Chemical analysis of callus extracts from toxic and non-toxic varieties of *Jatropha curcas* L.

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Abstract

Jatropha curcas L. belongs to Euphorbiaceae family, it synthesizes flavonoid and diterpene compounds that have showed antioxidant, anti-inflammatory, anticancer, antiviral, antimicrobial, antifungal and insecticide activity. Seeds of this plant accumulate phorbol esters, which are tigliane type diterpenes, reported as toxic and, depending on its concentration, toxic and non-toxic varieties has been identified. The aim of this work was to characterize the chemical profile of the extracts from seeds, leaves and callus of both varieties (toxic and non-toxic) of *Jatropha curcas*, to verify the presence of important compounds in dedifferentiated cells and consider the possibility of using these cultures for the massive production of metabolites. Callus induction was obtained using NAA (1.5 mg.L^{-1}) and BAP (1.5 mg.L^{-1}) after 21 d for both varieties. Thin layer chromatography analysis showed differences in compounds accumulation in callus from non-toxic variety throughout the time of culture, diterpenes showed an increase along the time, in contrast with flavonoids which decreased. Based on the results obtained through microQTOF-QII spectrometer it is suggested a higher accumulation of phorbol esters, derived from 12-deoxy-16-hydroxy-phorbol (*m/z* 365 [M+H]$^+$), in callus of 38 d than those of 14 d culture, from both varieties. Unlike flavonoids accumulation, the MS chromatograms analysis allowed to suggest lower accumulation of flavonoids as the culture time progresses, in callus from both varieties. The presence of 6 glycosylated flavonoids is also suggested in leaf and callus extracts derived from both varieties (toxic and non-toxic), including: apigenin 6-*C*-α-L-arabinopyranosyl-8-*C*-*β*-D-xylopyranoside (*m/z* 535 [M+H]$^+$), apigenin 4'-*O*-rhamnoside (*m/z* 417 [M+H]$^+$), vitexin (*m/z* 433 [M+H]$^+$), vitexin 4'-*O*-glucoside-2"-*O*-rhamnoside (*m/z* 741 [M+H]$^+$), vicenin-2 (*m/z* 595 [M+H]$^+$), and vicenin-2,6"-*O*-glucoside (*m/z* 757 [M+H]$^+$).

Introduction

38

39 *Jatropha curcas* L. (Euphorbiaceae) is a multipurpose plant native to Mesoamerica, it is important
40 because of its usefulness as raw material in biofuels production (Salvador-Figueroa *et al*., 2015),
41 as well as, in veterinary and human traditional medicine (Zhang *et al*., 2017). Several compounds
42 with different biological activities have been isolated from different species of *Jatropha* (Ferreira-
43 Rodriguez *et al*., 2016; Katagi *et al*., 2016). The identification of biologically active compounds
44 extracted from different organs of this plant has been reported (Prasad, Izam & Khan, 2012;
45 Sharma, Dhamija & Parashar, 2012). Isolated compounds or whole plant extracts have been
46 studied because of their potential pharmacological activity (Cocan *et al*., 2018). Biological effect
47 of *J. curcas* includes antibacterial (Rampadarath *et al*., 2016), cytotoxic (Katagi *et al*., 2016), anti-
48 inflammatory (Salim *et al*., 2018), and antifungal (Abdelgader, Suleiman & Ali, 2019; Srinivasan,
49 Palanisamy & Mulpuri, 2019). Most research on *J. curcas* have been done with toxic varieties;
50 toxicity is referred to phorbol esters content in seeds.

51 In Mexico, Brazil and India, it have been identified non-toxic varieties of this species with very
52 low or non-detectable levels of phorbol esters (PEs) in seeds (Laviola *et al*., 2010; Martínez-
53 Herrera, Chel-Guerrero & Martínez-Ayala, 2004; Kumar, Anand & Reddy, 2011). PEs are known
54 as *Jatropha* factors because each one of them has the same nucleus diterpene moiety, namely, 12-
55 deoxy-16-hydroxy-phorbol (DHP) which is coupled to unstables intramolecular diterpenes (named
56 C_1–C_6 factors) (Hirota *et al*., 2017).

57 Plants are the most successful source of chemical compounds, which potential mode of action
58 makes them an alternative phytomedicinal drug, since several natural products have shown
59 benefits against human diseases (Aye *et al*., 2019). Several compounds are tissue-specific
60 accumulated, and are usually structurally complex (Armaly *et al*., 2015). Therefore it is necessary
61 the use of chemical analysis techniques to isolate and identify the extracted plant metabolites
62 (Hernandez & Sarlah, 2019). There are a few cases where the use of plant cell culture of *Jatropha*
63 *curcas* has allowed the production of bioactive compounds (Alvero-Bascos & Ungson, 2012;
64 Mahalakshmi, Eganathan & Parida, 2013; Nassar, El-Ahmay & Al-Azizi, 2013; Zaragoza-
65 Martínez *et al*., 2016), the study of the culture at different stages of toxic and non-toxic varieties,
66 generate the opportunity to design biotechnological models for production of bioactive compounds
67 *i.e.* terpenoids, alkaloids, flavonoids (Abdelgavir & Van Staden, 2013; Sabandar *et al*., 2013)
68 providing opportunities for new drugs discovery.

69 Secondary metabolites are generally in complex matrices at very low concentrations in plant
70 organs, and lower in dedifferentiated cells. These compounds have a wide range of polarities,
71 therefore it is necessary the use of solvents with different polarity to obtain the extracts (Chemat
72 *et al*., 2019). The aim of this work was to characterize the chemical profile of the extracts from
73 callus of both varieties (toxic and non-toxic) of *Jatropha curcas*, through the cell culture, to verify
74 the presence of important compounds in dedifferentiated cells and consider the possibility of using
75 these cultures for the massive production of bioactive compounds.

Materials & Methods

Plant material

Seeds and young leaves of *Jatropha curcas* were collected. Non-toxic variety samples from Centro de Desarrollo de Productos Bióticos-IPN, Yautepec, Morelos, México (18º53'09"N, 99º03'38"W). The toxic variety samples were collected from Campo Experimental Zacatepec, Instituto Nacional de Investigaciones Agrícolas y Pecuarias (INIFAP), Zacatepec, Morelos, México (18º39'23"N, 99º11'28"W).

To induce cell dedifferentiation, two different explants were surface-sterilized according to Vanegas *et al*., 2002. Leaf blade of approximately 0.25 cm^2 and petiole of approximately 3 mm in length were cultured in MS medium (Murashige & Skoog, 1962) supplemented with sucrose (30 g.L^{-1}), phytagel (3 g.L^{-1}) (Sigma-Aldrich®). Since there are no reports of the induction of dedifferentiated cells in the varieties analyzed in this study, the combinations of three concentrations (0.0, 1.5 and 3.0 mg.L^{-1}) of naphthaleneacetic acid (NAA) and 6-benzyl-aminopurine (BAP) were evaluated according to Verma (2013), pH was adjusted to 5.7, media were sterilized at 121 °C for 15 min. Ten explants per Petri dish with 3 repetitions per treatment were incubated at 25 ± 2 °C, photoperiod of 16 h light/8 h darkness for 35 d (Kumar *et al*., 2015). Explants dedifferentiation was recorded every seven days using a stereoscopic microscope (Nikon, model SMZ 1500, Japan). In order to observe differences in accumulation of compounds during callus development, completely dedifferentiated cells were cultured under the above described conditions for 38 d, samples were taken on days 0, 2 and every 4 d thereafter.

Fresh washed leaves were indoors dried at 25 ± 2 °C during 3 weeks. Seeds without tegument and callus, were oven dried at 50 °C for 48 h, dried samples were ground with a mortar and sieved through a mesh size 53 μm.

Ultrasound assisted extraction (UAE)

UAE was performed with an ultrasound bath Branson (2510R-MTH, CT, USA) with automatic control of time and temperature and ultrasound frequency of 40 kHz. 500 mg of grounded biomass dry weight (dw) were placed into a 50 mL borosilicate glass conical Erlenmeyer flask, then 20 mL of ethanol 80% (v/v) were added, and sonicated at 40 ± 5 °C during 30 min (Bazaldúa *et al*., 2019; Pandey *et al*., 2018; Dumitraşcu *et al*., 2019). During sonication flasks were suspended in the water without contact with the bottom of the ultrasonic bath, subsequently they were vortexed. Supernatant was filtered, concentrated to dryness at 25 ± 2 °C, and solubilized in 500 μL of HPLC grade MeOH (Sigma-Aldrich®) for chromatographic analysis (Saeed *et al*., 2006; Liu *et al*, 2013).

Phorbol esters (PEs) rich defatted extract

500 mg of dried sample were packed in a filter paper cartridge and defatted in a Soxhlet equipment with petroleum ether (60-80 °C) (Sigma-Aldrich®) for 4 h. Petroleum ether (Fermont®) extract was concentrated using rotary evaporator at 40 °C, 90 rpm, and 900 mbar. The methyl esters in the

112   resulting oil, were extracted with MeOH, later filtered and concentrated to dryness at 25 ± 2 °C,
113   then solubilized in 500 μL of HPLC grade MeOH for chromatographic analysis (Demissie & Lele,
114   2010).

115   **Thin layer chromatography**

116   Extracts were applied on normal phase silica plates (Merck Millipore, 60 F_{254}, Germany).
117   Chloroform-methanol (94:6 and 75:25) were used as mobile phase, reference standards were
118   phorbol-12-myristate 13-acetate (PMA, Sigma-Aldrich®), quercetin, and vitexin (Sigma-
119   Aldrich®), both plates were revealed with anisaldehyde (Kathiravan & Raman, 2010).

120   To analyze extracts obtained by sonication-ethanol 80% and Soxhlet-methanol a mobile phase
121   consisting of chloroform-methanol (97:3) was used. The reference standard was PMA, and the
122   plates were cerium sulfate-revealed, then observed at 366 nm, and white light. Retention factor
123   (Rf) and color from the spots were compared with chromatographic terpenes profiles described by
124   Reich & Schibli (2007).

125   **MicrOTOF Q-II analysis**

126   Electrospray ionization analysis (ESI) was performed using a micrOTOF-Q II mass spectrometer
127   (Bruker Daltonics, Bremen, Germany) according to León-López *et al*. (2015). Samples were
128   solubilized in 500 μL of HPLC grade MeOH and filtered with a syringe filter (nylon membrane,
129   0.45 μm, Agilent Technologies, Santa Clara, CA, USA). The molecular ions related to the extracts
130   were analyzed in positive ion mode (ESI^+). 20 μL of sample were directly injected into the
131   evaporation chamber, capillary potential was -4.5 kV, gas temperature of 200 °C, drying gas flow
132   of 4 L min^{-1} and nebulizer gas pressure of 0.4 Bar. Detection was performed at 50-3000 *m/z*. The
133   predictive structures of the MS/MS partitioning profile were established utilizing the Competitive
134   Fragmentation Modeling for Metabolite Identification (CFM-ID. Version 3.0, 2019) platform from
135   Wishart-lab (http://cfmid3.wishartlab.com), which is referred to in the PubChem-NCBI site.
136   Relative abundance was calculated according to Scigelova *et al*. (2011).

137 # Results

138   **Establishment of callus culture**

139   Dedifferentiation cell was not observed in leaf blade explants. Petiole explants showed tissue
140   dedifferentiation since seventh day of culture and complete process was evident at the day 21 (Fig.
141   1). Friable and light green callus was obtained on MS media added with both combinations: NAA
142   (1.5 mg.L^{-1}), BAP (1.5 mg.L^{-1}), and NAA (3.0 mg.L^{-1}) and BAP (3.0 mg.L^{-1}).

143   **Thin layer chromatography (TLC) analysis**

144   TLC showed differences in compounds accumulation during time culture (2, 6, 10, 14, 18, 22, 26,
145   30, 34 and 38 d). Regard diterpenes, spots with Rf of 0.71 and 0.27 showed higher intensity along
146   this period (Fig. 2A), unlike flavonoids in which spots with Rf of 0.77 and 0.58, decreased

147   throughout the same culture period (Fig. 2B). These results suggest that the accumulation of
148   diterpenes and flavonoids was inversely related during callus development. To obtain diterpenes
149   the Soxhlet-methanol extraction was more efficient than sonication-ethanol 80%. TLC analysis of
150   extracts obtained by both methods evidenced differences in the size and intensity of spots in regard
151   to: extraction method, variety (toxic and non-toxic), and plant material (seeds, leaves and callus)
152   (Fig. S1).

153   **MicrOTOF Q-II and competitive fragmentation modeling for metabolite identification**
154   **platform (CFM-ID)**

155   **Phorbol esters (PEs) analysis**

156   Fragmentation profile analysis from seeds extract from both varieties showed several highs signals
157   one of them with m/z of 365 [M+H]$^+$ corresponding to 12-deoxy-16-hydroxy-phorbol (DHP),
158   which is the fundamental structural core of the PEs. The MS/MS analysis of this molecular ion
159   showed fragments with m/z of 295, 276, 234, 203, 185 and 127 [M+H]$^+$ which is similar to the
160   fragmentation profile of DHP presented in CFM-ID platform (Fig. 3), this suggests the
161   identification of that molecular structure in all of the extracts obtained from seeds and callus of
162   both toxic and non-toxic varieties. Based on signals intensities from 14 d and 38 d callus extracts
163   from both varieties, it is suggested that the accumulation of DHP is time-dependent. Since, the
164   corresponding signal was higher in callus of 38 d than in those of 14 d (Fig. 4). Furthermore, two
165   signals with m/z of 547 and 591 [M+H]$^+$ were observed, so it is proposed that they are related with
166   the fragmentation profile of the signal with m/z of 711 [M+H]$^+$ corresponding to any of the
167   *Jatropha* factors (C$_1$ or DHPB to C$_6$) which nucleus structure is DHP (Wink *et al*., 2000; Haas,
168   Sterk & Mittelbach, 2002) (Fig. S2). Table 1 shows the relative abundance of DHP molecular ion
169   (m/z 365 [M+H]$^+$) on 14 d and 38 d callus extracts from both varieties, evidencing the increment
170   of these compounds through the callus development.

171   **Flavonoids analysis**

172   On the other hand, the main group of compounds in *Jatropha* leaf extracts are flavonoids, among
173   them the apigenin, nevertheless, it is important to refer that the natural condition of flavonoids in
174   the plants is in glycosylated form. On this regard, another of the highest signals observed at the
175   chromatograms was the m/z of 381 [M+H]$^+$ ion, the MS-MS experiment of this signal and the
176   proposed structures obtained by CFM-ID platform allowed to relate that molecular ion (m/z 381
177   [M+H]$^+$) to the fragmentation profiles of apigenin 6-*C*-α-L-arabinopyranosyl-8-*C*-β-D-
178   xylopyranoside, and of apigenin 4'-*O*-rhamnoside (Fig. 5). The fragmentation signals and their
179   corresponding predictive structure were also related for vitexin (m/z 433 [M+H]$^+$), vitexin 4'-*O*-
180   glucoside-2 "-*O*-rhamnoside (m/z 741 [M+H]$^+$), vicenin-2 (m/z 595 [M+H]$^+$), and vicenin-2,6"-*O*-
181   glucoside m/z 757 [M+H]$^+$ (Fig. S3). Table 1 shows the relative abundance of six tentatively
182   identified compounds by relating their molecular ions on 14 d and 38 d callus extracts from both
183   varieties. Inversely to observed on DHP related signal (m/z 365 [M+H]$^+$), the intensity of the
184   molecular ion related with glycosylated apigenin (m/z 381 [M+H]$^+$) diminished (Fig. 6).

185 **Discussion**

186   The highest callus induction (95.5%) was observed in petiole explants on MS medium added with
187   NAA (3.0 mg.L^{-1}) and BAP (3.0 mg.L^{-1}), the second best result (87.7%) was obtained with NAA
188   (1.5 mg.L^{-1}) and BAP (1.5 mg.L^{-1}), in contrast to reported by Nassar *et al*. (2013), who observed
189   dedifferentiation with NAA and BAP at 0.5 mg.L^{-1} of each one plant growth regulator. Explants
190   dedifferentiation reported in this work was similar to reported by Kumar *et al*. (2015). The follow
191   up of the explants dedifferentiation process, every 7 d showed callus formation on explants starting
192   on the seventh day. Dedifferentiation began at the cutting sites as expected (Sujatha, Makkar &
193   Becker 2005; Nogueira *et al*., 2011; Ovando-Medina *et al*., 2016). The callus obtained was light
194   green and friable, similar to reported by Hernández *et al*. (2015). It has been reported that high
195   auxins concentrations could affect production and accumulation of secondary metabolites (Kim *et*
196   *al*., 2007), hence, according to our results, it is suggested the use of the lowest effective
197   concentration, 1.5 mg.L^{-1} for both growth regulators. Muñoz-Valverde *et al*. (2003) concluded that
198   BAP is determinant to induce callus formation in foliar explants of *J. curcas*. Likewise, Suárez &
199   Salgado (2008) reported that the presence of NAA induce callus formation in *Stevia rebaudiana*,
200   and this effect could be increased when adding BAP. On the other hand, Solange *et al*. (2002)
201   determined that the use of NAA and BAP in equal proportion induces callus formation from leaf
202   explants of *Tridax procumbens*. Coutiño-Cortés *et al*. (2013) reported the callus induction in *J.*
203   *curcas* leaf explants at 10 d of culture, and total explant-cell dedifferentiation at 20 d using 2, 4-
204   D, BAP and KIN, while in this work, petioles dedifferentiation started at 7 d and total explant-cell
205   dedifferentiation was achieved at 21 d. These results support that synergy between NAA and BAP
206   is essential to achieve a high dedifferentiation degree. Stable callus culture conditions for the two
207   varieties of *Jatropha curcas* were established.

208   The PEs are responsible for the toxicity in the plant (Devappa, Makkar & Becker, 2011; Sabandar
209   *et al*., 2013; Zhang *et al*., 2017). There are varieties of *Jatropha curcas* denominated as toxic and
210   non-toxic (Makkar *et al*., 1997). The non-toxic varieties have PEs concentration lower than 0.86
211   mg/g of seed on dry basis (He *et al*., 2011). Martínez-Herrera *et al*. (2006) detected high levels of
212   PEs in seed oil from the municipality of Coatzacoalcos, Veracruz, México, but did not detect PEs
213   in seeds from the municipality of Yautepec, Morelos, México. This corroborates the differences
214   between the seeds of the two varieties used in this study.

215   Regard, to TLC profile analysis, it has been reported that methanolic extraction from seed-oil
216   facilitates separation and availability of methyl ester type compounds, mainly phorbol esters (PEs)
217   (Demissie & Lele, 2010; Devappa, Bingham & Khanal, 2013). The detection by TLC of PEs in
218   seed methanolic extracts from toxic and non-toxic *J. curcas* varieties was reported (Devappa,
219   Makkar & Becker (2012), they reported higher spots intensity from toxic variety than from non-
220   toxic, when plates were observed at 366 nm UV light, this result is similar to that observed in this
221   work (Fig. S1). Makkar & Becker (2009) detected higher PEs accumulation in seeds than in leaves
222   extracts. Similar results were obtained in this work, even with different method of extraction.
223   Nevertheless, these results are different of that obtained by Martínez-Herrera, Chel-Guerrero &

224 Martínez-Ayala, 2004, because they reported 96% of PEs extraction through hydroalcoholic
225 extraction, quantified by HPLC; while, in this work the intensity of the spots was higher on
226 Soxhlet-methanol extracts than hydroalcoholic extraction (Fig. S1). Using TLC, differences
227 between dedifferentiated cell extracts of both varieties of *Jatropha curcas* were evidenced.

228 On the other hand, Hirota *et al*. (2017) reported the identification of DHP as the fundamental
229 structural core which is derived from 12-deoxy-16-hydroxy-phorbol-4'-[12',14'-butadienyl]-6'-
230 [16',18',20'-nonatrienyl]- bicycle [3.1.0] hexane-(13-*O*)-2'- [carboxylate]- (16-*O*)-3'- [8'-butenoic-
231 10']ate (DHPB or *Jatropha* factor C_1), identified as DHPB-Na adduct *m/z* 733 $[M+Na]^+$.
232 Furthermore, DHPB *m/z* 711 $[M+H]^+$ and DHP *m/z* of 365 $[M+H]^+$ were also reported in *J. curcas*
233 seeds (Wink *et al*., 2000). Even more so, Haas, Sterk & Mittelbach, (2002) reported the
234 identification of diterpenes named *Jatropha* factors C_2 to C_6 through ESI-MS *m/z* 711 $[M+H]^+$ and
235 of DHP (*m/z* of 365 $[M+H]^+$). Furthermore, Nishshanka *et al*. (2016) identified six phorbol esters
236 in *J. curcas* seeds by LC-MS, which have the same core (DHP), at the so named *Jatropha* factors
237 (C_1 to C_6).

238 Regard to PEs identification by ESI-MS analysis, Verardo *et al*. (2019) identified six phorbol
239 esters in *J. curcas* seeds with *m/z* of 711 $[M+H]^+$, which have the same fundamental structural core
240 (DHP) *m/z* of 365 $[M+H]^+$ which is coupled to diterpenes of 24 carbon structures named *Jatropha*
241 factors from C_1 (DHPB) to C_6. The relative intensity of the molecular ion *m/z* 365 $[M+H]^+$ was
242 higher in seeds extracts from toxic variety, than in seed extracts from non-toxic variety (Data not
243 shown). While in callus, the relative intensity is higher in toxic and non-toxic varieties callus of
244 38 d of culture (Fig. 4B, and 4D), than in toxic and non-toxic varieties callus of 14 d of culture
245 (Fig. 4A, and 4C). These results could suggest the presence of PEs coupled to DHP in the samples
246 analyzed and that their accumulation is differential in regard to the variety-derived cell culture and
247 throughout the time of culture. These results suggest that their accumulation of DHP is time
248 dependent. This ESI-MS analysis allowed to corroborate the results obtained by TLC (Fig. 2A).
249 Nevertheless, the relative intensity of the signals observed in extracts from callus were lower than
250 that obtained from seeds extracts as reported by Demissie & Lele (2010). By ESI-MS, differences
251 in the relative intensity of the signal corresponding to DHP were observed between the callus
252 extracts of both varieties, being higher in the toxic variety in addition to that in calluses at 38 d of
253 culture, it was higher than in the 14 d.

254 By other hand, phenolic compounds are ubiquitously produced by plants (Kumar & Goel, 2019),
255 the main role of phenols in plants is to protect them from biotic or abiotic stress (Pereira, 2016).
256 These properties include antimicrobial, insecticidal, antiparasitic, antiviral, anti-ulcerogeBynic,
257 cytotoxic, antioxidant, anti-hepatotoxic, anti-hypertensive and anti-inflammatory activities
258 (Oskoueian *et al*., 2011; Papalia, Barreca & Panuccio, 2017). Flavonoids are recognized as
259 polyphenols. Several of them have been identified in *Jatropha* genus, such as apigenin glycosides,
260 vitexin, and isovitexin which have been considered as chemiotaxonomic compounds from the
261 genus (Abdelgadir &Van Staden, 2013; Huang *et al*., 2014).

262   The tentative identification of glycosylates-flavonoids through microQTOF-QII has been already
263   reported (Pezzini *et al*., 2019). In this regard Xie *et al*. (2003) reported the apigenin 6-*C*-α-L-
264   arabinopyranosyl-8-*C*-*β*-D-xylopyranoside *m/z* 535 [M+H]⁺. Likewise, this result may be related
265   to that obtained by Abd-Alla *et al*. (2009) who identified apigenin and its aglycone as majoritarian
266   flavonoids in *J. curcas* leaves, as well as, that obtained by Reena, Nand & Sharma, (2008) who
267   reported to apigenin as major flavonoid in the same species. Those reports differ from that
268   published by Papalia, Barreca & Panuccio, (2017) who identified to vitexin and vicenin-2 as the
269   majoritarian flavonoids.

270   The results obtained by microQTOF-QII of the molecular ion *m/z* 381 [M+H]⁺ through the
271   MS/MS experiment, and the predictive structures obtained through the CFM-ID platform allowed
272   to suggest the relation of the structures from the molecular ion *m/z* 381 [M+H]⁺ with the
273   fragmentation profile from apigenin 6-*C*-α-L-arabinopyranosyl-8-*C*-*β*-D-xylopyranoside *m/z* 535
274   [M+H]⁺, which was identified through ESI-MS in *Viola yedoensis* (Xie *et al*., 2003) and apigenin
275   4'-*O*-rhamnoside *m/z* 417 [M+H]⁺, which was identified in *Olea europaea* (Pieroni *et al*., 1996).

276   Based on the molecular ion, MS-MS fragmentation profile and the predictive structures obtained
277   by CFM-ID platform, it is suggested the tentative identification of vitexin *m/z* of 433 [M+H]⁺,
278   vicenin-2 *m/z* of 595 [M+H]⁺, and vitexin 4'-*O*-glucoside-2 "-*O*-rhamnoside *m/z* of 741 [M+H]⁺ in
279   leaves and callus from both varieties. These results are similar to obtained by Huang *et al*. (2014)
280   who identified vitexin *m/z* of 433 [M+H]⁺ in *J. curcas* leaves. This flavonoid was also identified
281   by ESI-MS in *Parkinsonea aculeata m/z* of 431 [M-H]⁻ (Hassan, Abdelaziz & Al Yousef., 2019).
282   In this work it is also suggested the tentative identification of vicenin-2,6"-*O*-glucoside *m/z* 757
283   [M+H]⁺ which has not been reported to *Jatropha curcas*, but to *Stellaria holostea* (Bouillant *et al*.,
284   1984) (Fig. S2). By the MS-MS fragmentation profile, the identification of six glycosylated
285   flavonoids is suggested, it was observed that relative intensities signals related to flavonoid related
286   molecular ion *m/z* 381 [M+H]⁺ in callus of 14 d was higher than callus of 38 d, differences that
287   were not observed between calluses of the different varieties.

Conclusions

289   Stable dedifferentiated cells culture from petiole explants of *Jathopha curcas*, were stablished from toxic
290   and non-toxic varieties on MS medium added with NAA and BAP. Thin layer chromatography and mass
291   spectrometry, suggest an inverse relationship between phorbol esters and flavonoids accumulation in callus
292   throughout the time of culture. The tentative identification of diterpene type compounds such as 12-deoxy-
293   16-hydroxy-phorbol and *Jatroph*a factors by ESI-MS in seed and callus (14 and 38 d), as well as, the
294   presence of six flavonoids glycosides in leaf and callus, in extracts from both toxic and non-toxic varieties
295   of *J. curcas*, is suggested. Both of them, groups of compounds reported with bioactive activity with
296   pharmaceutic/agroindistrial potential.

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517

518   **Figures description**

519   **Figure 1. Cell dedifferentiation of petiole explants from both toxic and non-toxic varieties of**
520   ***Jatropha curcas.*** (A-D) Explants from non-toxic variety throughout dedifferentiation experiment
521   (0, 7, 14, and 21 d, respectively), (E-H) Explants from toxic variety throughout dedifferentiation
522   experiment (0, 7, 14, and 21 d, respectively). Both induced on MS culture medium added with
523   NAA (1.5 mg.L⁻¹) and BAP (1.5 mg.L⁻¹).

524   **Figure 2. Identification of both diterpenes-type (A), and flavonoids-type (B) compounds in**
525   **seeds, leaves, and callus of *Jatropha curcas*, through thin layer chromatography.** Lanes from
526   2 to 38 correspond to extracts of **c**allus of non-toxic variety throughout 38 d of culture, NTS= Non-
527   toxic variety-seeds, PMA= Phorbol-12-myristate-13-acetate (Sigma) reference standard (Rf 0.42),
528   IV= Isovitexin (Sigma) reference standard (Rf 0.42), and Q= Quercetin (Sigma) reference standard
529   (Rf 0.37). **A)** The spots intensity increased throughout to culture time (Rfs 0.71, and 0.27), mobile
530   phase chloroform-methanol (94:6). **B)** The spots intensity decreased throughout to culture time
531   (Rfs 0.77, and 0.58), mobile phase chloroform-methanol (75:25). Plates were revealed with
532   anisaldehyde.

533   **Figure 3. Spectrophotometrical analysis of phorbol esters in extracts of *Jatropha curcas***
534   **seeds.** MS/MS fragmentation profile of the molecular ion *m/z* 365 [M+H]⁺ related to 12-deoxy-
535   16-hydroxy-phorbol, which is the structural core from *Jatropha curcas*-phorbol esters (referred as
536   *Jatropha* factors). Predictive structures obtained through CFM-ID platform from each ionized
537   fragment.

538   **Figure 4. Mass spectra from callus extracts of *J. curcas* showing the relative intensity of the**
539   **molecular ion *m/z* 365 [M+H]⁺ related to the structural core of the *Jatropha*-phorbol esters.**
540   Callus extracts from toxic variety: (A) 14 d of culture; (B) 38 d of culture; non-toxic variety: (C)
541   14 d of culture, (D) 38 d of culture. The relative intensity from molecular ion *m/z* 365 [M+H]⁺
542   increased throughout culture time.

543   **Figure 5. Fragmentation profile (MS/MS) of the molecular ion *m/z* 381 [M+H]⁺, observed in**
544   **leaves extracts, and related to fragmentation of two glycosylated apigenin (apigenin**
545   **(apigenin 6-C-α-L-arabinopyranosyl-8-*C*-*β*-D-xylopyranoside *m/z* 535 [M+H]⁺, apigenin 4'-**
546   ***O*-rhamnoside *m/z* 417 [M+H]⁺).** Structures predicted to each molecular ion (381, 355, 335, and
547   219 *m/z*), obtained from CFM-ID platform.

548   **Figure 6. Mass spectra of callus extracts from both toxic and non-toxic varieties of *J. curcas***
549   **at 14 and 38 d culture, showing the relative intensity of the molecular ion *m/z* 381 [M+H]⁺**
550   **related to the fragmentation profile from two glycosylated apigenin.** A, and C) Extracts of *J.*
551   *curcas* callus from *J. curcas*-toxic variety (14 and 38 d, respectively). B, and D) Extracts of *J.*
552   *curcas* callus from non-toxic variety (14 and 38 d, respectively). The relative intensity from
553   molecular ion *m/z* 381 [M+H]⁺ diminished throughout culture time.

554   **Table 1.** Tentative compounds identified by ESI-MS in hydroalcoholic extracts from seeds, leaves,
555   and callus of 14 and 38 d of culture from both toxic and non-toxic *Jatropha curcas* L.varieties.

556

557   Supplementary material description

558   **Figure S1. Thin layer chromatogram of extracts obtained from seeds, leaves, and callus of**
559   **two *J. curcas*-varieties with two extraction methods for the identification of phorbol esters**
560   **(Rf's 0.81, 0.53, and 0.38).** The extracts obtained with ethanol 80% - sonication are referred
561   with numbers (1 – 8). The extracts obtained with Soxhlet – methanol are referred with letters (A
562   – H). PMA: Phorbol-12-myristate-13-acetate Rf 0.22 (Sigma, PE reference standard). Toxic
563   variety seed (1 and A), Non-toxic variety seed (2 and B), Toxic variety leaves (3 and C), Non-
564   toxic variety leaves (4 and D), Toxic variety-callus 14 d (5 and E), Toxic variety-callus 38 d (6
565   and F), Non-toxic variety-callus 14 d (7 and G), Non-toxic variety-callus 38 d (8 and H). Mobile
566   phase chloroform-methanol (97:3), cerium sulfate-revealed, observed at 366 nm UV light.

567   **Figure S2. Predicted structures related with the fragmentation profile from six flavonoids**
568   **identified through ESI-MS from calluses extracts of non-toxic *Jatropha curcas*.** It is included
569   the predictive structure corresponding to vicenin-2,6"-*O*-Glucoside m/z 757 [M+H]$^+$ which is not
570   reported to *Jatropha curcas*, but it is to *Stellaria holostea*.

Figure 1

Figure 1

Cell dedifferentiation of petiole explants from both toxic and non-toxic varieties of *Jatropha curcas*.

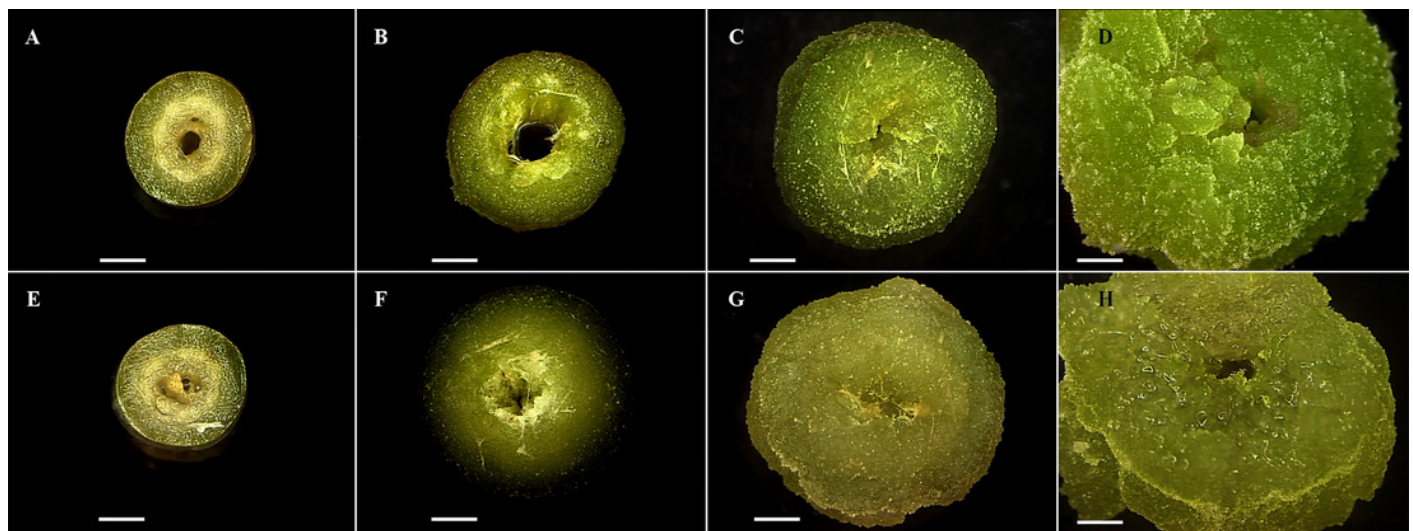



Figure 2

Figure 2

Identification of both diterpenes-type (A), and flavonoids-type (B) compounds in seeds, leaves, and callus of *Jatropha curcas*, through thin layer chromatography

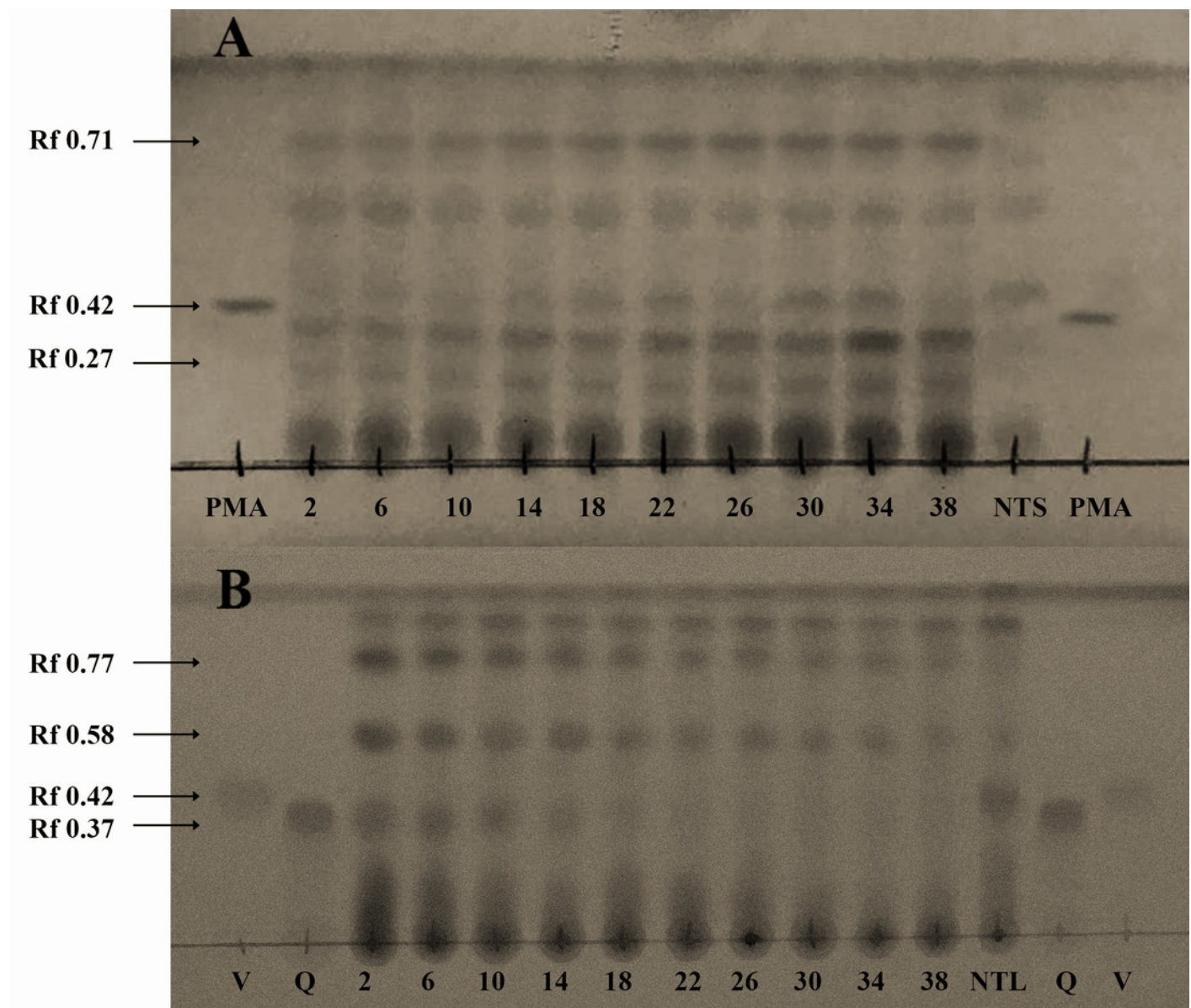



Figure 3

Figure 3

Spectrophotometrical analysis of phorbol esters in extracts of *Jatropha curcas* seeds

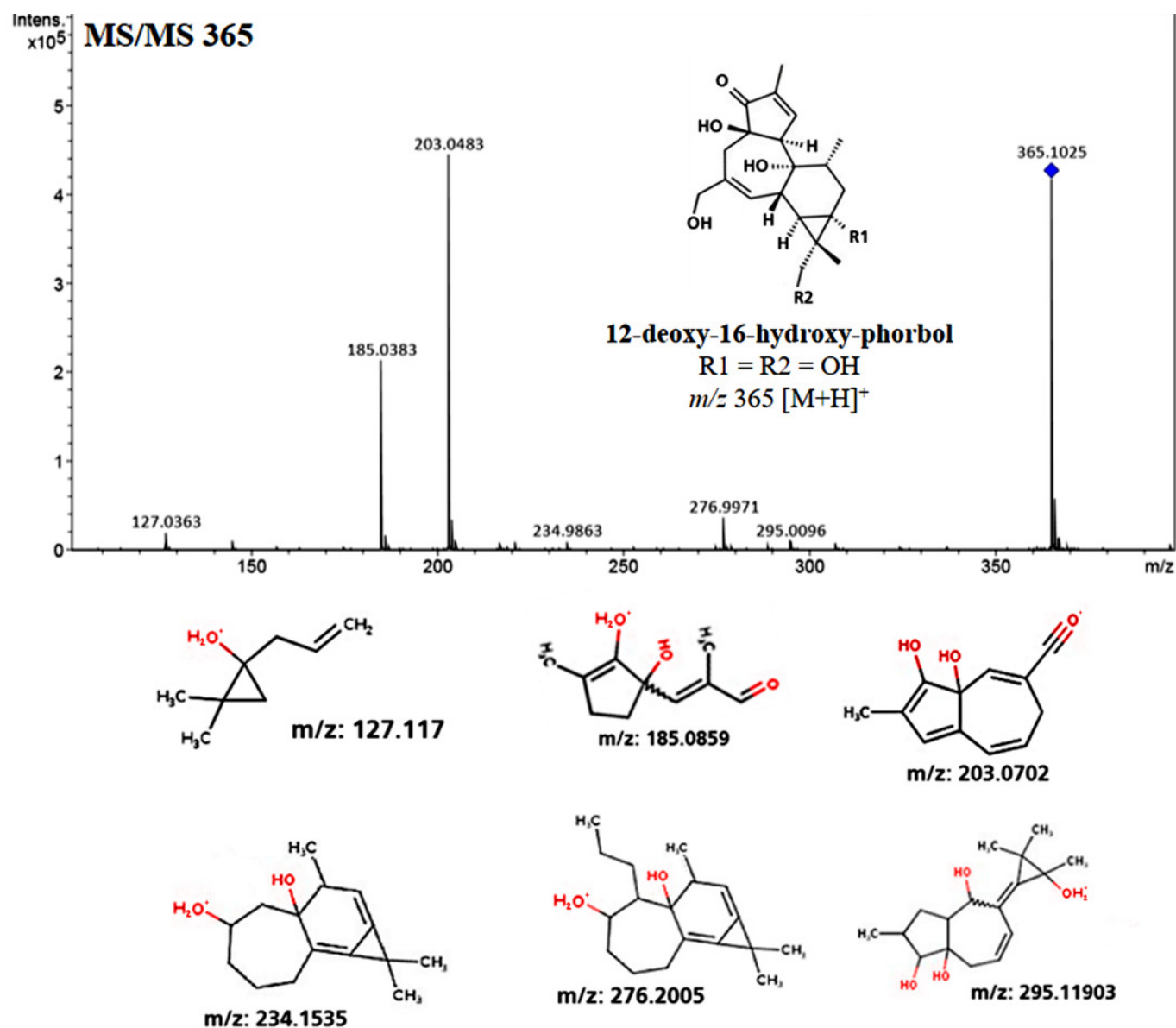



Figure 4

Figure 4

Mass spectra from callus extracts of *J. curcas* showing the relative intensity of the molecular

ion *m/z* 365 [M+H]$^+$ related to the structural core of the *Jatropha*-phorbol esters

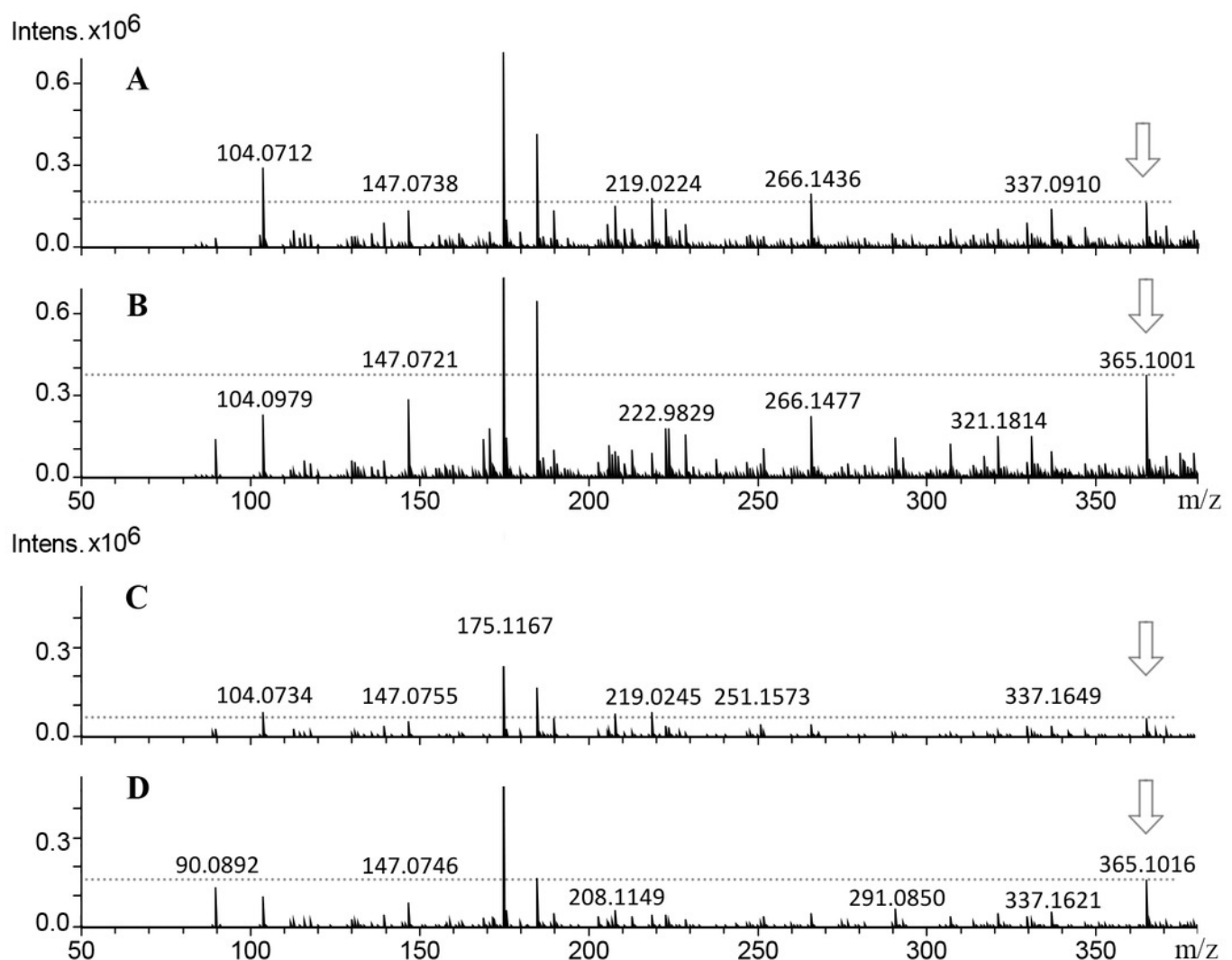



Figure 5

Figure 5

Fragmentation profile (MS/MS) of the molecular ion *m/z* 381 [M+H]⁺, observed in leaves extracts, and related to fragmentation of two glycosylated apigenin (apigenin (apigenin 6-C-α-L-arabinopyranosyl-8-C-β-D-xylopyranoside *m/z* 535 [M+H]⁺, apigenin 4'-O-rhamnoside *m/z* 417 [M+H]⁺)

![MS/MS spectrum and chemical structures figure]

Apigenina 6-C-α-L-arabinopiranosil-8-C-β-D-xilopiranósido *m/z*: 535 [M+H]⁺

m/z 335.1688   *m/z* 355.1917   *m/z* 381.0764   Apigenina 4'-O-ramnosido *m/z* 417 [M+H]⁺

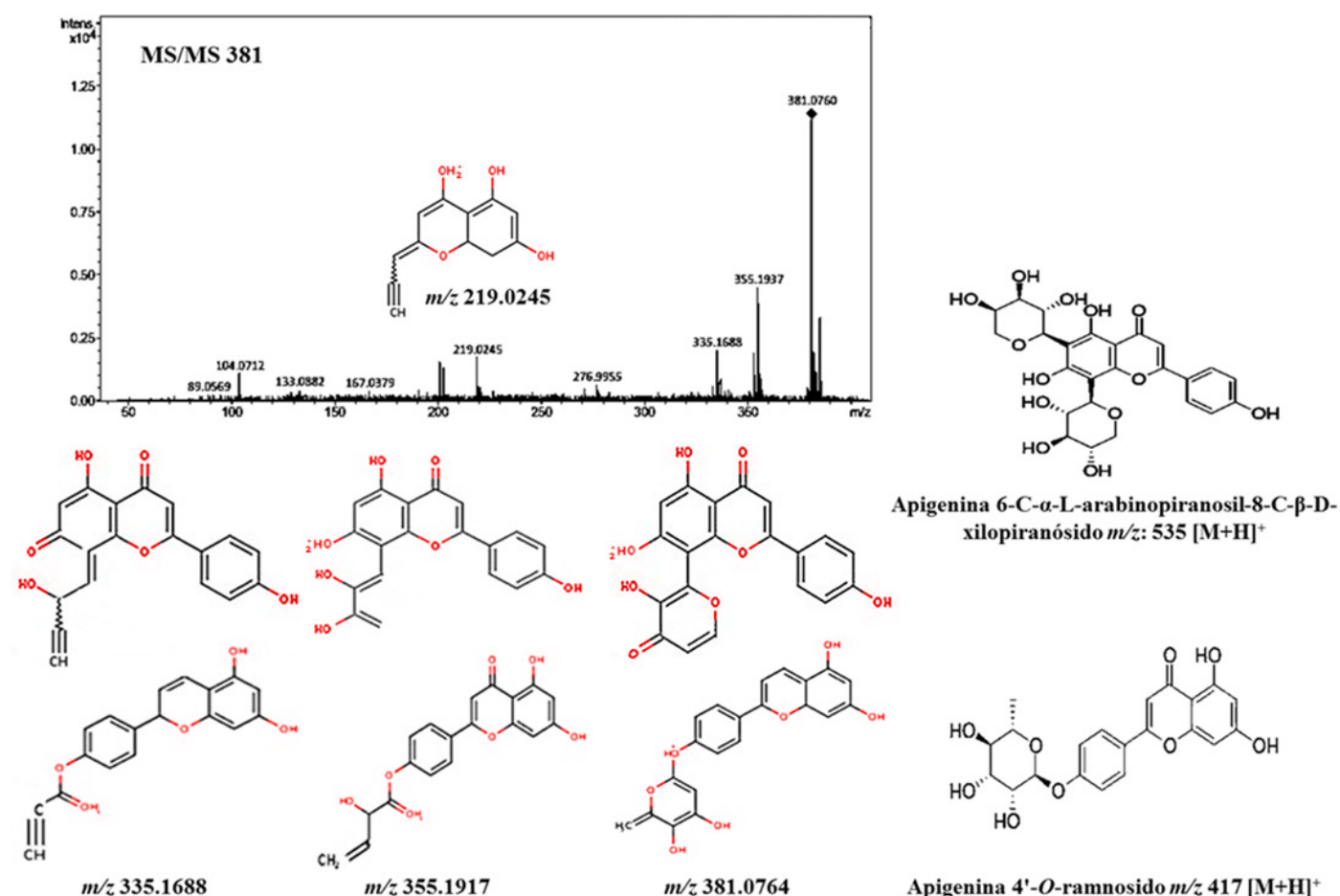
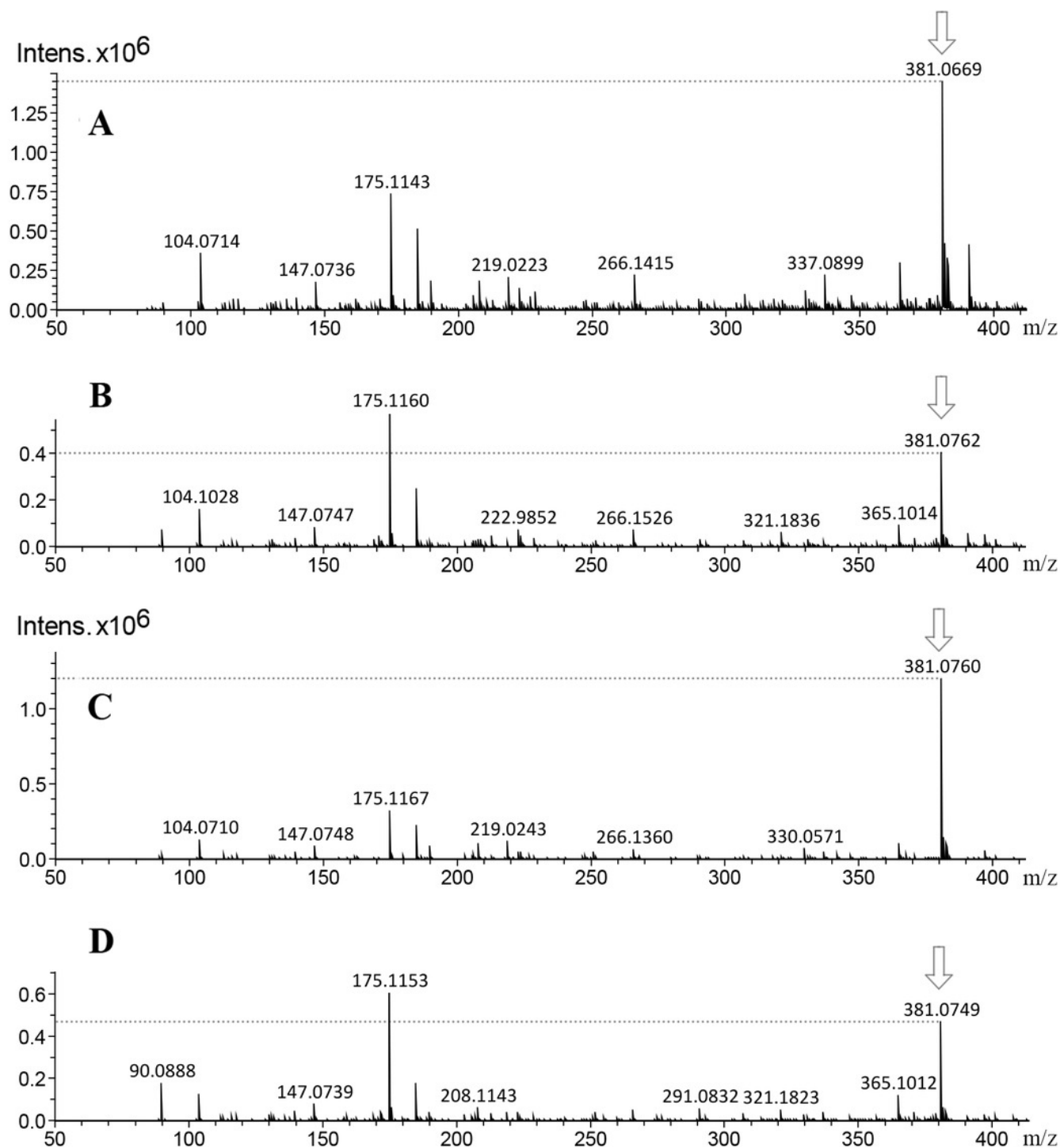



Figure 6

Figure 6

Mass spectra of callus extracts from both toxic and non-toxic varieties of *J. curcas* at 14 and 38 d culture, showing the relative intensity of the molecular ion *m/z* 381 $[M+H]^+$ related to the fragmentation profile from two glycosylated apigenin

Intens. x10^6

A

381.0669

175.1143

104.0714

147.0736

219.0223

266.1415

337.0899

1.25
1.00
0.75
0.50
0.25
0.00

50   100   150   200   250   300   350   400   m/z

B

175.1160

381.0762

104.1028

147.0747

222.9852

266.1526

321.1836

365.1014

0.4

0.2

0.0

50   100   150   200   250   300   350   400   m/z

Intens. x10^6

C

381.0760

175.1167

104.0710

147.0748

219.0243

266.1360

330.0571

1.0

0.5

0.0

50   100   150   200   250   300   350   400   m/z

D

175.1153

381.0749

90.0888

147.0739

208.1143

291.0832 321.1823

365.1012

0.6

0.4

0.2

0.0

50   100   150   200   250   300   350   400   m/z

Table 1

Tentative compounds identified by ESI-MS in hydroalcoholic extracts from seeds, leaves, and callus of 14 and 38 d of culture from both toxic and non-toxic *Jatropha curcas* L. varieties

Table 1. Tentative compounds identified by ESI-MS in hydroalcoholic extracts from seeds, leaves, and callus of 14 and 38 d of culture from both toxic and non-toxic *Jatropha curcas* L. varieties.

Compound type/name	Elemental Composition	Mass	Fragment Ions in Positive Ion Mode (*m/z*)	Plant material	Time of culture (d)	Variety	Relative abundance (%)
Phorbol							
12-deoxy-16-hydroxy-phorbol	$C_{20}H_{28}O_6$	364.4	127, 185, 203, 234, 276, 295	Seeds		T	64.70
						NT	21.05
				Callus	14	T	**14.28**
						NT	**8.69**
					38	T	**30.00**
						NT	**25.00**
Glycosylated Flavonoids							
Apigenin 6-C-α-L-arabinopyranosyl-8-C-*β*-D-xylopyranoside (*m/z* 535 [M+H]⁺) and Apigenin 4'-*O*-rhamnoside (*m/z* 417 [M+H]⁺)	$C_{25}H_{26}O_{13}$ and $C_{21}H_{20}O_9$	534.47 and 416.4	381	Leaves		T	45.83
						NT	100
				Callus	14	T	**100**
						NT	**100**
					38	T	**100**
						NT	**62.50**
Vitexin (*m/z* 433 [M+H]⁺)	$C_{21}H_{20}O_{10}$	432.37	415	Leaves		T	27.27
						NT	70.00
				Callus	14	T	**10.34**
						NT	**4.76**
					38	T	**11.11**
						NT	**<12.50**
Vitexin 4'-O-glucoside-2"-O-rhamnoside (*m/z* 741 [M+H]⁺)	$C_{33}H_{40}O_{19}$	740.7	577	Leaves		T	33.33
						NT	42.84
				Callus	14	T	**1.42**
						NT	**6.36**
					38	T	**12.50**
						NT	**8.75**
Vicenin-2 (*m/z* 595 [M+H]⁺)	$C_{27}H_{30}O_{15}$	594.5	503	Leaves		T	25.00
						NT	29.16
				Callus	14	T	**7.14**
						NT	**4.00**
					38	T	**6.25**
						NT	**3.75**
Vicenin-2,6"-*O*-glucoside (*m/z* 757 [M+H]⁺)	$C_{33}H_{40}O_{20}$	756.7	757	Leaves		T	**<9.09**
						NT	8.00
				Callus	14	T	**1.42**
						NT	**1.73**
					38	T	**2.22**
						NT	**1.25**

*T= Toxic, NT= Non-toxic