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Isolation, stability and bioactivity of *Jatropha curcas* phorbol esters

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ABSTRACT

Jatropha curcas seed oil, which can be utilized for biodiesel production upon transesterification, is also rich in phorbol esters (PEs). In this study, PEs from *J. curcas* oil (*Jatropha* factors C_1 and C_2 (purified to homogeneity), *Jatropha* factors C_3 and ($C_4 + C_5$) (obtained as mixtures) and PE-rich extract (containing all the above stated *Jatropha* factors) were investigated. The concentrations of *Jatropha* PEs were expressed equivalent to *Jatropha* factor C_1. In the snail (*Physa fontinalis*) bioassay, the order of potency (EC_{50}, μg/L) was: PE-rich extract < factor C_3 mixture < factor C_2 < factor C_1 < factor ($C_4 + C_5$). In the *Artemia salina* bioassay, the order of potency (EC_{50}, mg/L) was: factor C_2 < factor C_3 mixture < factor C_1 < factor ($C_4 + C_5$) mixture. In addition, *Jatropha* PEs exhibited platelet aggregation (ED_{50}, μM, factor C_2 < factor C_3 mixture < factor C_1 < factor ($C_4 + C_5$) mixture. The stability of a PE-rich extract was evaluated and found to be low at room temperature but favourable in ethanol over a range of temperatures. By integrating the isolation methodology developed in this study in the *Jatropha* biodiesel industry, PEs could be obtained as value-added co-products.

1. Introduction

Jatropha curcas L. is a promising feedstock of oil for biodiesel production. *J. curcas* is one of approximately 175 species in its genus and a member of the Euphorbiaceae family [1]. It is a perennial shrub or small tree native to South and Central America but cultivated in many tropical regions, including Africa and Asia. It thrives in poor, stony soils and under adverse climatic conditions. *J. curcas* has a variety of uses. In many parts of the world it is used as a live fence and for erosion control. The seed kernel of the plant contains up to 60% oil [2], which can be transesterified to biodiesel. The oil is also incorporated in cosmetics and soap production. The seed kernel meal remaining after oil extraction is rich in nutrients and is used as an organic fertilizer [3,4]. Although high in protein the use of the defatted kernel meal as livestock feed is precluded by the presence of phorbol esters (PEs), which are the major toxic principles in *J. curcas* seeds [3].

Jatropha oil cannot be used for edible purposes without detoxification, making it attractive for biodiesel production. During mechanical extraction, the majority of PEs (~70%) present in the seed is extracted with the oil fraction [5]. During biodiesel production, the oil is subjected to many treatments (stripping, degumming and esterification), which leads to partial or complete destruction of the PEs [5]. Instead of losing the PEs, if a suitable method can be adopted to extract and isolate these esters before biodiesel production, the PEs could be valued co-products, which would contribute to enhancing economic viability and sustainability of the *Jatropha* oil-based biodiesel production chain. This is subject to the condition that the extraction of PEs from the oil does not adversely affect the quality of biodiesel produced.

Haas et al. [6] identified six *J. curcas* PEs, namely *Jatropha* factors C_1–C_6 (Fig. 1). The reason for choosing this group of compounds (PEs) for this study was that they are highly bioactive both *in vitro* and *in vivo*, but they are currently considered to be merely toxic, unwanted biomaterial in the *Jatropha* biodiesel production chain [7]. The recent increase in the cultivation of *Jatropha* means that there are potentially huge quantities of PEs that could be used for various applications. In this paper, we provide an optimized method for obtaining a *J. curcas* PE-rich extract and purified *Jatropha* PEs. In

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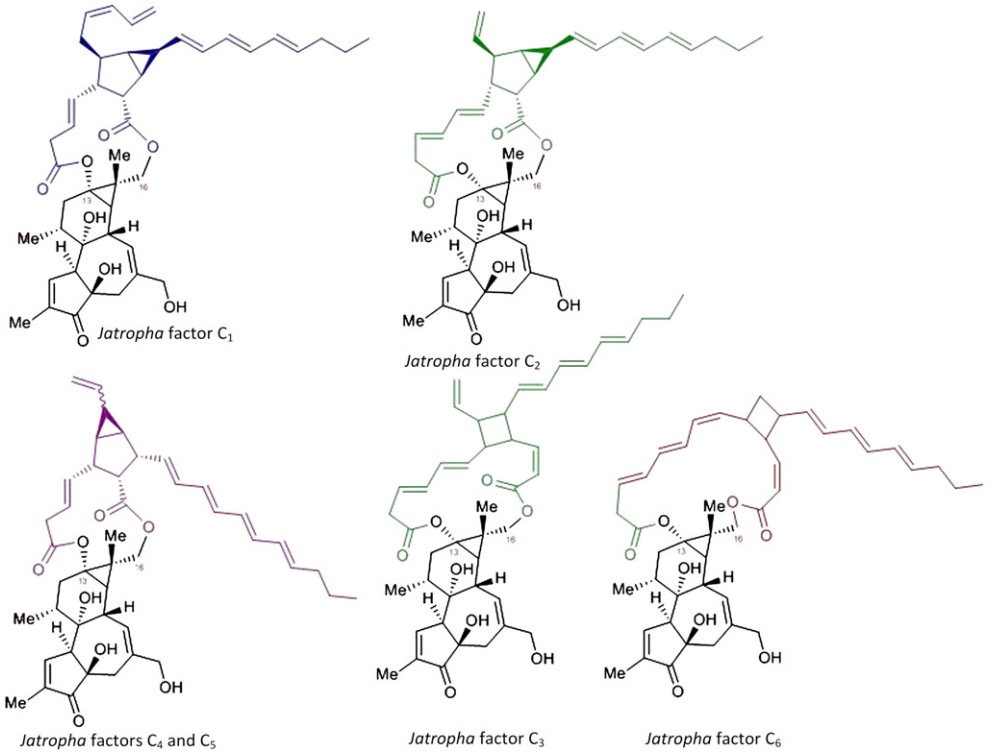

Fig. 1. Phorbol esters present in *Jatropha curcas* oil (Haas et al. [6]).

addition, the storage stability of the PE-rich extract and the biological activity of purified PEs are also presented.

2. Materials and methods

2.1. Processing of J. curcas oil

J. curcas seeds (Jaipur, India; Jaipur; geographical coordinates: 26°55′0″ N, 75°49′0″ E) were mechanically pressed and the resulting oil was centrifuged at 3150×*g* for 20 min to remove solid material. Clear oil was collected and stored at room temperature (23 °C) for further studies.

2.2. Phorbol ester-rich extract preparation and Jatropha factors C_1–C_5 isolation

Approximately 1 L of *J. curcas* seed oil (904.1 g) was extracted with methanol (MeOH, 750 ml) by stirring (300 rpm) in a 60 °C water bath for 15 min. The layers were allowed to separate then the MeOH layer was collected. The extraction process was repeated three times on the remaining oil. Combined MeOH extracts were concentrated *in vacuo* (60 °C) to yield a yellow oily residue (41.8 g). The crude MeOH extract was subjected to flash chromatography on 50 g of silica gel (40–63 μm, Merck), which had been preconditioned with dichloromethane (DCM). The column was eluted successively with DCM (500 ml), 1% DCM in MeOH (500 ml) then 5% DCM in MeOH (250 ml, collected in 50 ml fractions). Fractions containing PEs eluted with the 5% DCM in MeOH mixture and were concentrated *in vacuo* to yield a yellow PE-rich residue (0.26 g). The PEs were identified by a gradient elution high performance liquid chromatography (HPLC) method described by Makkar et al. [3,8]. Briefly, samples were injected on a reverse phase C_{18} Licrospher 100, 5 μm (250×4 mm) from Merck (Darmstadt, Germany), protected by a guard column of the same material. Starting with 60% solvent A (1.75 ml of 85% *o*-phosphoric acid in 1 L water and 40% solvent B (acetonitrile), B was increased to 50% over 10 min before increasing again to 75% over the next 30 min. From 30 to 45 min, B was increased to 100% before washing the column with solvent C (2% tetrahydrofuran in MeOH). Monitoring at 280 nm, four PEs peaks appeared between 25.5 and 30.5 min.

Additional oil was extracted, 1 L at a time, to yield a combined 2.0 g of the PE-rich product. This was subjected to semi-preparative HPLC on a reverse-phase C_{18}, 5 μm (250×10 mm) column from Phenomenex (California, USA). The column was eluted with 75% acetonitrile–water (0.01% trifluoroacetic acid) at a flow rate of 4 ml/min, monitored at 280 nm. Four prominent peaks were observed, which corresponded to *Jatropha* factors C_1 (18.3 min), C_2 (20.5 min), C_3 (23.4 min) and a C_4+C_5 mixture (25.2 min). Each of the four samples was subjected to open column chromatography on Sephadex LH-20 for purification. *Jatropha* factors C_1 and C_2 obtained were confirmed by 1D (^{1}H, ^{13}C) and 2D (HSQC, COSY, TOCSY, HMBC) NMR and comparison with data published by Haas et al. [6]. *Jatropha* factors C_3 and the factor (C_4+C_5) mixture needed further purification.

2.3. Bioassay for toxicity in Artemia salina

The assay was carried out by a slight modification of the method described by Kinghorn et al. [9]. The *A. salina* cysts