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BIOASSAY-ASSISTED IDENTIFICATION OF PHORBOL ESTER FROM JATROPHA CURCAS (LINN.) TISSUE CULTURE

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ABSTRACT

The study reported bio-assay assisted isolation of phorbol ester in optimized solid and liquid system tissue culture. The effect of the compound on human leukemic cell line (Jurkat J6) was conformed using MTT and SRB assays. The extract was analyzed using HPTLC in standard phorbol 12-myristate 13-acetate equivalent. Phorbol ester composition in seed oil was found to be 1.78 $\pm$ 0.34%. The composition from callus and suspension cultures sources was 0.145 $\pm$ 0.11% and 0.173 $\pm$ 0.15% respectively. The HPTLC profile of isolated PMA found to be Rf= 0.38 $\pm$ 0.02. The biological activity of phorbol ester was observed significantly proliferate the human leukemia cell line (Jurkat J6) even at 5µg/ml. This result provides the evidence that phorbol ester can be produced through plant cell *In vitro*. Thus, this bioassay-guided method to identify phorbol ester is reliable and reproducible for further bioprocess, biopharmaceutics and biotechnological studies.

KEWORDS

Bioactive molecules, *Jatropha curcas*, phytochemicals, Phorbol ester.

INTRODUCTION

The genus *Jatropha curcas* is one among many in Euphorbiace family with wide range of medical importance. The plant has multiple ethno-botanical values and flexible ecological requirements. In India, Africa and Latin America various parts of *J. curcas* have been used as a folk medicine. In recent years the plant becomes the major focus owing to its high non-edible oil source. The seed cake has also been reported as animal feeds for its high protein and essential amino acid contents [1-4]. However, phorbol esters remain the principle of the toxicity that averts utilization of such cake and the oil as food ingredients.

The phorbol esters are a family of naturally occurring diterpenes distributed in families Euphorbiaceae. They are highly toxic natural compounds, cathartic and skin irritant[5].

The ester groups of these compounds are recognized as being essential; besides, their undesirable features. Accidental ingestion of *Jatropha* seeds caused marked giddiness, vomiting, and diarrhea, nausea, gastro-intestinal irritation, abdominal pain in human [6]. Toxicity to sheep, goats, calves, chicks, and fish by consumption of *Jatropha* seed or seed meal has been reported [7, 8]. It has also the highest molluscicidal activity [9].Such undesirable features of this compound can also be used as pharmacological probe in cancer cell signal transduction process studies. It has strong affinity towards Protein kinase C (PKC) which is involved in several cell signaling pathways. Functionally

phorbol esters are analogues of diacylglycerol competing for the same binding site on PKC [10-12]. It is reversibly bounded to PKC in a similar fashion to DG-containing membranes via interactions at the membrane interface and act on proliferation of cell [13, 14]. Unlike DGs phorbol esters are metabolized very fast and able to persist long in the cell [15].

Phorbol esters are co-carcinogens which intensify cancer cell growth following exposure to subcarcinogenic dose of carcinogen [14, 16]. From these, Phorbol 12-myristate 13-acetate (PMA) induced protein kinase activation protects Jurkat cells from apoptosis [17]. Owing to its high pleiotropic effects on cells in culture; PMA has be used in immunology for activation of T-cells and polyclonal B-cell *in vitro*. Six phorbol esters have been characterized from the seed oil of *J. curcas* [18, 19]. Such toxic principle have been isolated from intact plant parts specifically seed oil for various studies. Till to date there is no report on isolation of PMA from plant cell cultures grown *In vitro*. Here we report bioassay- assisted isolation phorbol ester from *Jatropha curcas* tissue culture *In vitro*. This in vitro system may give an insight for further studies on biogenesis and production of this compound for various applications.

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MATERIALS AND METHODS

Plant material, callus and suspension culture initiation

Establishment of aseptic seedlings and callus induction: Seeds of *Jatropha curcas* L. was washed in running tap water for 10min to remove adherent particles and kept overnight immersed in distilled water (dH_2O). The husk was removed from the kernel using scissor and gently washed in dH_2O containing few drops of levermed and Tween20 for 5min. Thereafter, the seed was rinsed twice with dH_2O and ready for aseptic operation in a laminar hood. Therein, the seed was surface sterilized in 75% ethanol for 3-5mins and 4% sodium hypochlorite for 18-20min consecutively followed by three times dH_2O wash to remove all traces of sterilant. The sterilized seeds were transferred to petridish and blotted on sterile filter paper. The cotyledon leaf disc were excised and inoculated in a sterile MS media supplemented with 3% sucrose, 3mg/l BAP, 2 mg/l TDZ and 0.5mg/l IBA for germination. The 0.35% bacteriological agar, 0.12% gelrite (Gelrite® Gelan Gum, sigma) was used for solid phase cultures. The pH of the media was adjusted to 5.8±0.2 before autoclaving. After 15-days the first emerging leaflets were excised from in vitro seedlings and used as explants for callus induction in the aforementioned media and growth hormone composition and incubated in 16/8hrs photoperiods for one month. All chemicals were purchased from Hi Media, India unless and otherwise mentioned.

***Suspension culture*:** Cell suspension cultures were initiated from calluses grown in a solid agar media for 4weeks of same medium composition. The cultures were kept in orbital shaker maintained for 100rpm and 16/8 light/dark photoperiods. Henceforth, the stock suspension culture was subcultured after every10days to fresh medium in 1:1 ratio. The rest fifty percent of the culture was used for various analyses.

***Extraction and isolation*:** The *Jatropha* plant parts both the intact and the *In vitro* biomass was dried over night at 50℃ in an oven, ground, sieved and then taken for the study. Each time 50g powder were packed in soxhlet and defatted with petroleum ether (60-80℃) for 30-35 complete cycles. The petroleum ether extract was concentrated using Buchi-Rotavapor R-124 at reduces pressure and temperature. Thereafter the oil was suspended in methanol to separate methyl esters from glycerin. The pooled methyl esters were filtered and concentrated. The solid yellow oily product was collected for bioactivity tests and other analysis.

***Analytical method*:** Thin layer chromatography was performed on 20 cm × 10 cm high performance thin layer chromatography (HPTLC) silica gel $G60F_{254}$ plates. The plate was prewashed with methanol and activated 50^0C for 5min. Sample were applied at 15mm from the base of the plate using CAMAG Linomat 5 automated spray-on applicator. Plates were developed to a distance of 80mm with acetone: petroleum ether, 4:6 (v/v) mobile phases. Before development the chamber was saturated with mobile phase for 10mins. The chromogram was

obtained after densitometric scanning was performed with a Camag TLC scanner3 controlled under Camag winCATS planar chromatography software.

Bioassay and toxicity study

Cell culture preparation: The human leukemic cell line Jurkat Jb were prepared and maintained in Roswell park memorial institute medium (RPMI1640) supplemented with 10% fetal calf serum (FCS), genetamisin (100 IU/ml). All cultures were incubated at 37^0C in a humidified atmosphere with 10% CO_2. The stock cultures were sub-cultured every 3days. Every time 5-10X 10^5 cells/well was used for assay. This leukemic cell line was obtained from NCCS, Pune, India.

Tetrazolium (M TT) assay: We used a modified MTT assay to determine degree of cytotoxicity [21]. Cells were harvested and 100µl cell/wl was plated in microtitre plates in cell density between 5-10 X 10^5 cells/well. Experiments were performed in triplicate. After 24 hrs these cells attained exponential growth and 5-flouro uracil, plant extract, and 5% DMSO (2.5µl, 5µl, 7.5µl, 10µl, 12.5µl, 15µl) were added to the respective wells. 72 hrs after addition, 20 µl MTT in PBS to a final concentration of 0.5 mg/ml was added to the wells. Then after 8 hrs incubation at 37℃ the medium was centrifuged, flicked out and the formazan crystals formed were dissolved with 100 µl DMSO. The optical density (OD) was measured at 620nm using microplate reader (ELISA).

Sulforhodamine B (SRB) assay: A modified SRB assay was performed [22]. The method cell culturing conditions, cell density, treatments and maintenance was similar to the MTT assay. After 3days of incubation unlike MTT, the cells were fixed in 50 µl/w of 30% trichloroacetic acid at 4℃ for 1hr. Thereafter, the media flicked out followed by water wash, the cells were stained for 30min with 0.4% SRB. Then the stain were dissolved in 1% acetic acid 50µl /w and subsequently washed four times with 1% acetic acid to remove unbound stain. The plates were air-dried and bound protein stain was solubilised with 100µl/w of 10mM unbuffered Tris base. The OD was measured at 540 nm using microplate reader (ELISA).

Data analysis:The results were analyzed statistically using SPSS vers. 10 (SPSS Inc., Chicago, IL, USA). The mean comparison between values of the treatment was at 5% level of significance.

RESULTS AND DISCUSIONS

Phorbol ester extraction procedures:Isolation of phorbol esters from *Jatropha curcas* through tissue culture were carried out for the first time in our study. The cell mass cultivated in solid and liquid system was dried at 50^0C and extracted with petroleum ether by soxhlet. The extract was filtered and concentrated using rotary evaporator at a reduced temperature and pressure. Thereafter, the concentrate was subjected to methanol extract to obtain crude methyl esters where phorbol ester is the major component. Conventionally, methanol was used for extraction purposes from different part of the plant since it has high affinity towards phorbol ester [14, 19]. Hence, *In vitro* grown biomass was used for determination of phorbol ester in triplicates.

Phorbol ester composition in seed oil was found to be 1.78 $\pm$ 0.34%. However, the composition in oil from callus and suspension cultures sources was 0.145 $\pm$ 0.11% and 0.173 $\pm$ 0.15% respectively. The content of phorbol ester from *In vitro* grown sources is shown in table 1. The fractionation procedure for phorbol was carried out using petroleum ether: ethyl acetate (20:0→10:10) gradient elution on silica gel column chromatography (60-120 mesh size). Such fractions were conformed using thin layer chromatography in PMA equivalent.

The biological activity was monitored using leukemia cell line (Jurkat J6). The phorbol ester is a toxic biologically active element which can be used as a pharmacological probe for many immunological and pharmacological studies [23-26]. Such uses of plant biotechnology could be able to ensure bio-availability and sustainability of raw material for continuous production of such compound for cancer cell signal probing. The toxicity of the compound on non-target animals can be easily controlled; *In vitro* detoxification or *In vitro* selection of the genotype in such a system.

Table 1

Phorbol ester content in Jatropha plant material grown In vitro and intact seed

Source	% Oil content[a]	% crude phorbol ester in oil[a]
Seed	21±0.21	1.78 ± 0.34
Callus	9±0.16	0.145 ± 0.11
Suspension cells	8±0.23	0.173 ± 0.15

[a]Values shown are means ± standard deviation (%); *n* = 3.

HPTLC Analysis of phorbol esters: We used HPTLC for subsequent analysis owing to its simplicity and require short time to determine phorbol ester of different sources. The isolated phorbol esters were analyzed using such system on PMA equivalent. The analysis was carried out on silica gel TLC plate of 20cm X10cm size and mobile phase petroleum ether: acetone (6:4). The system profiles of phorbol esters extracted are shown in figure 1a and b. The crude of phorbol esters were observed between Rf= 0.36- 0.50 (Fig. 1b). The standard PMA peak was observed Rf= 0.38 ± 0.02 (Fig. 1a). In our study the Rf value of the fraction was also matched with the standard PMA. HPTLC method has been reported as a method of choice to standardize various herbal extracts containing biologically active compounds [27]. We used this method for the first time to determine phorbol ester from *Jatropha* tissue culture.

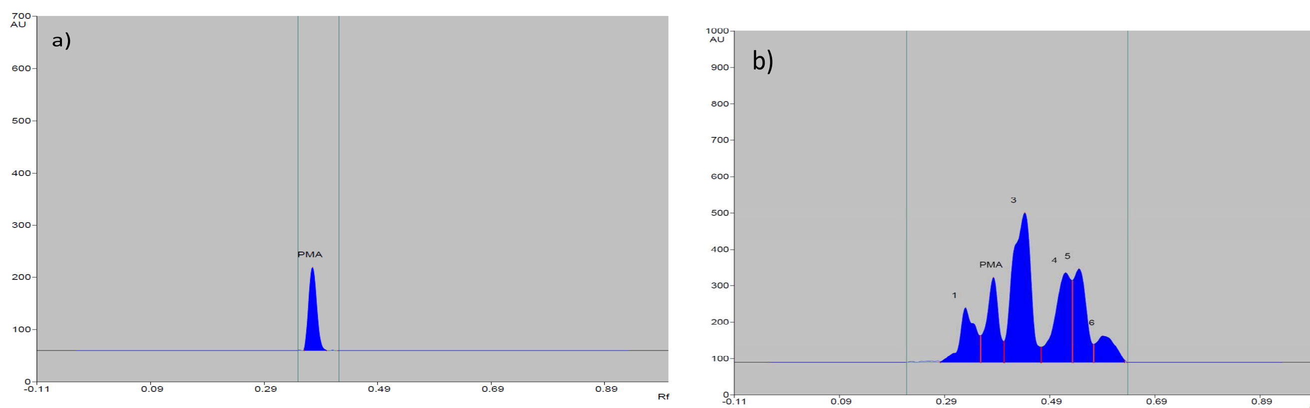

Fig 1. HPTLC profile of phorbol ester in PMA equivalent: a) pure PMA after column chromatography; b) crude phorbol esters

Chemo-sensitivity study using SRB: SRB and MTT assays were selected for rapid bioactivity test on leukemia cell lines. The OD of the cyto-toxicity and/or proliferative activity of compound were determined using microplate ELISA reader. The MTT assay showed the biological activity of PMA at 2.5 µl concentration and 168.09% cell proliferation percentage (Table 2). The SRB assay also confirmed that PMA is effective at 2.5 µl concentrations which was 5µg/ml of the final volume showed 146.71% cell survival percentage (Table 3). However, we also observed a decrease in percent of proliferation with increasing concentration of PMA. That may be due to DMSO concentration increase with increasing PMA which leads to inhibition. On the other hand the cells may enter to the stationary phase and produces toxic substance which limit the growth. All the results on the effect of phorbol ester were monitored using PMA standard equivalents. The readout of the MTT and SRB assay profile is shown in table 2 and 3. This further confirmed the isolated compound can have same biological activity which can be equivalent to the standard PMA. Hence the production of this compound for various applications in industries and pharmaceutics through plant biotechnology can

provide significant values over the conventional means. The regulatory effect of PMA on jurkat cell line to avert Fas-mediated apoptosis has been reported by several authors [25, 26]. This showed that the PMA is important in cancer cell signal probing studies and protection of immune cells from apoptosis.

Table 2.
Percentage proliferation of Jurkat Jb leukemic cell line using MTT assay

Concentration (µl)	DMSO[a*]	5FU[+]	PMA[b*]	PMA Proliferation%	PMA Inhibition%
2.5	0.95± 0.07	0.53 ±0.01	1.19 ± 0.07	168.09	-68.09
5	0.93±0.36	0.47 ±0.04	1.14 ±0.03	131.65	-31.65
7.5	0.92±0.13	0.45 ±0.02	1.20 ±0.09	160.03	-60.03
10	0.93±0.44	0.40 ±0.03	1.03 ±0.001	83.92	16.07
12.5	0.84±0.17	0.39 ±0.07	0.97 ±0.04	66.98	33.02
15	0.91±0.25	0.38 ±0.04	0.98 ±0.14	71.40	28.59

*Optical density: 620nm;**5 Flouro Uracil; *[+]Values shown are means ± standard deviation; n=3; [a] Dimethyl sulfoxide, [b]Phorbol 12-myristate 13-acetate

Table 3.
Percentage proliferation of Jurkat Jb leukemic cell line using SRB assay

Concentration (µl)	DMSO[a*]	5FU[+]	PMA[b*]	PMA proliferation %	PMA Inhibition%
2.5	0.61	0.40	0.85	146.71	-46.70
5	0.60	0.28	0.769	130.21	-30.21
7.5	0.57	0.22	0.654	106.79	-6.78
10	0.52	0.17	0.646	104.08	-4.08
12.5	0.49	0.15	0.73	117.62	-17.62
15	0.43	0.11	0.637	102.63	-2.63

*Optical density=540nm; [+]5 Flouro Uracil; *[+]Values shown are means; n=3; [b] Dimethyl sulfoxide, [c]Phorbol 12-myristate 13-acetate

In conclusion our study provides a proof to produce PMA using plant biotechnology for pharmaceutical activities. The determination of PMA from plant parts was easy and rapid using HPTLC. The biologically activity of PMA produced through tissue culture either in liquid or solid state was equivalent to the standard compound. However, different media components, various biological and physiochemical parameters were the major components to make a strong conclusion.

It can be considered from the present work that the significant associations of the culture conditions are vital points in laboratory and/or large scale production of PMA. The method established here can be helpful for further studies of bio-processing, pharmaceutics and biotechnology of *J. curcas* at cellular level for various agricultural/industrial applications.

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