Chemical analysis of seeds, leaves and callus extracts of toxic and non-toxic varieties of *Jatropha curcas* L. (#45707)

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Chemical analysis of seeds, leaves and callus extracts of toxic and non-toxic varieties of *Jatropha curcas* L.

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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 establish the cellular dedifferentiated culture of toxic and non-toxic varieties of *Jatropha curcas* L., and to analyze the chemical profile variation in extracts of seeds, leaves and callus of both varieties. Callus induction was obtained using NAA (1.5 mg/L) and BAP (1.5 mg/L) 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 5 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 $[M+H]^+$).

Chemical analysis of seeds, leaves and callus extracts of 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) and BAP (1.5 mg/L) 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

Jatropha curcas L. (Euphorbiaceae) is a multipurpose plant native to Mesoamerica, it is important because of its usefulness as raw material in biofuels production (Salvador-Figueroa *et al*., 2015), as well as, in veterinary and human traditional medicine (Abdelgadir & Van Staden, 2013). Several compounds with different biological activities have been isolated from different species of *Jatropha* (Devappa, Makkar & Becker, 2012b). The identification of biologically active compounds extracted from different organs of this plant has been reported (Prasad, Izam & Khan, 2012; Sharma, Dhamija & Parashar, 2012). Isolated compounds or whole plant extracts have been studied because of their potential pharmacological activity (Cocan *et al*., 2018). Biological effect of *J. curcas* includes antibacterial (Igbinosa, Igbinosa & Aiyegoro, 2009), antitumor (Lin *et al*., 2003), anti-inflammatory, and antifungal (Saetae & Suntornsuk, 2010; Srinivasan, Palanisamy & Mulpuri, 2019). Most research on *J. curcas* have been done with toxic varieties; toxicity is referred to phorbol esters content in seeds.

In Mexico, it has been identified a non-toxic variety of this species with very low or non-detectable levels of phorbol esters (PEs) (Martínez-Herrera, Chel-Guerrero & Martínez-Ayala, 2004). The highest accumulation of PEs is at the seeds. PEs are known as *Jatropha* factors because each one of them has the same nucleus diterpene moiety, namely, 12-deoxy-16-hydroxy-phorbol (DHP) which is coupled to unstables intramolecular diterpenes (named C_1–C_6 factors) (Hirota *et al*., 1988; Haas, Sterk & Mittelbach, 2002; Baldini *et al*., 2014; Nishshanka *et al*., 2016).

Plants are the most successful source of chemical compounds, which potential mode of action makes them an alternative phytomedicinal drug, since several natural products have shown benefits against human diseases (Briskin, 2000). Several compounds are tissue-specific accumulated, and are usually structurally complex (Balunas & Kinghorn, 2005). Therefore it is necessary the use of chemical analysis techniques to isolate and identify the extracted plant metabolites. There are a few cases where the use of plant cell culture has allowed the production of active compounds, even more biotechnological production either as pure compounds or as standardized extracts, provides unlimited opportunities for new drug discoveries due to the great chemical diversity (Karuppusamy, 2009).

Secondary metabolites are generally in complex matrices at very low concentrations in plant organs, and lower in dedifferentiated cells. These compounds have a wide range of polarities, therefore it is necessary the use of solvents with different polarity to obtain the extracts (Amita & Shalini, 2014). 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.

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).

~~Callus obtaining~~

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®). Nine treatments, as result of the combination of three concentrations (0.0, 1.5 and 3.0 mg.L-1) of both naphthaleneacetic acid (NAA) and 6-benzyl-aminopurine (BAP) were evaluated (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 evolution~~ was recorded every seven days using a stereoscopic microscope (Nikon, model SMZ 1500, Japan).

~~Sample preparation~~

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.

~~Extract obtaining~~

20 mL of ethanol 80% (v/v) were added to 500 mg of biomass dry weight (dw) and sonicated at 40 ± 5 °C during 30 min (Bransonic Ultrasonic Cleaner, 2510R-MTH, CT, USA) (Bazaldúa *et al*. 2019), subsequently 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 resulting oil, were extracted with MeOH, later filtered and concentrated to dryness at 25 ± 2 °C, then solubilized in 500 µL of HPLC grade MeOH for chromatographic analysis (Demissie & Lele, 2010).

Thin layer chromatography

Extracts were applied on normal phase silica plates (Merck Millipore, 60 F_{254}, Germany). Chloroform-methanol (94:6 and 75:25) were used as mobile phase, reference standards were paclitaxel (TX, Sigma-Aldrich®), and quercetin (Sigma-Aldrich®), both plates were revealed with anisaldehyde (Kathiravan & Raman, 2010).

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

MicrOTOF Q-II analysis

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

Results

Establishment of callus culture

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

Thin layer chromatography (TLC) analysis

TLC showed differences in compounds accumulation during time culture (2, 6, 10, 14, 18, 22, 26, 30, 34 and 38 d). Regard diterpenes, spots with Rf of 0.76 and 0.24 showed higher intensity along this period (Fig. 2A), unlike flavonoids in which spots with Rf of 0.84, 0.73 and 0.55, decreased throughout the same culture period (Fig. 2B). These results suggest that the accumulation of diterpenes and flavonoids was inversely related during callus development. To obtain diterpenes the Soxhlet-methanol extraction was more efficient than sonication-ethanol 80%. TLC analysis of extracts obtained by both methods evidenced differences in the size and intensity of spots in regard to: extraction method, variety (toxic and non-toxic), and plant material (seeds, leaves and callus) (Fig. S1).

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

Phorbol esters (PEs) analysis

Fragmentation profile analysis from seeds extract from both varieties showed several highs signals one of them with *m*/*z* of 365 $[M+H]^+$ corresponding to 12-deoxy-16-hydroxy-phorbol (DHP), which is the fundamental structural core of the PEs. The MS/MS analysis of this molecular ion showed fragments with *m*/*z* of 295, 276, 234, 203, 185 and 127 $[M+H]^+$ which is similar to the fragmentation profile of DHP presented in CFM-ID platform (Fig. 3), this suggests the identification of that molecular structure in all of the extracts obtained from seeds and callus of both toxic and non-toxic varieties. Based on signals intensities from 14 d and 38 d callus extracts from both varieties, it is suggested that the accumulation of DHP is time-dependent. Since, the corresponding signal was higher in callus of 38 d than in those of 14 d. Furthermore, two signals with *m*/*z* of 547 and 591 $[M+H]^+$ were observed, so it is proposed that they are related with the fragmentation profile of the signal with *m*/*z* of 711 $[M+H]^+$ corresponding to any of the *Jatropha* factors (C_1 or DHPB to C_6) which nucleus structure is DHP (Wink *et al*., 2000; Haas, Sterk & Mittelbach, 2002) (Fig. 4C).

Flavonoids analysis

On the other hand, the main group of compounds in *Jatropha* leaf extracts are flavonoids, among them the apigenin, nevertheless, it is important to refer that the natural condition of flavonoids in the plants is in glycosylated form. On this regard, another of the highest signals observed at the chromatograms was the *m*/*z* of 381 $[M+H]^+$ ion, the MS-MS experiment of this signal and the proposed structures obtained by CFM-ID platform allowed to relate that molecular ion (*m/z* 381 $[M+H]^+$) (Fig. 5) to the fragmentation profiles of apigenin 6-*C*-α-L-arabinopyranosyl-8-*C*-*β*-D-xylopyranoside, and of apigenin 4'-*O*-rhamnoside (Fig. 6). Table 1 shows six tentatively identified compounds by relating their molecular ion *m*/*z* of 381 $[M+H]^+$ with the fragmentation signals and their corresponding predictive structure 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 $[M+H]^+$ (Fig. S2). Inversely to observed on DHPB related signal (*m/z* 365 $[M+H]^+$), the intensity of the molecular ion related with flavonoids diminished, but there was not difference between type of extracts, leaves or callus from both varieties.

Discussion

The highest callus induction (95.5%) was observed in petiole explants on MS medium added with NAA (3.0 $mg.L^{-1}$) and BAP (3.0 $mg.L^{-1}$), the second best result (87.7%) was obtained with NAA (1.5 $mg.L^{-1}$) and BAP (1.5 $mg.L^{-1}$), in contrast to reported by Nassar *et al*. (2013), who observed dedifferentiation with NAA and BAP at 0.5 $mg.L^{-1}$ of each one plant growth regulator. Explants dedifferentiation reported in this work was similar to reported by Kumar *et al*. (2015). The follow up of the explants dedifferentiation process, every 7 d showed callus formation on explants starting

on the seventh day. Dedifferentiation began at the cutting sites because of the high response capacity of cells as expected (Sujatha, Makkar & Becker 2005; Nogueira *et al*., 2011; Ovando-Medina *et al*., 2016). The callus obtained was light green and friable, similar to reported by Hernández *et al*. (2015). It has been reported that high auxins concentrations could affect production and accumulation of secondary metabolites (Kim *et al*., 2007), hence, according to our results, it is suggested the use of the lowest effective concentration, 1.5 $mg.L^{-1}$ for both growth regulators. Muñoz-Valverde *et al*. (2003) concluded that BAP is determinant to induce callus formation in foliar explants of *J. curcas*. Likewise, Suárez & Salgado (2008) reported that it is necessary the presence of NAA in the culture medium to induce callus formation in *Stevia rebaudiana*, and that this effect could be increased when adding cytokinins like BAP. On the other hand, Solange *et al*. (2002) determined that the use of NAA and BAP in equal proportion induces callus formation from leaf explants of *Tridax procumbens*. Coutiño-Cortés *et al*. (2013) reported the callus induction in *J. curcas* leaf explants at 10 d of culture, and total explant-cell dedifferentiation at 20 d using 2, 4-D, BAP and KIN, while in this work petioles dedifferentiation started at 7 d and total explant-cell dedifferentiation was achieved at 21 d. These results support that synergy between NAA and BAP is essential to achieve a high dedifferentiation degree.

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

Regard, to TLC profile analysis, it has been reported that methanolic extraction from seed-oil facilitates separation and availability of methyl ester type compounds, mainly phorbol esters (PEs) (Demissie & Lele, 2010; Devappa, Bingham & Khanal, 2013). The identification by TLC of PEs in seed methanolic extracts from toxic and non-toxic *J. curcas* varieties was reported (Devappa, Makkar & Becker (2012a), they reported higher spots intensity from toxic variety than from non-toxic, when plates were observed at 366 nm UV light, this result is similar to that observed in this work (Fig. S1). Makkar & Becker (2009) identified higher PEs accumulation in seeds than in leaves extracts. Similar results were obtained in this work, even with different method of extraction. Nevertheless these results are different of that obtained by Martínez-Herrera, Chel-Guerrero & Martínez-Ayala, 2004, because they reported 96% of PEs extraction through hydroalcoholic extraction, while, in this work the intensity of the spots was higher on Soxhlet-methanol extracts than hydroalcoholic extraction (Fig. S1).

On the other hand, Hirota *et al*. (1988) reported the identification of DHP as the fundamental structural core which is derived from 12-deoxy-16-hydroxy-phorbol-4'-[12',14'-butadienyl]-6'-[16',18',20'-nonatrienyl]- bicycle [3.1.0] hexane-(13-*O*)-2'- [carboxylate]- (16-*O*)-3'- [8'-butenoic-10']ate (DHPB or *Jatropha* factor C_1), identified as DHPB-Na adduct *m/z* 733 $[M+Na]^+$.

Furthermore, DHPB *m/z* 711 $[M+H]^+$ and DHP *m/z* of 365 $[M+H]^+$ were also reported in *J. curcas* seeds (Wink *et al*., 2000). Even more so, Haas, Sterk & Mittelbach, (2002) reported the identification of diterpenes named *Jatropha* factors C_2 to C_6 through ESI-MS *m/z* 711 $[M+H]^+$ and of DHP (*m/z* of 365 $[M+H]^+$). Furthermore, Nishshanka *et al*. (2016) identified six phorbol esters in *J. curcas* seeds by LC-MS, which have the same core (DHP), at the so named *Jatropha* factors (C_1 to C_6).

Regard to PEs identification by ESI-MS analysis, Baldini *et al*. (2014) identified six phorbol esters in *J. curcas* seeds with *m/z* of 711 $[M+H]^+$, which have the same fundamental structural core (DHP) *m/z* of 365 $[M+H]^+$ which is coupled to diterpenes of 24 carbon structures named *Jatropha* factors from C_1 (DHPB) to C_6. The relative intensity of the molecular ion *m/z* 365 $[M+H]^+$ was higher in seeds extracts from toxic variety, than in seed extracts from non-toxic variety (Fig. 4A, and 4B). While in callus, the relative intensity is higher in toxic and non-toxic varieties callus of 38 d of culture (Fig. 4E, and 4G), than in toxic and non-toxic varieties callus of 14 d of culture (Fig. 4D, and 4F). These results could suggest the presence of PEs coupled to DHP in the samples analyzed and that their accumulation is differential in regard to type of organ, variety, and in cel cultures, throughout the time of culture. This results suggest that their accumulation of DHP is time dependent. This ESI-MS analysis allowed to corroborate the results obtained by TLC (Fig. 2A). Nevertheless, the relative intensity of the signals observed in extracts from callus were lower than that obtained from seeds extracts as reported by Demissie & Lele (2010).

By other hand, phenolic compounds are ubiquitously produced by plants (Boudet, 2007), the main role of phenols in plants is to protect them from biotic or abiotic stress (Clé *et al*., 2008). These properties include antimicrobial, insecticidal, antiparasitic, antiviral, anti-ulcerogenic, cytotoxic, antioxidant, anti-hepatotoxic, anti-hypertensive and anti-inflammatory activities (Oskoueian *et al*., 2011; Papalia, Barreca & Panuccio, 2017). Flavonoids are recognized as polyphenols. Several of them have been identified in *Jatropha* genus, such as apigenin glycosides, vitexin, and isovitexin which have been considered as chemiotaxonomic compounds from the genus (Abdelgadir &Van Staden, 2013; Huang *et al*., 2014).

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

The results obtained by microQTOF-QII of the molecular ion *m/z* 381 $[M+H]^+$ through the MS/MS experiment, and the predictive structures obtained through the CFM-ID platform allowed to suggest the relation of the structures from the molecular ion *m/z* 381 $[M+H]^+$ with the

fragmentation profile from apigenin 6-*C*-α-L-arabinopyranosyl-8-*C*-*β*-D-xylopyranoside *m*/*z* 535 $[M+H]^+$, which was identified through ESI-MS in *Viola yedoensis* (Xie *et al*., 2003) and apigenin 4'-*O*-rhamnoside *m/z* 417 $[M+H]^+$, which was identified in *Olea europaea* (Pieroni *et al*., 1996).

Based on the molecular ion, MS-MS fragmentation profile and the predictive structures obtained by CFM-ID platform, it is suggested the tentative identification of vitexin *m*/*z* of 433 $[M+H]^+$, vicenin-2 *m*/*z* of 595 $[M+H]^+$, and vitexin 4'-*O*-glucoside-2 "-*O*-rhamnoside *m*/*z* of 741 $[M+H]^+$ in leaves and callus from both varieties. These results are similar to obtained by Huang *et al*. (2014) who identified vitexin *m*/*z* of 433 $[M+H]^+$ in *J. curcas* leaves. This flavonoid was also identified by ESI-MS in *Vigna radiata* and *Luehea divaricata* (Tanaka *et al*., 2005; Peng *et al*., 2008). In this work it is also suggested the tentative identification of vicenin-2,6"-*O*-glucoside *m/z* 757 $[M+H]^+$ which has not been reported to *Jatropha curcas*, but to *Stellaria holostea* (Bouillant *et al*., 1984) (Fig. S2).

Interestingly it was observed that relative intensities signals related to PEs (*m*/*z* 365 $[M+H]^+$) in callus of 38 d was higher than callus of 14 d, but the flavonoid related molecular ion *m*/*z* 381 $[M+H]^+$ decreased at same period.

Conclusions

During cell dedifferentiation NAA and BAP at the same concentration induced the highest amount of callus in petiole explants from both toxic and non-toxic varieties of *Jatropha curcas*. The variety of the species did not influenced the cell dedifferentiation. Soxlet-methanol extraction was more efficient than sonication-ethanol 80% to obtain phorbol esters type compounds from seeds and callus. Thin layer chromatography and mass spectrometry, suggest an inverse relationship between phorbol esters and flavonoids accumulation in callus throughout the time of culture. The tentative identification of diterpene type compounds such as 12-deoxy-16-hydroxy-phorbol and *Jatropha* factors by ESI-MS in seed and callus (14 and 38 d) extracts of *J. curcas*, from both toxic and non-toxic varieties, as well as, the presence of six flavonoids glycosides in leaf and callus, from both toxic and non-toxic varieties, is suggested.

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Figures description

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

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. Lanes from 2 to CNT correspond to extracts of: 2 – 38= Callus of non-toxic variety throughout 38 d of culture, SNT= Non-toxic variety-seeds, ST= Toxic variety-seeds, CT= Callus (15 d culture) from toxic variety, CNT= Callus (15 d culture) from non-toxic variety, Tx= paclitaxel (Sigma) reference standard. **A)** The spots intensity increased throughout to culture time (Rfs 0.76, and 0.24), mobile phase chloroform-methanol (94:6). **B)** The spots intensity decreased throughout to culture time (Rfs 0.73, and 0.55), mobile phase chloroform-methanol (75:25). Plates were revealed with anisaldehyde.

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

Figure 4. Mass spectra of seeds and 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. Seeds extracts of (A) toxic, and (B) non-toxic, varieties; (C) predictive structures related to the structural nucleus of phorbol esters and their ionized fragments. Callus extracts from toxic variety: (D) 14 d of culture; (E) 38 d of culture; non-toxic variety: (F) 14 d of culture, (G) 38 d of culture.

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 $[M+H]^+$). Structures predicted to each molecular ion (381, 355, 335, and 219 *m/z*), obtained from CFM-ID platform.

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. A, and C) Extracts of *J. curcas* callus from *J. curcas*-toxic variety (14 and 38 d, respectively). B, and D) Extracts of *J.*

curcas callus from non-toxic variety (14 and 38 d, respectively). The relative intensity from molecular ion *m/z* 381 $[M+H]^+$ diminish throughout culture time.

Table 1 Tentative flavonoids identified by ESI-MS in hydroalcoholic extracts of leaves and callus of *Jatropha curcas*.

Supplementary material description

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

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

Figure 1

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

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


A
1 mm
B
1 mm
C
1 mm
D
1 mm
E
1 mm
F
1 mm
G
1 mm
H
1 mm

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.

Identification of both diterpenes-type (A), and flavonoids-type (B) compounds in seeds, leaves, and callus of *Jatropha curcas*, through thin layer chromatography. Lanes from 2 to CNT correspond to extracts of: 2 – 38= Callus of non-toxic variety throughout 38 d of culture, SNT= Non-toxic variety-seeds, ST= Toxic variety-seeds, CT= Callus (15 d culture) from toxic variety, CNT= Callus (15 d culture) from non-toxic variety, Tx= paclitaxel (Sigma) reference standard. **A)** The spots intensity increased throughout to culture time (Rfs 0.76, and 0.24), mobile phase chloroform-methanol (94:6). **B)** The spots intensity decreased throughout to culture time (Rfs 0.73, and 0.55), mobile phase chloroform-methanol (75:25). Plates were revealed with anisaldehyde.

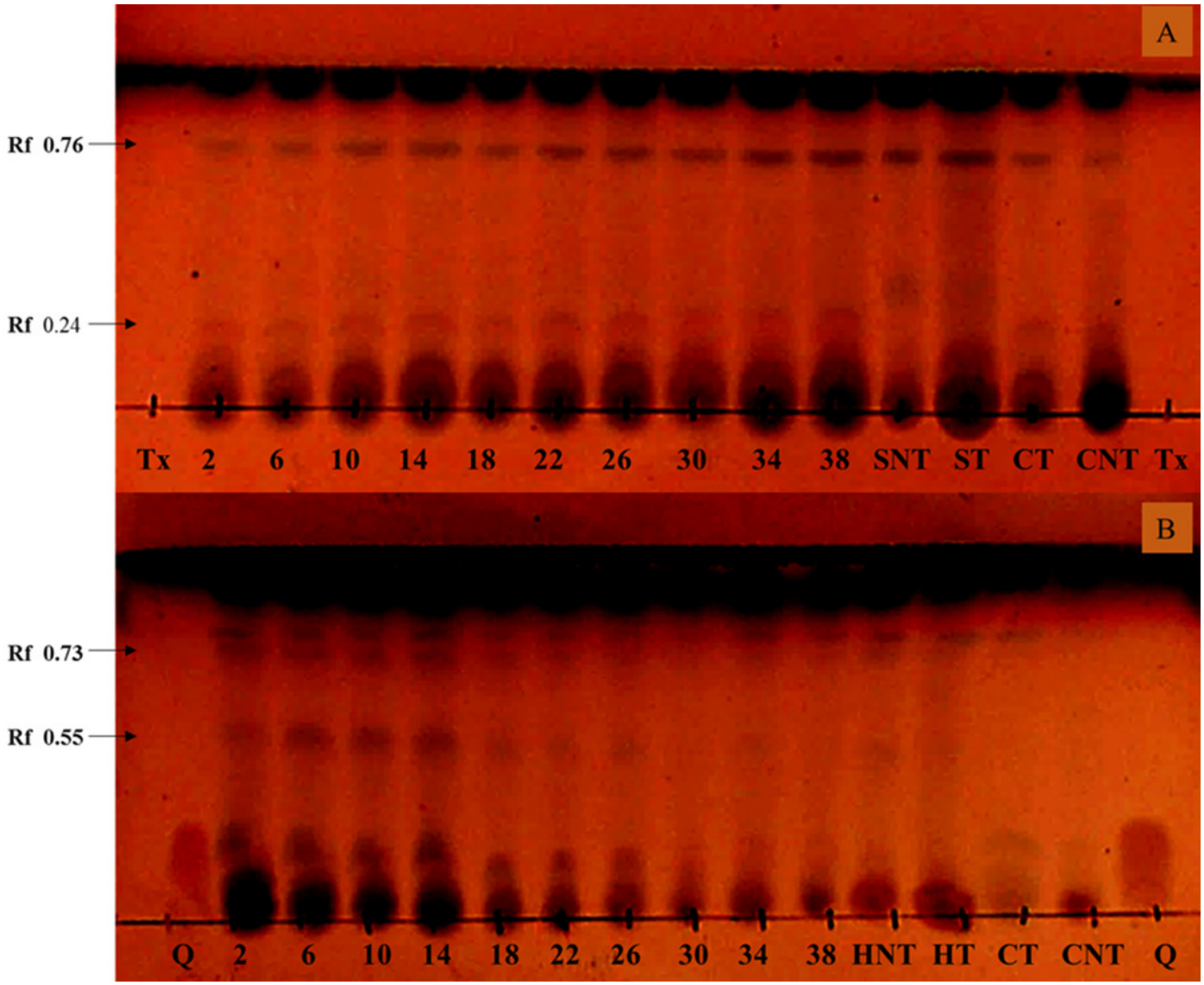
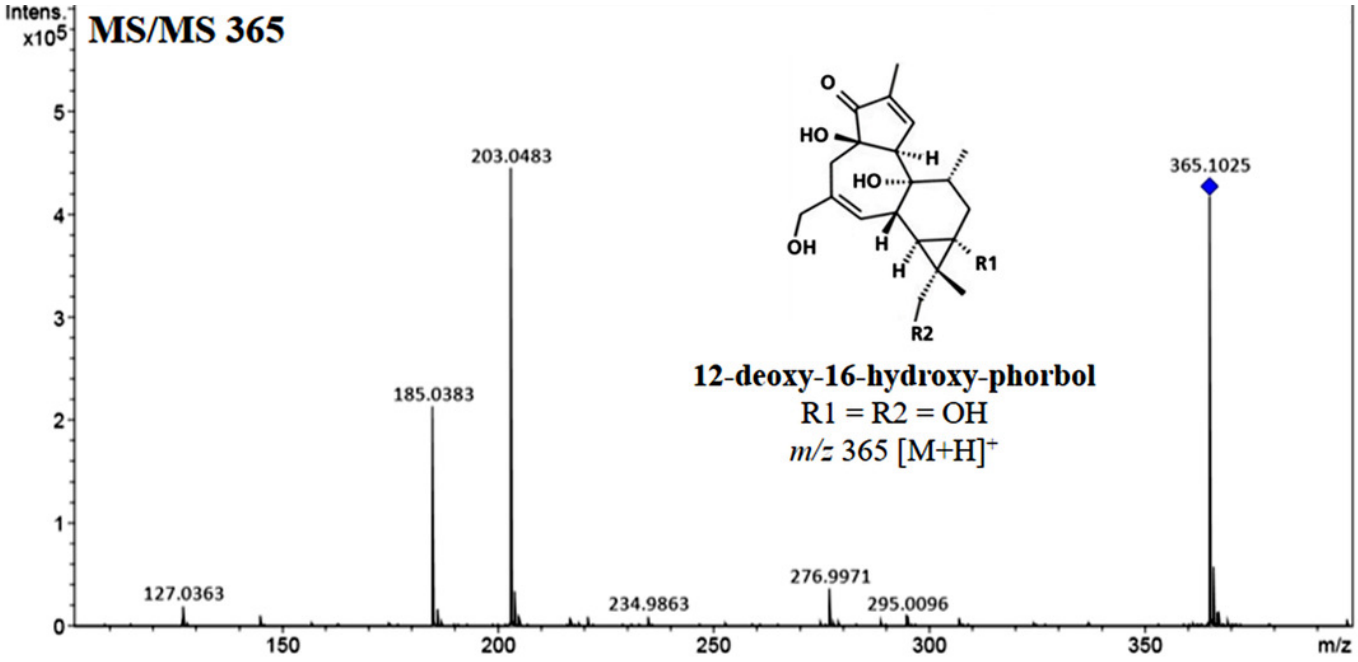

A
Rf 0.76
Rf 0.24
Tx 2 6 10 14 18 22 26 30 34 38 SNT ST CT CNT Tx
B
Rf 0.73
Rf 0.55
Q 2 6 10 14 18 22 26 30 34 38 HNT HT CT CNT Q

Figure 3

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

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


Intens.
x10^5
MS/MS 365
203.0483
365.1025
185.0383
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234.9863
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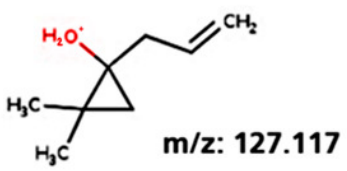
295.0096
m/z
12-deoxy-16-hydroxy-phorbol
R1 = R2 = OH
m/z 365 [M+H]+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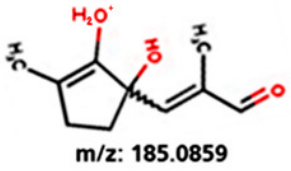
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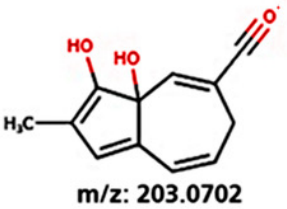
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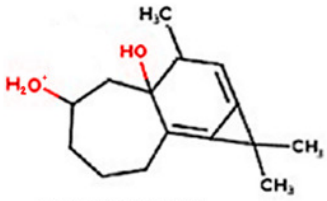
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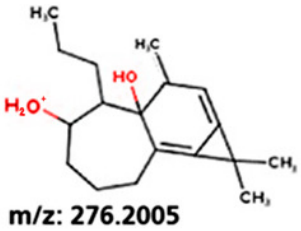
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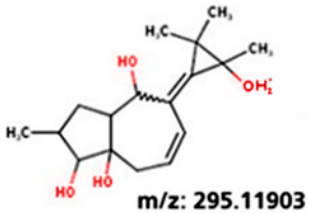
m/z: 295.11903

Figure 4

Mass spectra of seeds and 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.

Mass spectra of seeds and 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. Seeds extracts of (A) toxic, and (B) non-toxic, varieties; (C) predictive structures related to the structural nucleus of phorbol esters and their ionized fragments. Callus extracts from toxic variety: (D) 14 d of culture; (E) 38 d of culture; non-toxic variety: (F) 14 d of culture, (G) 38 d of culture.

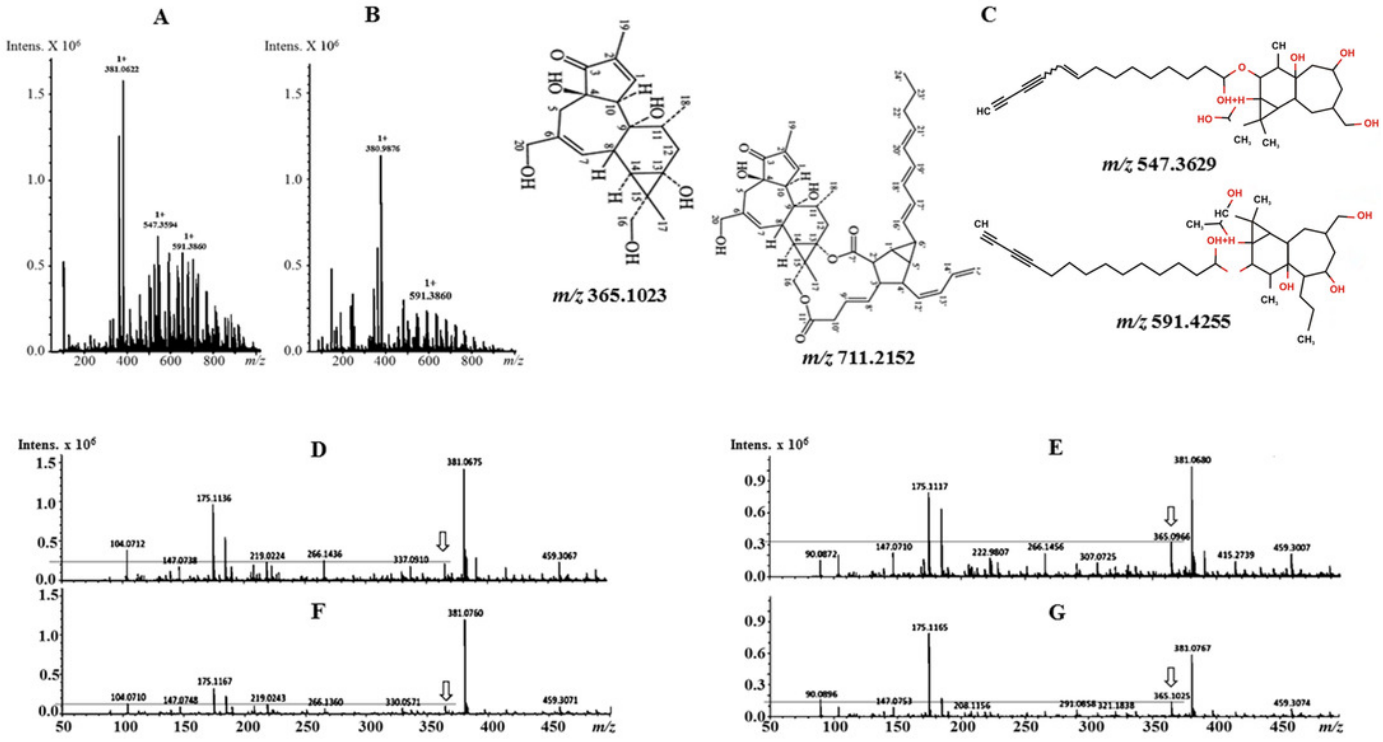

Figure 5

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 [i]m/

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 $[M+H]^+$). Structures predicted to each molecular ion (381, 355, 335, and 219 *m/z*), obtained from CFM-ID platform.

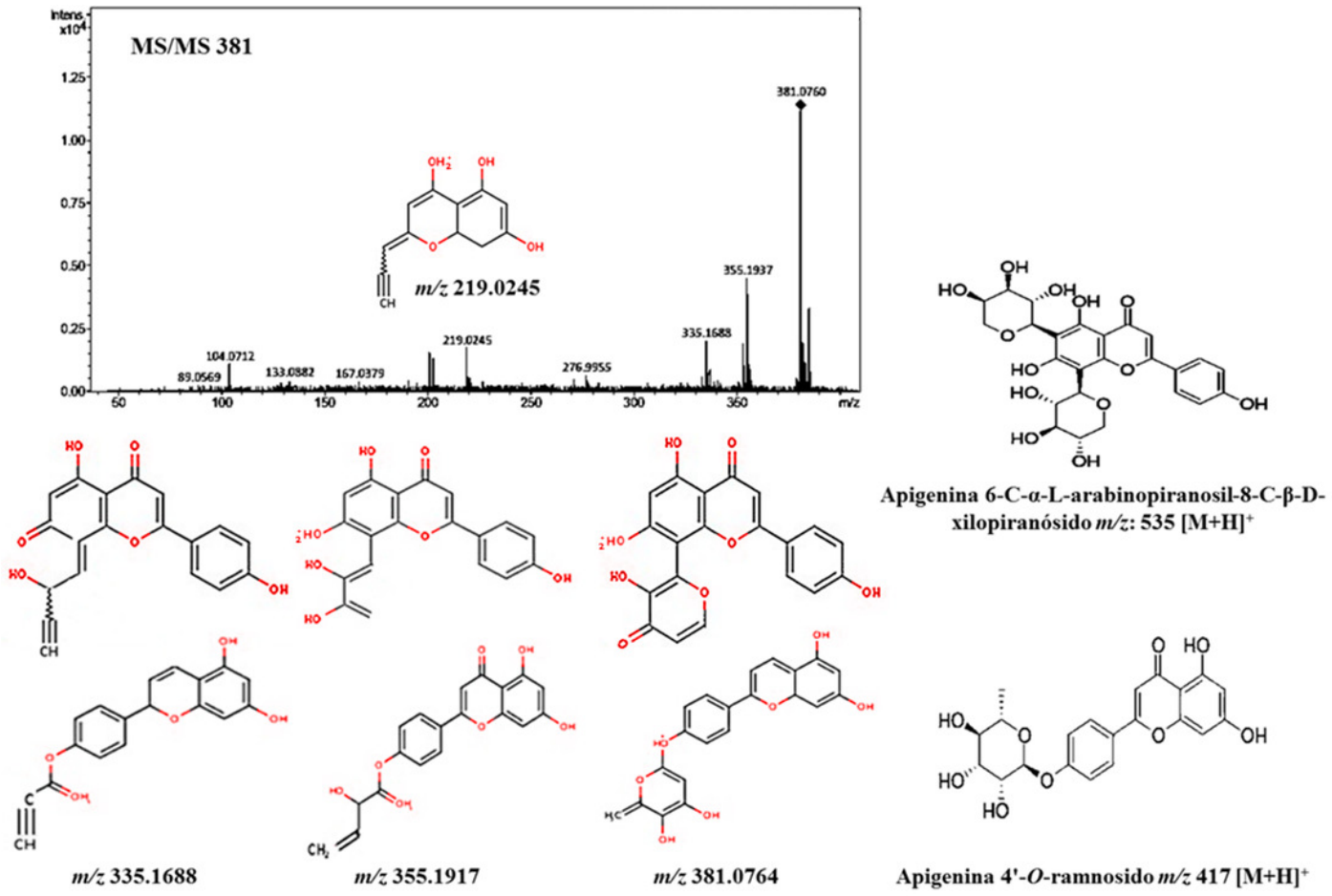

Figure 6

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 glycosylate

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. A, and C) Extracts of *J. curcas* callus from *J. curcas*-toxic variety (14 and 38 d, respectively). B, and D) Extracts of *J. curcas* callus from non-toxic variety (14 and 38 d, respectively). The relative intensity from molecular ion *m/z* 381 $[M+H]^+$ diminish throughout culture time.

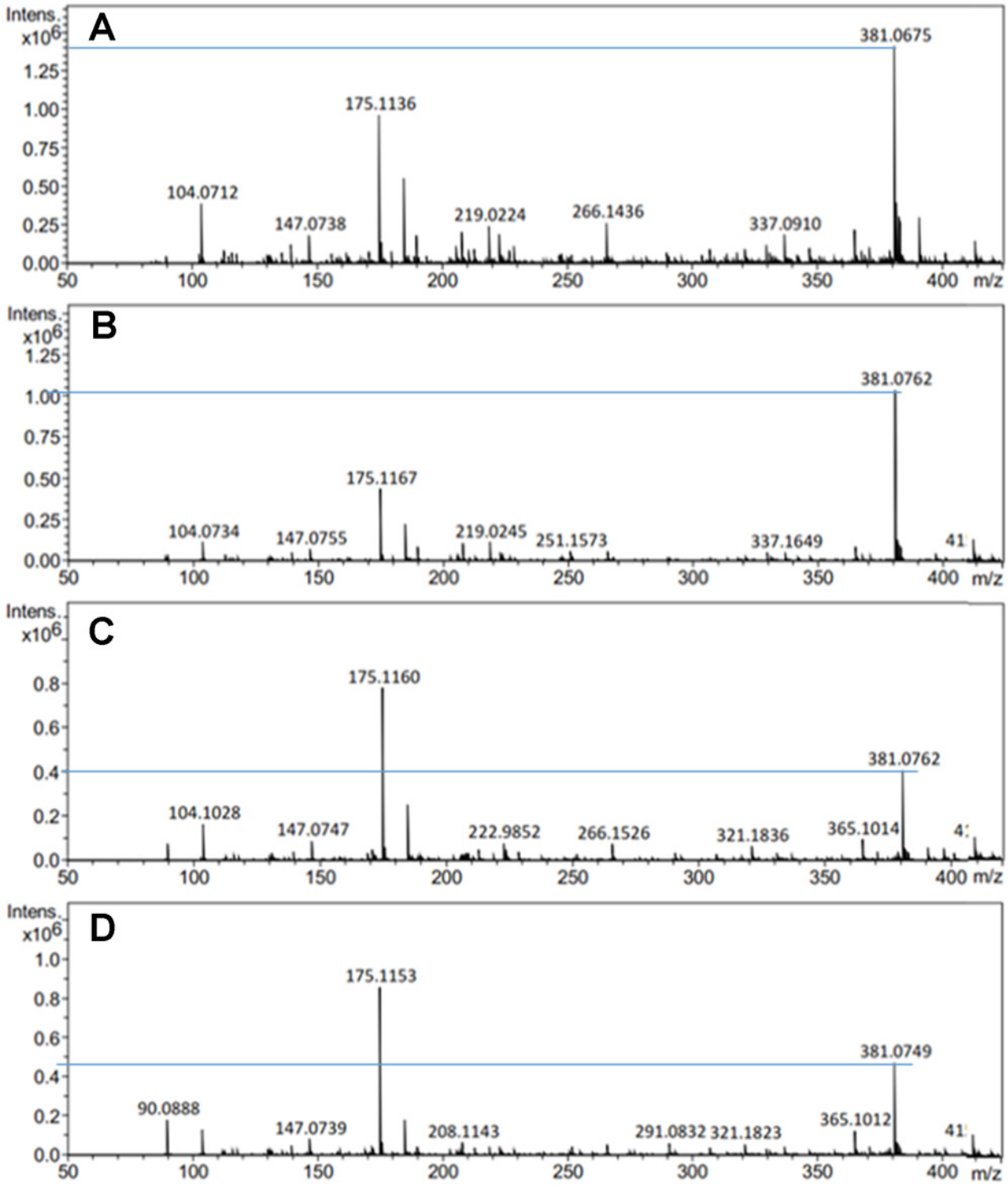
A
Intens.
x10^6
1.25
1.00
0.75
0.50
0.25
0.00
381.0675
175.1136
104.0712
147.0738
219.0224
266.1436
337.0910
50
100
150
200
250
300
350
400
m/z
B
Intens.
x10^6
1.25
1.00
0.75
0.50
0.25
0.00
381.0762
175.1167
104.0734
147.0755
219.0245
251.1573
337.1649
41
50
100
150
200
250
300
350
400
m/z
C
Intens.
x10^6
0.8
0.6
0.4
0.2
0.0
175.1160
381.0762
104.1028
147.0747
222.9852
266.1526
321.1836
365.1014
4:
50
100
150
200
250
300
350
400
m/z
D
Intens.
x10^6
1.0
0.8
0.6
0.4
0.2
0.0
175.1153
381.0749
90.0888
147.0739
208.1143
291.0832
321.1823
365.1012
41
50
100
150
200
250
300
350
400
m/z

Table 1(on next page)

Tentative flavonoids identified by ESI-MS in hydroalcoholic extracts of leaves and callus of *Jatropha curcas*.

[a] The predictive structures related with vitexin, vicenin-2, and their glycosides are included as supplementary material (Fig. S2). [b] The predictive structures related to the fragmentation profile from apigenin 6-*C*-alpha-L-arabinopyranosyl-8-*C*-*β*-D-xylopyranoside, and from apigenin 4'-*O*-rhamnoside (*m/z* 381, 355, 335, and 219), are included at Figure 5. [c] Vicenin-2,6''-*O*-glucoside has not been reported to *Jatropha curcas*, but to *Stellaria holostea*.

Table 1. Tentative flavonoids identified by ESI-MS in hydroalcoholic extracts of leaves and callus of *Jatropha curcas*.

Tentative identified flavonoid	Condensed formula	*m/z* [M+H]+	Fragments related to each corresponding fragmentation profile [a]		
Apigenin 6-*C*-alpha-L-arabinopyranosyl-8-*C*-*β*-D-xylopyranoside [b]	$C_{25}H_{26}O_{13}$	534	381	355	219
Apigenin 4'-*O*-rhamnoside [b]	$C_{21}H_{20}O_9$	417	381	355	219
Vitexin	$C_{21}H_{20}O_{10}$	433	415	401	397
Vitexin 4'-*O*-Glucoside-2''-*O*-Rhamnoside	$C_{33}H_{40}O_{19}$	741	723	577	561
Vicenin-2	$C_{27}H_{30}O_{15}$	595	565	503	445
Vicenin-2,6''-*O*-glucoside [c]	$C_{33}H_{40}O_{20}$	757	729	695	621

[a] The predictive structures related with vitexin, vicenin-2, and their glycosides are included as supplementary material (Fig. S2).

[b] The predictive structures related to the fragmentation profile from apigenin 6-*C*-alpha-L-arabinopyranosyl-8-*C*-*β*-D-xylopyranoside, and from apigenin 4'-*O*-rhamnoside (*m/z* 381, 355, 335, and 219), are included at Figure 5.

[c] Vicenin-2,6''-*O*-glucoside has not been reported to *Jatropha curcas*, but to *Stellaria holostea*.