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Development of a sensitive *in vitro* assay to quantify the biological activity of pro-inflammatory phorbol esters in Jatropha oil.

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Running head: Assessment of Jatropha oil's bioactivity

Introduction

The use of biodiesel is promoted worldwide as it can contribute to a reduction of fossil fuel dependency and greenhouse gas emissions (Adler et al. 2007; Huo et al. 2009). Biodiesels are generally less toxic than fossil fuels (Poon et al. 2007; Poon et al. 2009), but potential human health hazards arising from their increasing use also need to be properly assessed, especially for biodiesels manufactured from non-edible or toxic feedstock (Poon et al. 2013).

Biodiesel production from *Jatropha curcas* seed oil may avoid competition with food crop and reduce pressure on arable land, as this shrub can withstand harsh conditions and poor soils otherwise unsuitable for agriculture (Brittaine and Lutaladio 2010; Makkar and Becker 2009). Numerous toxins including curcin, lectins, trypsin inhibitors, phytates and saponins are found in Jatropha seeds, but most of Jatropha oil's toxicity is attributed to the inflammatory and co-carcinogenic properties of phorbol esters (Devappa et al. 2010b; Furstenberger et al. 1981; Hirota et al. 1988; Makkar et al. 1998). So far, six different phorbol esters (named C1, C2, C3, C4, C5 and C6) have been characterized in Jatropha oil and their bioactivity assessed (Haas et al. 2002; Roach et al. 2012).

Total phorbol ester concentration in Jatropha oil generally varies from 2 to 4 mg/g. Although Jatropha oil refining and esterification in a small-scale laboratory setting appears to remove or degrade phorbol esters (Haas and Mittelbach 2000; Ichihashi et al.

Jatropha oil triggered the same characteristic alterations of MDCK cellular morphology observed following exposure to TPA (Fey and Penman 1984). We then confirmed that similarly to TPA, Jatropha phorbol esters' effects were mediated (at least in part) through activation of Protein Kinase C (Griner and Kazanietz 2007). Finally, we selected cyclooxygenase-2 (*COX-2*), a well-known, highly inducible gene involved in inflammation (Langenbach et al. 1999) to assess MDCK transcriptional response to Jatropha phorbol esters. This transcriptional response was then compared to a TPA dose-response curve and phorbol ester biological activity in Jatropha oil was expressed as TPA toxic equivalent (TEQ), a well-known approach (Van den Berg et al. 2006) that represents a convenient way to quantitatively report the pro-inflammatory potential of Jatropha oil.

Material and Methods

Material and reagents

MDCK (NBL-2, Catalog No. CCL-34) cell line was purchased from American Type Culture Collection (Manassas, VA, USA) in 2002, grown for a few passages and then cryopreserved in liquid nitrogen. Thawed cell aliquots used in this study were cultured for a maximum of 15 passages. Jatropha oil was obtained from Agroils (Firenza, Italy, 50129) whereas corn oil (Mazola brand, ACH Food Companies, Oakville, ON, Canada) was purchased from a local grocery store. Fatty acid profiles for these two oils

penicillin-streptomycin solution (Catalog No. 15140-122, Life Technologies, Burlington, ON, Canada) in a humidified incubator at 37EC and 5% CO_2. MDCK cells seeded at 70,000 cells/cm^2 were allowed to attach to the bottom of a petri dish and to grow for 24 hours. The cell culture media was then removed and the test substance added in fresh culture media. TPA was dissolved in dimethyl sulfoxide (DMSO) and added to culture media at 0, 0.003, 0.015, 0.075, 0.375, 1.875 and 9.375 nM, keeping final DMSO concentration constant at 0.1% (v/v). Jatropha and corn oils were emulsified directly in cell culture media by 5 ultrasonic bursts of 5 seconds, using an Ultrasonic Processor equipped with a microtip (Cole Parmer, Vernon Hills, IL, USA). Although lactate dehydrogenase assay (Roche Diagnostics, Laval, QC, Canada) suggested that MDCK cells can easily withstand up to 6 µl/ml oil exposures (data not shown), oil emulsions above 1.5 µl/ml proved unstable as an oily phase quickly reformed. In order to avoid such dispersion issue, oil exposure was limited to 1.5 µl/ml. At this exposure level, corn oil did not affect transcriptional response of MDCK cells to TPA (data not shown). MDCK cells were therefore exposed to 0, 0.0015, 0.015, 0.15 and 1.5 µl/ml Jatropha oil, keeping oil volume constant at 1.5 µl/ml cell culture media across control and treatment groups using corn oil. Cells were exposed to TPA or Jatropha oil for 24 hours. For the assessment of PKC activity, MDCK cells were harvested after two hours of exposure. For the qualitative assessment of cellular morphology, cells were seeded at a lower density (approximately 30,000 cells/cm^2), stained with Sigma-Aldrich's modified Giemsa stain according to the manufacturer's protocol and observed under light microscopy.

Immunoblot analysis

185 sample from all four treatment groups loaded in duplicate and each gel was also run in
186 duplicate. The intensity of each sample was normalized against the reference sample
187 present on the same gel and the four normalized values generated from each individual
188 sample were averaged.
189
190 *Gene expression analysis*
191
192 Total RNA was isolated and purified using RNeasy Kit (Qiagen, Toronto, ON,
193 Canada) according to the manufacturer's instructions. RNA quality and quantity were
194 determined using a 2100 Bioanalyzer (Agilent Technologies) and a Nanodrop 1000
195 spectrometer (Thermo Scientific, Waltham, MA, USA). Five micrograms of total RNA
196 were used for first strand cDNA synthesis, using Superscript III reverse transcriptase
197 (Life Technologies) according to the manufacturer's protocol. The resulting first-strand
198 cDNA was diluted 10 times to 200 µl to be used as templates for qPCR analyses. Given
199 the fact that exposure to phorbol esters alters cellular morphology (Fey and Penman
200 1984) and beta-actin expression (Gerstenfeld et al. 1985), hypoxanthine
201 phosphoribosyltransferase 1 (*HPRT1*) and glyceraldehyde 3-phosphate dehydrogenase
202 (*GAPDH*) were selected as housekeeping genes to monitor the relative expression of
203 *COX-2*. Information on the primers used for the amplification of those genes is provided
204 in Table 1. Quantitative PCR was performed with an iCycler iQ5 Real-Time Detection
205 System (Bio-Rad) using SYBR-Green I dye (Qiagen) in a reaction volume of 25 µl
206 containing 5 µl of the diluted cDNA synthesis reaction and a primer concentration of 0.4
207 µM. The RT-qPCR reaction mix was denatured at 95EC for 3 min. and then submitted to

231 Owing to their acute sensitivity, MDCK cells have often been used to study the
232 effects of TPA on cellular functions (Daniel et al. 1999). Figure 1 clearly shows that
233 direct exposure to Jatropha oil triggered the typical deformation of MDCK epithelial
234 polygonal geometry and the appearance of extensive neurite-like processes observed
235 following exposure to TPA (Fey and Penman 1984).
236
237 Based on the molecular mechanisms underlying the biological effects of TPA
238 (Fig. 2), we then assessed the phosphorylation state of Myristoylated Alanine-Rich C-
239 Kinase Substrate (MARCKS), a substrate and surrogate biomarker of Protein Kinase C
240 activity. MDCK cell exposure to 9.375 nM TPA resulted in a 3.5-fold increase of
241 phosphorylated MARCKS signal. A very similar increase was observed following
242 exposure to 1.5 µl Jatropha oil/ml, while exposure to 1.5 µl canola oil/ml had essentially
243 no effect (Fig. 3).
244
245 *COX-2* is among the first genes induced following exposure to TPA (Coyne et al.
246 1990; Sciorra and Daniel 1996). Significant induction of *COX-2* gene expression which
247 was observed following exposure to 0.375 nM TPA appeared to reach a plateau around
248 1.875 nM TPA (Fig. 4). Light microscopy observation of MDCK cells also confirmed
249 that the thresholds for *COX-2* gene induction and alteration of MDCK cellular
250 morphology were approximately similar.
251
252 Although MDCK cells can tolerate up to 6 µl/ml of vegetable oil (data not
253 shown), exposure to Jatropha oil was limited to 1.5 µl/ml in the concentration-response

277 (Devappa et al. 2010b). Consequently, we first confirmed that the acute sensitivity of
278 MDCK cells to TPA (Blumberg 1980; Ohuchi and Levine 1978) is also observed
279 following exposure to Jatropha oil phorbol esters.
280
281 Direct exposure of MDCK cells to Jatropha oil unequivocally triggered the typical
282 alteration of cellular morphology observed following exposure to TPA (Fig. 1). This
283 observation confirmed that MDCK cells can respond to the presence of Jatropha phorbol
284 esters presenting only a small fraction of TPA's activity (Beutler et al. 1989).
285 Incidentally, this experiment also demonstrated that direct exposure to vegetable oil did
286 not mute MDCK cellular response to phorbol esters. Although it is possible to directly
287 measure and quantify the alteration of cellular morphology resulting from exposure to
288 phorbol esters (Penman and Fey 1986), this approach is time-consuming, labour-
289 intensive, prone to artefacts and not easily amenable to high throughput applications.
290
291 The exceptional sensitivity of MDCK cells to TPA was described well before the
292 elucidation of the molecular mechanisms involved (Blumberg 1980; Nishizuka 1984;
293 Ohuchi and Levine 1978). As indicated in Figure 2, TPA directly binds and activates
294 Protein Kinase C (Griner and Kazanietz 2007). As a first step toward the development or
295 an *in vitro* bioassay, we confirmed that the effects of Jatropha oil are mediated at least in
296 part through PKC activation (Fig. 3). The very similar 3.5-fold increase in the phospho-
297 MARCKS signal observed following exposure to 9.375 nM TPA and to the much less
298 potent 1.5 µl/ml Jatropha oil (see Figures 4 and 5) suggests that activation of PKC had
299 already plateaued.

323 report claiming that the absorption at 280 nm of the most abundant phorbol ester in
324 Jatropha oil is about 40 times greater than TPA on a mass basis (Roach et al., 2012), our
325 results may underestimate the potency of Jatropha oil phorbol esters.
326
327 In spite of the relatively weak potency of phorbol esters present in Jatropha oil,
328 we were able to detect a significant induction of *COX-2* gene expression following direct
329 exposure of MDCK cells to 0.15 µl/ml Jatropha oil. Hence, in addition to routine testing,
330 the described bioassay may prove useful for many other applications such as selection of
331 *Jatropha curcas* strains presenting lower toxicity, assessment of phorbol ester extraction
332 or deactivation procedures, detection of Jatropha oil blended in other feedstock or
333 detection of pro-inflammatory phorbol esters from other sources. Although the ability to
334 quantify the biological activity of phorbol esters directly in vegetable oil without pre-
335 concentration significantly improves the convenience and throughput of this assay, pre-
336 fractionation and purification steps may also be added in order to measure more dilute
337 samples or assess phorbol ester biological activity in other matrices. Of course, the
338 development of a stable MDCK reporter cell line would further improve the convenience
339 and throughput of this bioassay, providing that the exceptional sensitivity of the parental
340 cell line can be retained.
341
342 **Conclusions**
343
344 With the increasing production and use of Jatropha oil and the development of
345 economically viable methods to detoxify the remaining protein-rich seed cake for animal

360 **References**

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Table 1: Primer sequences used for qRT-PCR amplification of the *COX-2*, *GAPDH* and *HPRT1* genes.

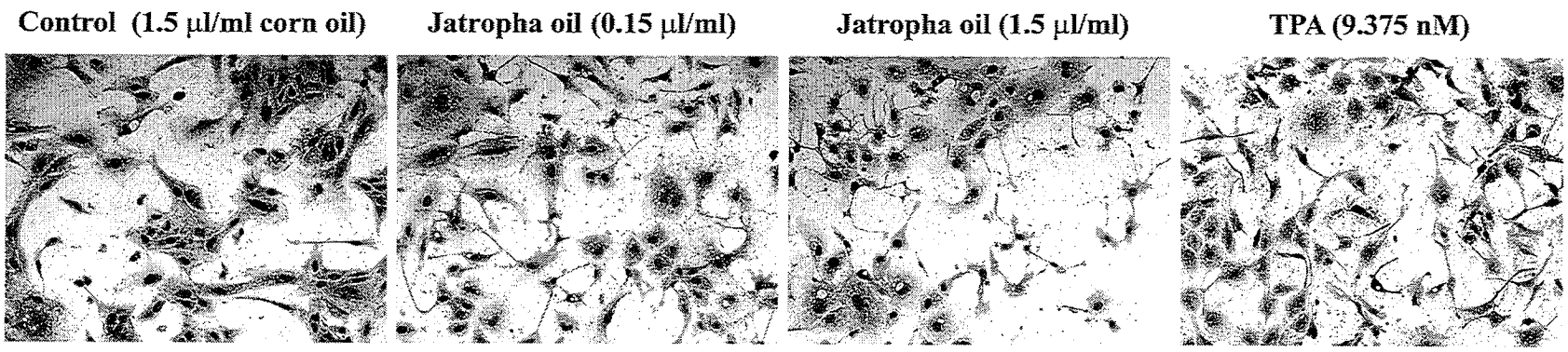
Gene	Accession	Primer sequence	Location	Amplicon size (bp)
PTGS2/ COX-2	NM_001003354	F-GTTCATTCCTGATCCCCAAG	Exon 6	186
		R-TTGAAAAGGCGCAGTTTATG	Exon 7	
HPRT1	NM_001003357	F-TGACACTGGGAAAACAATGCAGACT	Exon 6	110
		R-AGCCAACACTTCGAGGGGTCCT	Exon 7	
GAPDH	NM_001003142	F-AAGGTCATCCCTGAGCTGAA	Exon 7	192
		R-GACCACCTGGTCCTCAGTGT	Exon 9	

Figure legends:

Figure 1: Illustration of the effects of Jatropha oil on MDCK cellular morphology at 0.15 and 1.5 µl/ml and comparison with exposure to 9.375 nM TPA.

Figure 2: Simplified representation of the molecular pathways involved in the production of inflammatory prostaglandins following exposure to TPA. Dark gray arrows indicate mRNA and protein synthesis independent events, while the light gray arrow is dependent on *COX-2* gene transcription and protein synthesis.

Figure 3: Exposure to Jatropha oil activates Protein Kinase C *in vitro*. The relative phospho-MARCKS immunoblot signal of unexposed (control) MDCK cells is presented along with the signal from cells exposed to Canola oil, Jatropha oil (1.5 µl/ml) and TPA (9.375 nM). Errors bars represent standard deviation and * indicates statistically significant difference from control group values ($n = 6$, $p<0.05$). Inset: illustration of a typical immunoblot where the 87 kDa phospho-MARCKS signal (indicated by an arrow)


Control (1.5 µl/ml corn oil)
Jatropha oil (0.15 µl/ml)
Jatropha oil (1.5 µl/ml)
TPA (9.375 nM)