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# Determination of phorbol esters in seeds and leaves of *Jatropha curcas* and in animal tissue by high-performance liquid chromatography tandem mass spectrometry

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ABSTRACT

In order to improve the economic sustainability of the Jatropha-biofuel chain, seed cake detoxification and utilization of 'non toxic' *Jatropa curcas* accessions are the main activities pursued with the aim of using *J. curcas* seed cake in animal feed. Given this growing interest, a robust and reliable method for phorbol esters (PEs) determination is necessary. HPLC-UV is a well-established method to detect and quantify the PEs content in Jatropha seeds and related products, but it seems to be unsuitable for more complex matrices like Jatropha leaves and animal tissues, due to the presence of interfering compounds. The objective of this work was to develop and optimize a LC–MS/MS method for the quantitative determination of PEs in seeds and leaves of *J. curcas* L. plants from Ghana and Mexico and in liver (as an organ with the function of accumulation) from goats fed with PEs in their diet. The HPLC-UV analysis evidenced five chromatographic peaks in the toxic seed kernels corresponding to the factors C1, C2, C3, C6 and C4–C5, respectively, with a PEs concentration of about 5100 μg/g (as TPA equivalent). No PEs related peaks were detected in Mexican kernel seeds while in the case of leaves and liver the analysis was hampered by the presence of interfering compounds. The toxic kernel seed extract was used as a standard solution for the PEs quantitation in leaves and liver samples by LC–MS/MS, with the standard addition method. The most intense MRM transitions used to quantify and qualify the PEs were: 675 → 311, 693 → 311, and 293 → 265 *m/z*. The LC–MS/MS method with a LOD and a LOQ of 0.07 and 0.21 μg/g, respectively, resulted in more sensitivity and selectivity than the HPLC-UV method. All three MRM transitions were present in Ghanaian toxic kernel seed, while no peaks were present in the supposed non-toxic Mexican kernel seed. PEs concentration in the leaves of toxic Ghanaian accession resulted in about 1/10 of that in the kernel, while no PEs peaks were found in the *J. curcas* leaves from Mexico and in liver samples.

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## 1. Introduction

*Jatropha curcas* L. is a plant suitable for the production of biofuels (biodiesel) without any evident bottlenecks in the technological-industrial aspects related to its production and use. This fact, coupled with sometimes over-emphasized resistance of the plant to biotic and abiotic stresses, has turned the crop in few years from marginal plant to a very promising raw material for biofuel production, as evidenced by the projects funded by public and private bodies, policy-makers and non-government organisations (NGOs) (Baldini et al., 2012). But most of large-scale biofuel projects have shown their uncertain economic sustainability without significant government subsidies, confirming that business expectations regarding Jatropha as a biofuel crop, are still too high (Ribeiro and Matavel, 2009; Friends of the Earth, 2010; Quintero et al., 2012).

In order to improve the economic sustainability of the Jatropha-biofuel chain, the development of high-yielding varieties and

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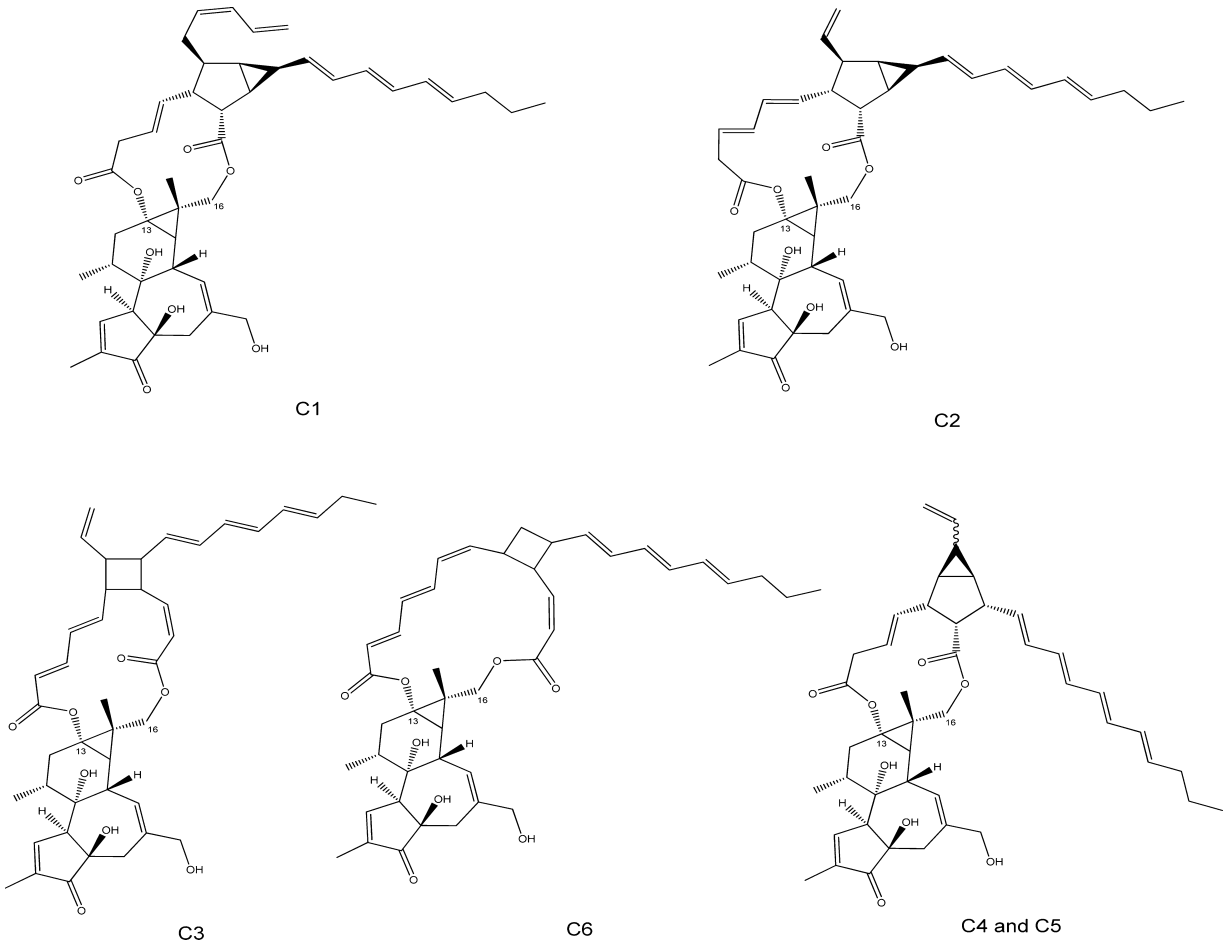


**Fig. 1.** Molecular structures of phorbol esters.

adapted to wasteland areas remains the main goal, but it may be not enough without a suitable exploitation of the seed cake, as this represents about 70% (w/w) of the processed seed and has a high nutritional value and a higher level of essential amino acids than the FAO reference protein for a growing child (Makkar and Becker, 2009). Unfortunately, its use as a source of protein for animals and humans (Makkar and Becker, 1997; Makkar et al., 1997; King et al., 2009) is prevented by the presence of important anti-nutritional factors and toxic compounds such as phorbol esters (PEs), which are very toxic for animals and humans (Abdu-Aguye et al., 1986; Castagna, 1987; Gandhi et al., 1995; Becker and Makkar, 1998). In fact, PEs exhibit biological activities such as tumour promotion, platelet aggregation, apoptosis, cell differentiation and other adverse metabolic effects (Goel et al., 2007). The characterization of PEs was carried out by Hass et al. (2002) who isolated six unstable intramolecular diterpene esters (named C1–C6 factors) from the seed oil, and all compounds possess the same diterpene moiety, namely, 12-deoxy-16-hydroxyphorbol (Fig. 1). To date, seed cake detoxification and utilization of 'non toxic' *J. curcas* accessions, present in some areas of Mexico, are the main activities pursued with the aim of using *J. curcas* seed cake in animal feed (Makkar et al., 1998a,b; Rakshit et al., 2008; Kumar et al., 2010; Wang et al., 2011). Many approaches have been proposed to detoxify the seed cake, either through removal of the PEs by solvent washing treatments or by biotransformation (by means of fungus/yeast) (Haas and Mittelbach, 2000; Aregheore et al., 2003; Makkar et al., 2009; Joshi et al., 2011; de Barros et al., 2011). Given the growing numbers of experiments aimed at studying the use of jatropha products in animal diets, interest in a robust and reliable method for PEs determination is increasing. As reported by many authors (Makkar et al., 2009; Devappa et al., 2013), High Performance Liquid Chromatography coupled with a UV detector (HPLC-UV, $\lambda = 280$ nm) is a well-established method to detect and quantify the PEs content in Jatropha seeds and related products (kernel, meal, seedcake and oil).

Today the need to determine the PEs content in other matrices such as the leaves of *J. curcas*, in which the PEs presence has always been deduced, but never proven, could be of great interest. This knowledge would facilitate the breeding activities aiming to transfer the non-toxic character into adapted and performing toxic accessions; in this case the time saved by analyzing the leaves of each segregating progeny instead of the seeds would be considerable. Other matrices of great interest might be livestock tissues or organs, designed for substance accumulation, permitting to assess whether an animal, even if fed with PEs, would be safe or not for human consumption.

Although the determination of PEs by HPLC-UV is currently applied to *J. curcas* L. seed and oil, its application to more complex matrices like leaves and animal tissues seems to be unsuitable due to the presence of interfering compounds which make the PEs identification and quantitation difficult. Also the use of time-consuming purification procedures seems not to be resolutive. These problems could be overcome by using a more selective technique such as HPLC coupled with Mass Spectrometry detection (LC–MS/MS) The LC–MS/MS methods have been already utilized to detect PEs and their derivatives in *Croton tiglium* seed oil by Vogg et al. (1999) and