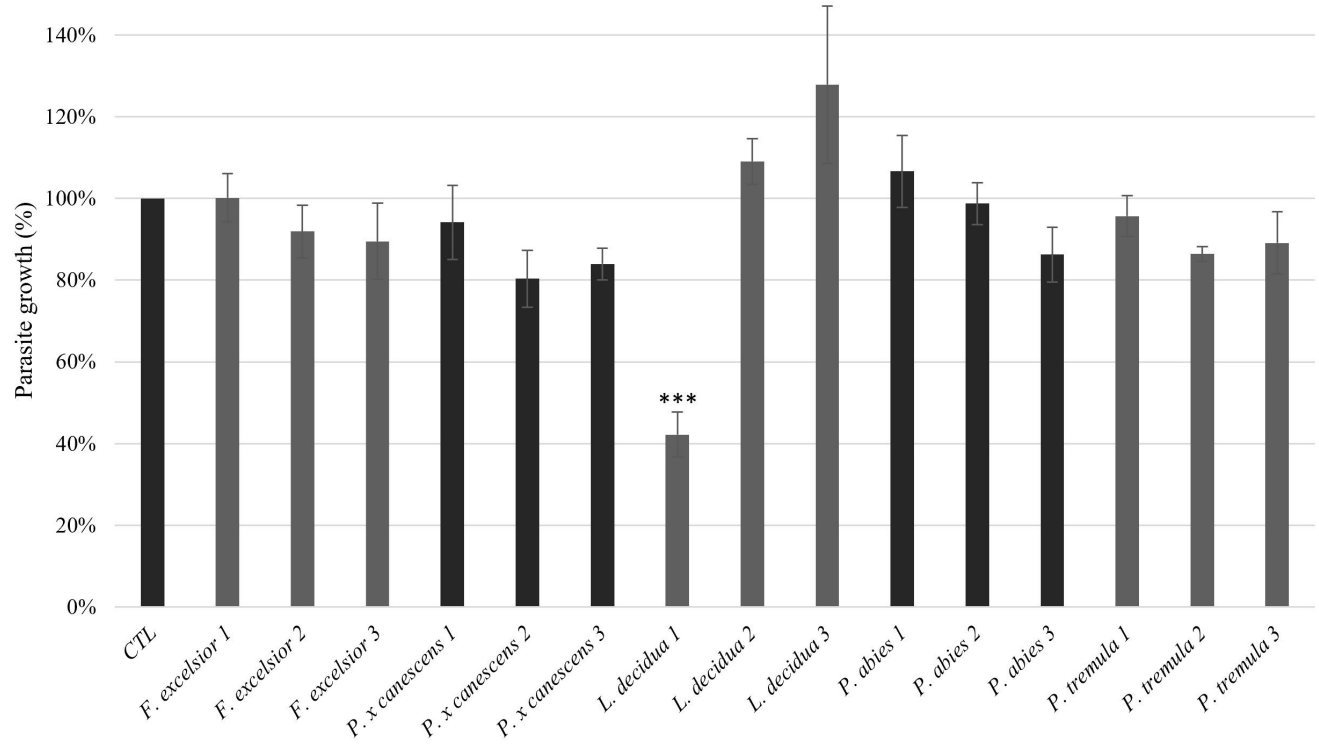
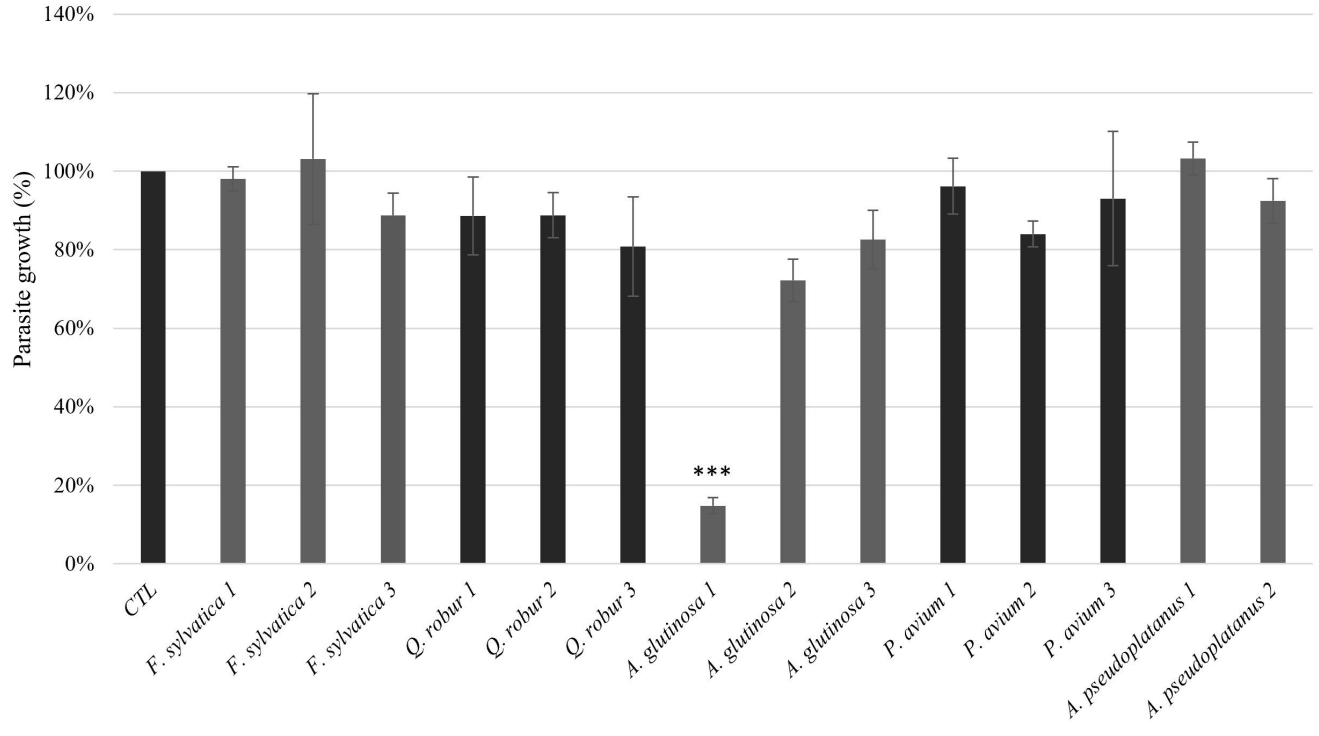
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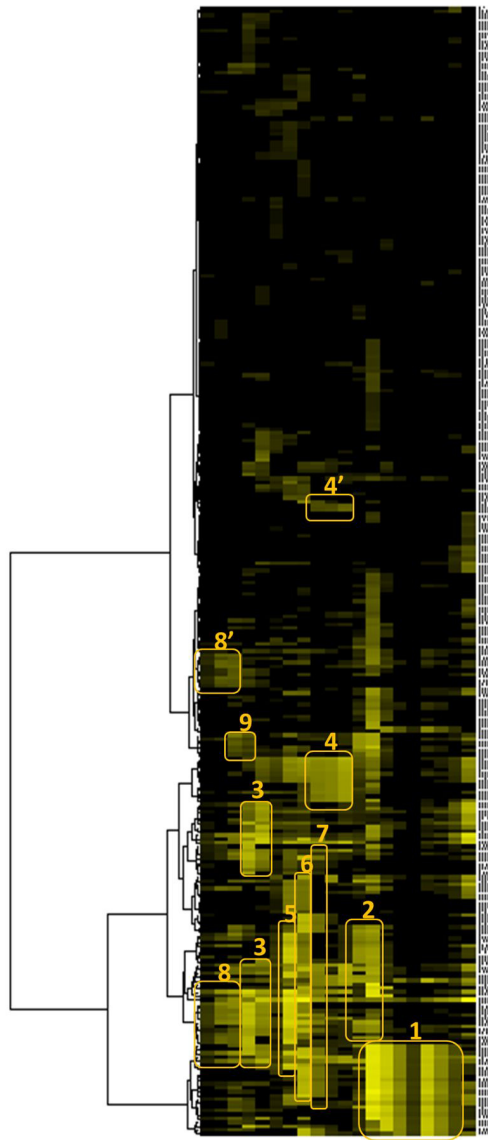
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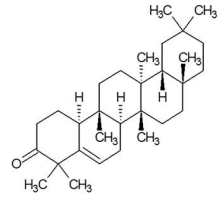
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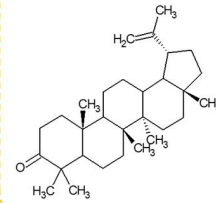




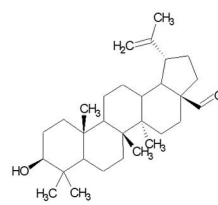
Cluster 1

Alusenone

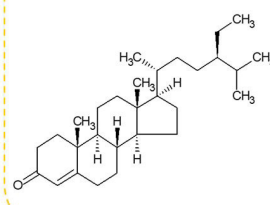
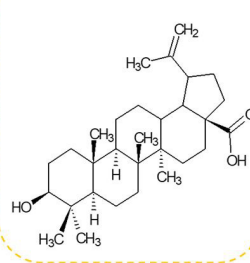
Cluster 2

Lupenone

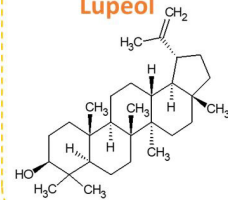
Cluster 3+3'

Betulinaldehyde

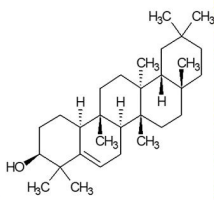
Cluster 4+4'

 β -sitostenone**Betulinic acid**

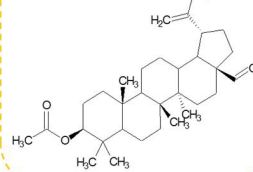
Cluster 5

Lupeol

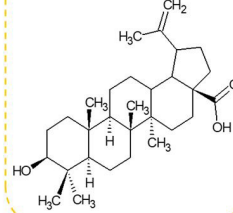
Cluster 6

Glutinol

Cluster 7

3-O-acetylbetulin aldehyde

Cluster 8+8'

Betulin

Cluster 9

Betulone