4.1 Phorbol Esters of *J. curcas* - Biological Activities and Potential Applications

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Abstract

Toxicity of *Jatropha curcas* seeds can be caused by several components, including saponins, lectins (curcin), phytates, protease inhibitors, curcalonic acid, and phorbol esters. Phorbol esters which activate the important cellular target protein kinase C (PKC) constitute the most active components which must be removed if oil or seed cake is being used for animal or human nutrition. *Jatropha* oil and phorbol esters exhibit insecticidal and molluscicidal activities over a wide range of organisms, suggesting their potential use in agriculture as biorational pesticides and as mollusc control agents (against water snails which transmit parasites, such as schistosomes or flukes). Phorbol esters are known tumor promotors, but are not mutagenic or carcinogenic themselves. Before using them in agriculture or health control certain precautions and toxicological studies on the fate of phorbol esters in water, soil and plants are necessary to assess the potential environmental risks.

Introduction

Jatropha curcas L. (family Euphorbiaceae), a shrub of 3 to 8 m height, originates from Central America but is presently cultivated in Central and South America, West- and South Africa, India and South-East Asia. *J. curcas* is well adapted to marginal areas with poor soils and low rainfall and is resistant to diseases and herbivores. Thus it does not compete with conventional food or feed crops for land and water and can also used as a live hedge (not browsed by livestock) [1].

J. curcas produces relatively big seeds (mean weight 0.64 ± 0.10 g). The kernels contain up to 53 % oil (range 43 - 59 %), 26 % protein (range 19 - 31 %), 5 % neutral detergent fibres (range 3.5 - 6.1 %), and 4.2 % ash (range 3.4 - 5 %). Trypsin inhibitor activity in the degreased kernels (meal) varies from 18.4 - 27.5 mg trypsin inhibited/g, saponins from 1.8 - 3.4 % as diosgenin equivalent, phytate from 6.2 - 10.1 % as phytic acid equivalent, and lectin activity from 0.85 - 6.85 using a latex agglutination test and 51.3 - 204 using a hemagglutination assay [2]. The level of phorbolesters ranged from 0.87 to 3.32 mg/g kernel [2]. Tannins, amylase inhibitor, glucosinolates and cyanogens were not detected [2].

The oil can be used as petrol for diesel motors to drive tractors or pumps. Furthermore, the oil can be exploited for the production of soap or candles [1]. Since the oil is rich in unsaturated fatty acids (C16:0 = 15.3 %, C18:0 = 6.6 %, C18:1 = 40.1, C18:2 = 35.9 %, and C18:3 = 0.2 %) it might be useful for human nutrition (if the purgative curcalonic acid has been removed). But the oil also contains up to 1 - 2 % phorbol esters (Fig. 1) which make it unpalatable and toxic if the phorbol esters are not completely removed. The oilseed cake (left after extraction of oil) is presently exploited as a fertiliser but it has potential to be used as livestock feed as it is rich in crude protein (50 - 58 % depending on the residual oil) [1].

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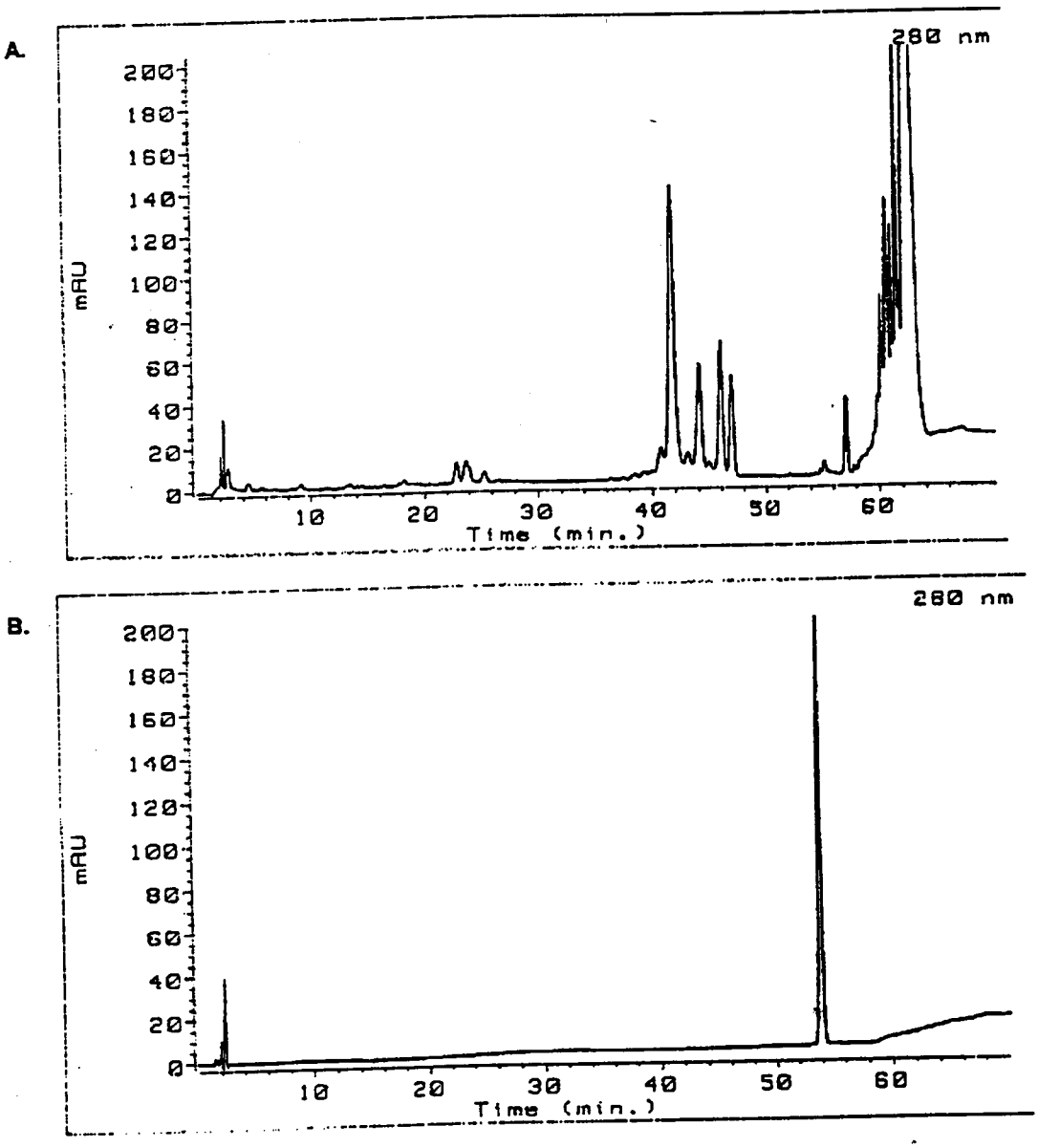

Fig. 1: Separation of phorbol esters from *J. curcas* oil by HPLC
A. HPLC profile from *Jatropha* oil; compounds which elute between 40 and 48 min are phorbol esters.
B. HPLC profile of a pure phorbol ester, phorbol-12-myristate 13-acetate (TPA), which is used as an external standard for quantification.

HPLC equipment: Hewlett Packard 1050 HPLC pump, Hewlett Packard 1040A photo diode array detector, and a Spark Holland-Basic Marathon autosampler.
Analytical column: Reversed phase C18 (LiChrospher 100, endcapped 5 µm), 250 x 4 mm I.D. (Lichrocart)
Solvents: (A) 1.75 ml o-phosphoric acid (85 %) in 1 liter distilled water, (B) acetonitrile and (C) tetrahydrofuran.
Gradient: Start with 60 % A and 40 % B; for 10 min decrease A to 50 % and increase B to 50 %; for the next 30 min a linear gradient to 25 % A and 75 % B; then increase of B to 100 % within the next 15 min. Then the column is washed with 100 % C and adjusted to the starting conditions with 60 % A and 40 % B. Separation is performed at room temperature (ca. 22°C) with a flow rate of 1.3 ml/min.

Results and Discussion

Toxicity of J. curcas

The seeds from *J. curcas* have been reported to be toxic to humans, rodents and livestock [1, 3]. Reports on the accidental intoxication of humans after ingestion of oil or seeds have appeared in Hawaii, Florida and the Philippines. The symptoms of intoxication in humans are: burning and pain in mouth and throat, vomiting, delirium, muscle shock, decrease of visual capacity and a high pulse [4]. A high mortality rate has been reported for rodents (mice, rats) and domestic animals (sheep, goats, zebu calves and chicks) when feeding *J. curcas* seeds [5 - 10]. 0.1 or 0.5 % seed meal in a diet for chicks caused growth depression, hepatonephropaties, hemorrhages and congestion [8]. When given to rats orally, oil exhibited an LD_{50} with 6 ml/kg body weight and an LD_{100} with 9 or 13 ml/kg body weight; symptoms were diarrhoea, gastro-intestinal inflammation, and hemorrhagic eyes [3].

In Nigeria and Burkina Faso seeds and seed oil are added to *Strophanthus* arrow poison. In Gabon seeds are grated with palm oil to kill rats [9]. The Shamba of the Usambara region of Tanzania used *Jatropha* seeds as an ordeal poison [3].

Toxicity of *J. curcas* seeds could be caused by several components, including saponins, lectins (curcin), phytates, protease inhibitors, curcalonic acid (which is a stronger purgative than ricinolic acid) and phorbol esters [2, 4, 9, 11, 12]. A variety from Mexico, which was non-toxic in feeding trials, still contained levels of saponins, lectins, protease inhibitors similar to those in toxic varieties, but was almost free from phorbol esters. Therefore, we have concluded that phorbol esters contribute predominantly to the toxicity of *Jatropha* seeds, seed cake and oil [2].

J. curcas has also been used in Traditional Medicine as a cathartic purgative, and to treat skin ailments, dropsy, gout, paralysis, and rheumatism [9]; often extracts from leaves and roots are preferred to those from seeds [9].

Biological activities of phorbol esters (PE)

Phorbolesters have been found to be responsible for skin-irritant effects and tumor promotion since they stimulate protein kinase C (PKC) [11, 12]. The natural substrate of PKC is diacylglycerate (DAG) whose structure is mimicked by phorbolesters. Since PKC is involved in signal transduction and developmental processes of most cells and tissues, a variety of biological effects should be expected over a wide range of organisms.

Insecticidal activities of oil containing phorbol esters or of concentrated phorbolester fractions have been recorded for *Manduca sexta* [13], *Helicoverpa armigera, Aphis gossypii, Pectinophora gossypiella, Empoasca biguttula, Callosobruchus chinensis, Sitophilus zeamays* [14], *Phthorimaea operculella* [15], *Culex* spec. (M. Wink, unpublished), *Sesamia calamistis, Busseola fusca* [16], *Periplaneta americana, Blatella germanica* and *Oncopeltus fasciatus* [17].

The effect of 0.1 % and 1 % oil on the survival of some insects is shown in Tab. 1 indicating that topical applications of phorbol ester containing oil have insecticidal properties over a wide range of insects. It is not a very strong activity, but it should be recalled that extracts and formulations had not been optimised for these trials.

Tab. 1: Insecticidal activity of phorbol ester containing oil of *Jatropha curcas* (in collaboration with laboratory Dr. Wachendorf; Bayer AG, Monheim)

Species	Mortality (% dead animals)			
	1 % oil		0.1 % oil	
	6 d	13 d	6 d	13 d
Phaedon cochliariae	33	50	n.d.	33
Plutella xylostella	33	60	n.d.	33
Spodoptera frugiperda	33	33	n.d.	n.d.
Mycus persicae	100	100	90	95
*Tetranychus urticae**	100	100	50	45

* this is a spider mite, not an insect

In larvae of the Tobacco horn moth (*Manduca sexta*), the effect of PE was studied in more detail: Larvae obtained an artificial diet to which different amounts of *Jatropha* oil or PE extracts were added. Larvae were weighted every day, so that the effect on the growth and development of the larvae could be compared with untreated controls. Fig. 2A shows that the addition of *Jatropha* oil at concentrations of 1 or 5 % to the diet inhibits the growth of the larvae which do not die immediately but stop growth and development. Methanolic extracts of *Jatropha* oil, which contain an enriched PE fraction show significant inhibitory effects (Fig. 2B) with concentrations higher than 250 ppm (= 0.025 %).

Substances were added to the artificial diet which was exchanged daily. 5 larvae were used for each treatment / concentration. In addition, phorbol ester containing oil and phorbol ester extracts exhibit a significant molluscidal activity against water snails, for example those which transmit parasites, such as *Biomphalaria glabrata*, and *Oncomelania hupensis* [18] thus confirming earlier reports [19]. Phorbol esters are probably responsible for this activity, since pure phorbol esters unambiguously showed this effect. When added to the water, PE (4ß-phorbol-13-decanoate) killed 100 % of the snails at concentrations of 0.001 % [18] and also the schistosomiasis causing parasite *Schistosoma mansoni* [20]. Molluscicidal activity against *Lymnaea auricularia* which transmits river fluke in the Philippines has also been recorded [21].

Potential applications of phorbol esters (PE)

Phorbol esters act as tumor promotors in mice which have been treated with a carcinogen beforehand but not in untreated animals. Therefore, PE are called co-carcinogens, although they are no carcinogens themselves (also the hormones present in the pill are co-carcinogens according to this definition).

Since PE or PE containing fractions might be used in agriculture as biorational pesticides [22] or to irradiate schistosomiasis transmitting snails, it is necessary to assess the risks of PE for the people who have to work with these compounds or for humans who come into contact with treated water or agricultural products. In the first instance precautions must be taken when handling the oil or PE containing fractions to avoid skin contact and ingestion because of skin irritance.

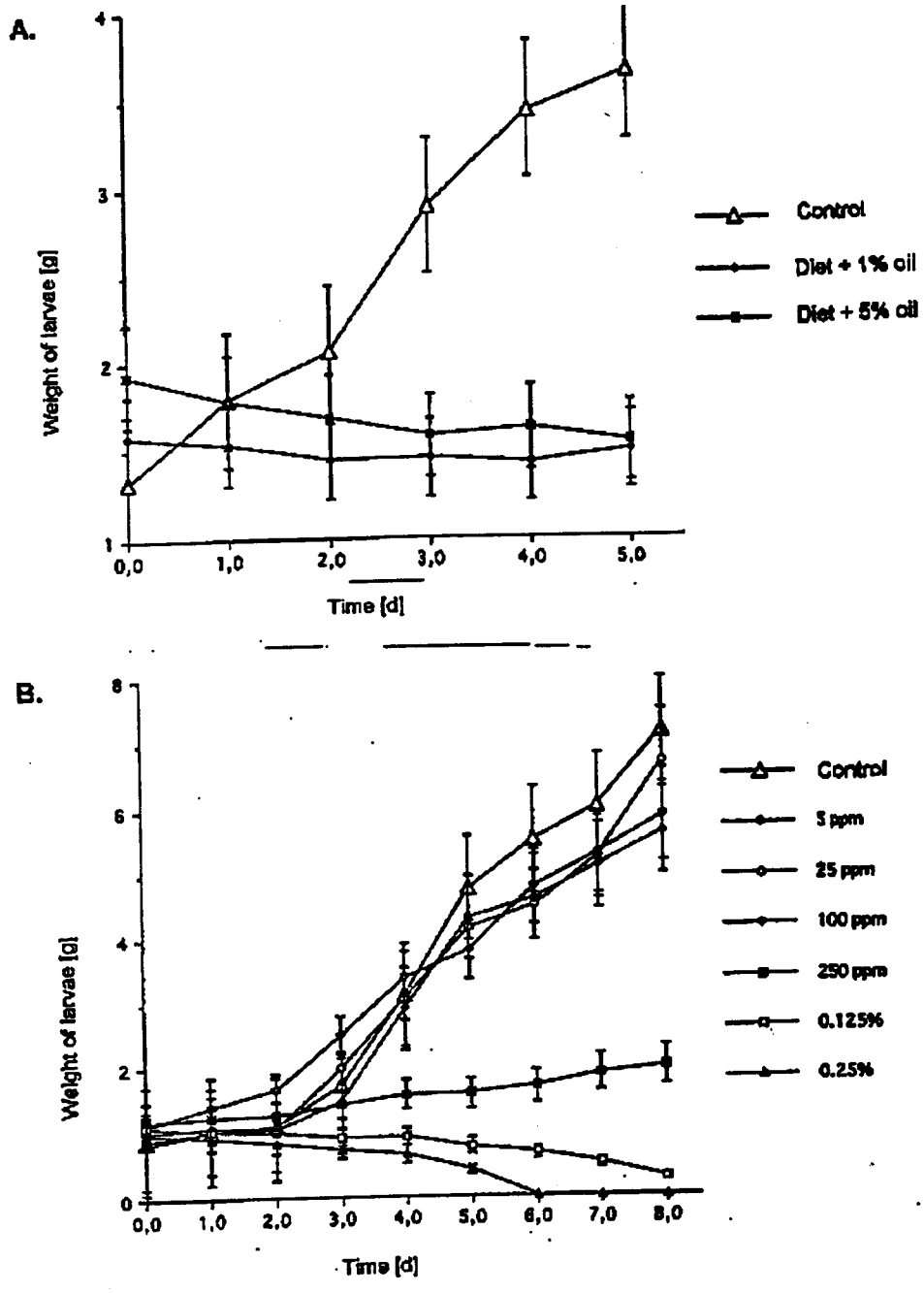

Fig. 2: Effect of *Jatropha* oil and PE fraction on the growth and development of *Manduca sexta* larvae
A. Effect of 1 or 5 % oil in the diet
B. Effect of different concentrations of the PE containing methanolic extracts from *J. curcas* oil

Secondly, any mutagenic or carcinogenic potential must be determined in detail. As a first measure into this direction, we have analysed whether *Jatropha* oil or isolated phorbolesters exhibit mutagenic effects in vertebrate cells. Human liver cells were selected as an assay system in order to obtain relevant data for human toxicology. It was assayed if single strand breakage occurs in the DNA of liver cells after treatment with *Jatropha* oil, phorbol ester extracts or a

positive mutagenic control $Na_2Cr_2O_7$. In a first set of experiments standard dilutions were chosen. Since no evidence for mutagenicity was found, higher concentrations were studied in a second set of experiments. Important is the last column which reflects the mutagenic potential: values between -10 and +10 are in the range of variation of this method. Values from 10 to 20 indicate that a very low mutagenic potential cannot be ruled out with certainty. Mutagenic compounds usually display values which are much higher than 20 even in very low concentrations (see our positive controls). In our experiments, only the PE fractions tested at high concentrations reached values around 20. Since the dosage which will be applied in agriculture or mollusc control will be several orders of magnitude lower than those used in these assays, the mutagenic risk should be very low if it exists at all. Besides DNA strand breakage, also the viability of the liver cells was analysed. As can be seen from Tab. 2 oil and PE result in a decrease of cell viability of maximally 27 % when the PE fraction was given in a 1 : 2 dilution. Further toxicological studies are needed to evaluate the fate of PE in plants, water, soil and the ecosystem.

PEs will be a side product of *Jatropha* oil production which might be exploited in tropical agriculture as a biorational pesticide or as a mollusc control agent. Since protein kinase C is such an important target, it is likely that PE could also be of use as therapeutic agents or as biochemical probes.

Tab. 2: Mutagenicity test for *Jatropha* oil and phorbol esters (in collaboration with P. Schmezer, DKFZ, Heidelberg)

Sample	concentration	vitality %	% DNA	Relative activity*
First experiment				
Control (C)	---	100	62	0
Mutagen	0.5 µmol	78	23	39
Oil	1:10	73	69	(-7)
	1:100	88	67	(-5)
	1:1000	88	69	(-7)
PE	1:10	75	47	15
	1:100	83	62	0
	1:1000	98	64	(-2)
Second experiment				
Control	---	100	82	0
Mutagen	0.5 µmol	77	53	29
Oil	1:2	73	74	8
	1:5	77	78	4
	1:10	97	71	11
PE	1:5	77	62	20
	1:10	92	61	21

*difference between control (C) and treatment

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