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Antifungal Activities of Ethanolic Extract from *Jatropha curcas* Seed Cake

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Phorbol ester extraction was carried out from *Jatropha curcas* seed cake, a by-product from the biodiesel fuel industry. Four repeated extractions from 5 g of *J. curcas* seed cake using 15 ml of 90% (v/v) ethanol and a shaking speed of 150 rpm gave the highest yield of phorbol esters. The ethanolic extract of *J. curcas* seed cake showed antifungal activities against important fungal phytopathogens: *Fusarium oxysporum*, *Pythium aphanidermatum*, *Lasiodiplodia theobromae*, *Curvularia lunata*, *Fusarium semitectum*, *Colletotrichum capsici*, and *Colletotrichum gloeosporioides*. The extract contained phorbol esters mainly responsible for antifungal activities. The extract could therefore be used as an antifungal agent for agricultural applications.

Keywords: Phorbol esters, *Jatropha curcas*, antifungal activity, seed cake

Jatropha curcas is an industrial crop that belongs to the family *Euphorbiaceae* and has a long history of cultivation in tropical America, Africa, and Asia [17]. Since its seed kernels contain a high amount of oil [58–60% (w/w)] [2], the seeds serve as a potential source of biodiesel fuel currently being used in India, Thailand, and other South East Asian countries. The seeds also contain high protein, antinutritional factors including trypsin inhibitor, lectin, saponin, and phytic acid, and toxic compounds called phorbol esters [24].

It has been known that parts of *J. curcas* can be used for a wide range of purposes. Extracts from various parts of *J. curcas*, such as seeds, seed oil, and leaves, have shown molluscicidal, insecticidal, and fungicidal properties [21, 25, 27, 30, 34]. *J. curcas* extracts were found to be able to inhibit the mycelial growth of *Colletotrichum musae* that causes anthracnose disease in bananas [35]. Its leaf extract was effective in controlling the fungal pathogen *Sclerotium* sp., which causes *Azolla* disease [11]. The chemicals responsible for those effects were suggested to be phorbol esters in the extract [14]. Goel *et al.* [12] also state that some derivatives of phorbol esters are known to have antimicrobial and antitumor properties, as well as molluscicidal and insecticidal effects.

Phorbol esters are diterpenes that contain 20 carbon atoms made up of four isoprene units [18]. They are generally found in plant species of the families *Euphorbiaceae* and *Thymelaeceae*. Recently, various forms of phorbol esters have been isolated from *J. curcas* aerial parts and seed oil [16, 26, 29]. Phorbol esters become toxic since they have biological effects that include skin inflammation, tumor promotion, tissue damage, activation of blood platelets, lymphocyte mitogenesis, prostaglandin production, and stimulation of degranulation in neutrophils in living cells [3, 12].

J. curcas seed cake is generated in considerable quantities as a by-product of *J. curcas* seed oil extraction. This by-product cannot be utilized owing to the presence of antinutritional factors and toxic compounds. These toxic compounds could be, however, isolated to be applied to agricultural applications. The compounds, especially phorbol esters, can be extracted from *J. curcas* seed by using methanol and dichloromethane as an extractant [23, 24]. Those solvents, however, are both harmful and relatively expensive. Ethanol is an organic solvent that is normally used for extractions from various plant parts, such as *Funtumia elastica* bark extract, *Mallotus oppositifolius* leave extract [1], *Casearia sylvestris* leave extract [32], and *Opuntia ficus-indica* stem extract [20]. It has never been, however, used for phorbol ester extraction from *J. curcas* parts. In addition, to our knowledge, the extract from *J. curcas* seed cake has not been studied for antifungal activities.

In this report, the exploitation of *J. curcas* seed cake for antifungal properties was investigated. We tested ethanolic extract of the seed cake containing phorbol esters to determine its natural antifungal properties against important fungal phytopathogens.

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Materials and Methods

J. curcas Seed Cake Samples

J. curcas seeds were obtained from four provinces of Thailand: Chiang Mai, Satun, Phitsanulok, and Phrae. They were ground and defatted by a screw press. The seed cake was stored in polyester plastic containers at -20°C prior to use.

Chemical Composition of *J. curcas* Seed Cake

J. curcas seed cake samples were analyzed for moisture, protein, fat, crude fiber, and ash by AOAC methods [7].

Phorbol Ester Extraction and Analysis

Phorbol esters in the samples were extracted and determined by the modified method of Hass and Mittelbach [15]. *J. curcas* seed cake samples (5 g) were ground by using a scientific blender (Waring, U.S.A.) and poured into flasks containing 20 ml of methanol (Fisher Scientific, U.K.). The mixture was stirred by using a shaker operated at 250 rpm for 5 min. It was then filtered using a Whatman No. 4 filter paper and a vacuum pump (Buchi, Switzerland). The residue on the filter paper and the extract were collected. This process was repeated and the residue was extracted four additional times. The extract fractions from all five extractions were combined and dried under vacuum at 40°C using a vacuum oven. The dried extract was dissolved in 5 ml of methanol and passed through a 0.2-μm membrane filter (ChroMex, U.K.). Twenty μl of extract solution was analyzed for phorbol esters by HPLC using an Agilent instrument 1100 (Agilent, U.S.A.). The analytical column used was a 150×3.9 mm ID, 4-μm particle size, Nova-Pak C18 (Waters, Ireland), with a SB-C18 guard column (12.5×4.6 mm ID), 5-μm particle size (Agilent, U.S.A.). The column was thermally controlled at 25°C. A mixture of acetonitrile (HPLC grade; Fisher Scientific, U.K.) and deionized water in the ratio of 80:20 (v:v) was used as the mobile phase at a flow rate of 1 ml/min. The detector wavelength was set at 254 nm. The results were expressed as equivalent to phorbol-12-myristate 13-acetate (PMA) (Sigma, U.K.) used as an external standard. PMA was dissolved in methanol (HPLC grade; Fisher Scientific, U.K.).

Study on Optimum Condition for Phorbol Ester Extraction

Five factors influencing phorbol ester extraction were studied at 25°C. They were extractant types and concentrations, extractant volume, number of extractions, extraction time, and shaking speed of extraction. An optimal condition for phorbol ester extraction giving the highest yield of phorbol esters from the seed cake was obtained from each factor.

Extractant type and concentrations. Methanol, ethanol, and dichloromethane (analytical grade; Fisher Scientific, U.K.) at various concentrations [50%, 70%, 90%, 95%, and 99.5% (v/v)], and distilled water, were used as extractants. The extraction was done according to the previous procedure. The extractant giving the highest phorbol ester yield (mg/g dry sample) was selected for the next experiment.

Extractant volume. Various volumes (15, 20, and 25 ml for each replication) of the selected extractant for phorbol ester extraction were investigated. The extraction was carried out as per the previous procedure. The volume of extractant that gave the highest content of phorbol esters (mg/g dry sample) was used for further study.

Number of Extractions

The number of phorbol ester extractions (1–5 replications) was investigated as previously described. The minimum replication of extraction providing the highest phorbol ester yield (mg/g dry sample) was selected for the next study.

Extraction time. Extraction times (5, 10, 15, and 30 min) for each replication were studied. The extraction procedure was carried out as per the previous procedure. The minimum time giving the highest content of phorbol esters (mg/g dry sample) was used for further study.

Shaking speed. Various shaking speeds (150, 200, 250, and 300 rpm) were tested for the best phorbol ester extraction with the procedure previously described. The minimum shaking speed that yielded the highest content of phorbol esters (mg/g dry sample) was selected.

The phorbol ester content of each treatment was statistically analyzed by one-way analysis of variance (ANOVA). The means were compared using the Duncan's multiple range test (DMRT) and were considered significantly different at $p \leq 0.05$ (SPSS Version 11.5; Chicago, IL, U.S.A.).

Test on Antifungal Activity of Crude Extract

Fungal isolates. Seven important fungal phytopathogens from the culture collection of the Plant Pathology Department, Kasetsart University, Bangkok, Thailand, were selected to be tested. They were *Fusarium oxysporum, F. semitectum, Colletotrichum capsici, C. gloeosporioides, Pythium aphanidermatum, Lasiodiplodia theobromae,* and *Curvularia lunata.* They were cultured on potato dextrose agar (PDA) plates at 25°C for 7 days prior to use.

Commercial fungicides. Five commercial fungicides (carbendazim, mancozeb, metalaxyl, imazalil, and prochloraz), kindly provided by Ladda Company (Thailand), were selected to test all phytopathogens as positive controls.

Preparation of crude extract from *J. curcas* seed cake. The crude extract was prepared using the optimum condition obtained above. It was dried using a vacuum oven at 40°C. The powder was dissolved in 95% (v/v) ethanol at various concentrations. The phorbol ester concentration in the crude extract was also determined by HPLC.

Effect of crude extract on fungal growth. Petri dishes containing sterile PDA cooled to 60°C were mixed well with various concentrations of the crude extract (0 to 10,000 mg/l). The agar was inoculated by a piece of each 7-day-old phytopathogen made with a 5-mm diameter cork borer and incubated at 25°C until it fully grew on a PDA plate. The medium mixed with 95% (v/v) ethanol was prepared as a negative control. For the positive controls, the commercial fungicides carbendazim mixed with mancozeb were used for *F. oxysporum, F. semitectum,* and *Curvularia lunata*; metalaxyl for *P. aphanidermatum*; imazalil for *L. theobromae*; and prochloraz for *C. capsici* and *C. gloeosporioides.* The diameters of fungal colonies on PDA plates were measured each day to determine the effective inhibitory concentration (EC_{50}). The experiments were done in triplicates for each crude extract concentration. Percentage inhibition of mycelial growth was calculated using the formula:

$$\% \text{ Inhibition} = \frac{(\text{Colony diameter of control} - \text{colony diameter of treatment})}{\text{Colony diameter of control}} \times 100$$

Table 1. Chemical composition of *J. curcas* seed cake samples (on dry matter basis).

Composition	Amount (%, w/w)
Dry solid	93.5–93.6
Protein	23.4–23.8
Fat	14.9–18.0
Crude fiber	10.0–10.9
Ash	7.2–8.4
Phorbol esters (mg/g dry sample)	0.21–0.47

RESULTS AND DISCUSSION

Chemical Composition and Phorbol Ester Contents in *J. curcas* Seed Cake

The chemical composition of *J. curcas* seed cake samples from the four provinces are shown in Table 1. The seed cake had high protein and fat contents. The protein and fat contents of seed cake in the present study were much different from the report of Martinez-Herrera [24] because of differences in sample preparation, especially an oil extraction method. However, other components, ash and crude fiber, were comparable to their report [24].

Phorbol esters were present in all *J. curcas* seed cakes. Their concentrations were in the range of 0.21–0.47 mg/g dry sample (Table 1). The content of phorbol esters varied in each sample, possibly caused by various amounts of residual oil left in the samples and variations in *J. curcas* due to differences in cultivation, soil, and climatic conditions, as reported by Makkar *et al.* [22]. The concentrations, however, were found to be much lower than those of unshelled seed cake from Zimbabwe (0.70 mg/g dry sample) [9] and Nicaragua (1.78 mg/g dry sample) [8]. In this study, the highest concentration of phorbol esters, at 0.47 mg/g dry sample, was found in the seed cake from Phrae. Thus, *J. curcas* seed cake from Phrae Province was chosen for the extraction studies.

Study on Optimum Conditions for Phorbol Ester Extraction

Extractant types and concentrations. Four types and concentrations of extractants were studied on extractions from 5-g seed cake, using 20 ml of each extractant, at a shaking speed of 250 rpm for 5 min, with 5 repeated extractions. The types and concentrations of extractants affected phorbol ester extraction, as shown in Table 2. Phorbol esters could not be extracted by distilled water. They were extracted by methanol, ethanol, and dichloromethane. However, 99.5% (v/v) methanol and 90% (v/v) ethanol showed much higher efficiencies of phorbol ester extraction than dichloromethane at all concentrations and methanol and ethanol at other concentrations. Generally, methanol was a good solvent for the extraction of bioactive compounds from plant parts. It was used for phorbol ester extraction from seeds, kernels, shells, and seed oil of *J. curcas* [15, 21, 24, 30]. Ninety percent (v/v) ethanol, however, was an alternative extractant used in this study and was selected for further studies because its extraction ability was similar to 99.5% (v/v) methanol. It is also safer for humans and animals. Importantly, this study is the first to demonstrate that 90% (v/v) ethanol is just as effective as methanol for phorbol ester extraction from *J. curcas* seed cake.

Extractant volume. Three volumes of 90% (v/v) ethanol were investigated on extractions from 5-g seed cake, at a shaking speed of 250 rpm for 5 min, with 5 repeated extractions. Results showed that differing volumes of ethanol had no effect on phorbol ester extraction from the seed cake. A range of 0.41–0.43 mg phorbol esters/g dry sample was extracted by 15–25 ml of 90% (v/v) ethanol. A minimum volume of 15 ml of 90% (v/v) ethanol was therefore selected for the next study.

Number of extraction. A number of repeated extractions using 15 ml of 90% (v/v) ethanol as a solvent on extractions from 5-g seed cake, at a shaking speed of 250 rpm for 5 min, were studied. Fig. 1 shows that 5 repeated extractions produced no significant difference in phorbol ester extraction from 4 repeated extractions and that both gave much

Table 2. Effect of types and concentrations of extractant on phorbol ester extraction from *J. curcas* seed cake (on dry matter basis).

Concentration (%, v/v)	Phorbol esters (mg phorbol esters[1]/g dry sample)*			
	Methanol	Ethanol	Dichloromethane	Distilled water
50	0.08^{a}±0.02	0.09^{a}±0.02	0.04^{a}±0.01	–
70	0.27^{b}±0.09	0.20^{b}±0.02	0.03^{a}±0.01	–
90	0.36^{b}±0.05	0.43^{d}±0.00	0.03^{a}±0.02	–
95	–	0.35^{c}±0.04	–	–
99.5	0.47^{c}±0.00	–	0.08^{b}±0.01	–
100	–	–	–	ND

*Mean±SD of triplicate analyses.
[1]Equivalent to phorbol-12-myristate-13-acetate.
ND: Not detected.
–: Not analyzed.
$^{a, b, c, d}$Mean values with different letters in the same column are significantly different ($p<0.05$).

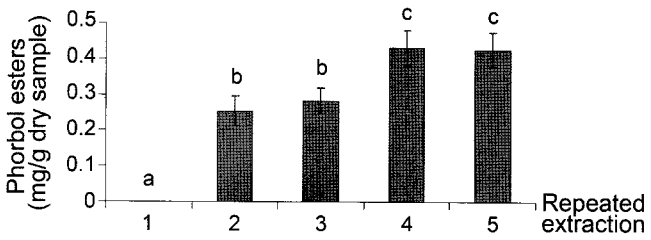

Fig. 1. Effect of number of extractions on phorbol ester extraction from *J. curcas* seed cake (on dry matter basis).
[a,b,c]Mean values with different letters are significantly different ($p<0.05$).

higher phorbol ester yields than fewer numbers of repeated extractions. Four repeated extractions were therefore selected for further study, as it was the minimum needed to obtain the highest yield of phorbol ester extraction.

Extraction time. Various extraction times were carried out for each extraction using 15 ml of 90% (v/v) ethanol as a solvent on extractions from 5-g seed cake, at a shaking speed of 250 rpm, and 4 repeated extractions. Results showed that 5–30 min/extractions had no significant difference on phorbol ester extraction, giving phorbol esters in the range of 0.43–0.45 mg /g dry sample. In this study, the minimum extraction time of 5 min/extraction was selected for the next study.

Shaking speed. Various shaking speeds (150–300 rpm) for each extraction were carried out using 15 ml of 90% (v/v) ethanol as a solvent on extractions from 5-g seed cake, for a 5-min extraction time, with 4 repeated extractions. The results showed that shaking speed had no significant difference on phorbol esters extraction with the phorbol ester contents of 0.43–0.44 mg/g dry sample. The minimum speed of 150 rpm was thus selected.

The optimal condition for phorbol ester extraction from 5 g of *J. curcas* seed cake was therefore obtained using 15 ml of 90% (v/v) ethanol, at a shaking speed of 150 rpm for 5 min, with 4 repeated extractions. These conditions were used to prepare crude extract for studies on antifungal activity.

Table 4. EC_{50} values (mg/l) of the crude extract and commercial fungicides.

Fungal isolates	EC_{50} values	
	Crude extract	Commercial fungicides
Fusarium oxysporum	2,800	37[a]
Pythium aphanidermatum	1,500	25[b]
Lasiodiplodia theobromae	950	7[c]
Curvularia lunata	1,000	56[a]
Fusarium semitectum	580	41[a]
Colletotrichum capsici	1,400	5[d]
Colletotrichum gloeosporioides	4,000	5[d]

[a]Carbendazim combined with mancozeb.
[b]Metalaxyl.
[c]Imazalil.
[d]Prochloraz.

Test on the Antifungal Activity of Crude Extract

Antifungal activities of crude extract on seven phytopathogens are shown in Table 3. The crude extract affected fungal growth of all species tested. *F. semitectum*, a species mainly responsible for crown rot of banana, and seed rot, collar rot, and root rot in legume crops, was found to be the most susceptible species to the extract, as indicated by the least EC_{50} value of 580 mg/l (Table 4). *P. aphanidermatum*, which causes damping off, root and stem rots, and blights of grasses and fruits, and *F. semitectum* were the species most sensitive to the extract, with complete inhibition of their mycelial growth at 3,000 mg/l (Table 3). They were also completely inhibited by 100 mg/l metalaxyl and 100 mg/l

Table 3. Effect of crude extract on *in vitro* mycelial growth of fungal phytopathogens.

Crude extract concentrations (mg/l)	Inhibition of mycelial growth (%)						
	Fusarium oxysporum	*Pythium aphanidermatum*	*Lasiodiplodia theobromae*	*Curvularia lunata*	*Fusarium semitectum*	*Colletotrichum capsici*	*Colletotrichum gloeosporioides*
0	0	0	0	0	0	0	0
500	12	4	38	34	48	26	4
1,000	26	6	51	50	61	48	10
2,000	29	90	71	61	70	53	15
3,000	53	100	82	71	100	61	31
4,000	66	100	88	86	100	100	51
5,000	74	100	91	100	100	100	70
6,000	100	100	100	100	100	100	80
7,000	100	100	100	100	100	100	87
10,000	100	100	100	100	100	100	100
Positive control	100[a]	100[b]	100[c]	100[d]	100[a]	100[e]	100[f]

[a]100 mg/l carbendazim combined with mancozeb. [b]100 mg/l metalaxyl.
[c]100 mg/l imazalil. [d]200 mg/l carbendazim combined with mancozeb.
[e]7 mg/l prochloraz. [f]50 mg/l prochloraz.

carbendazim combined with mancozeb, being used as commercial fungicidal agents. In contrast, *C. gloeosporioides*, which is responsible for anthracnose, dieback, root rot, leaf spot, blossom rot, and seedling blight on tropical fruit crops, was the species least affected by the crude extract, achieving complete inhibition of its mycelial growth at 10,000 mg/l or an EC_{50} value of 4,000 mg/l (Tables 3 and 4). There are, however, revealing reports on growth inhibition of *C. gloeosporioides* by natural plant extracts. Ogbebor *et al.* [28] found the extract of *J. curcas* leaves at 1,000 mg/l inhibited the mycelial growth of *C. gloeosporioides* by 50%, whereas *C. gloeosporioides* growth was reduced by 18% using 800 mg/l hexane extract of *Copaifera langsdorffii* leaves [5].

In addition, the mycelial growth of other fungal species, *F. oxysporum*, *L. theobromae*, *Curvularia lunata*, and *C. capsici*, was completely inhibited at 4,000–6,000 mg/l crude extract (Table 3). The results correlated to earlier reports of antifungal growth by natural plant extracts. Hexane extract from leaves of *Clerodendrum inerme* at 1,000 mg/l completely inhibited the growth of *Curvularia lunata*, but it could not inhibit *F. oxysporum* growth [6]. Methanol extract of *Azadirachta indica* (neem) oil at 1,000 mg/l reduced the growth of *Curvularia lunata* by 26% [13]. The extracts of *Ocimum sanctum* and *Azadirachta indica* inhibited the growth of *C. capsici* by 43% and 64%, respectively [33]. Shukla and Tripathi [31] reported that the oil of *Pimpinella anisum* at 1,000 mg/l exhibited total lethality on *C. capsici*, *Curvularia lunata*, *F. oxysporum*, and *F. semitectum*.

Antifungal and antimicrobial activities of extracts from parts of *Jatropha* species have been reported. Aiyelaagbe *et al.* [4] found moderate antifungal activity against *Candida albicans* by hexane, chloroform, and methanol extracts from roots of *Jatropha podagrica* at a concentration of 20,000 mg/l. Kumar *et al.* [19] reported that 500 mg/l crude extract from leaves of *Jatropha gossypifolia* L. completely inhibited eight microorganisms: *Bacillus cereus* var. *mycoides*, *B. pumilus*, *B. subtilis*, *Bordetella bronchiseptica*, *Staphylococcus epidermidis*, *Klebsiella pneumoniae*, *Streptococcus faecalis*, and *Candida albicans*.

This study is therefore the first to demonstrate that the extract from *J. curcas* seed cake has fungal activities against important fungal phytopathogens. Phorbol esters were found to be the main active components in the crude extract and were responsible for fungal growth inhibition as indicated by a HPLC chromatogram in Fig. 2. The chromatogram shows four major peaks of phorbol esters at retention times of 5.0–6.5 min, closely related to those reported by Hass and Mittelbach [15]. After removal of phorbol esters from the crude extract, the antifungal activity of the crude extract without phorbol esters was not significantly different from the negative control (data not shown). Our result is in agreement with a previous study that phorbol esters isolated from the fruits of *Sapium indicum*

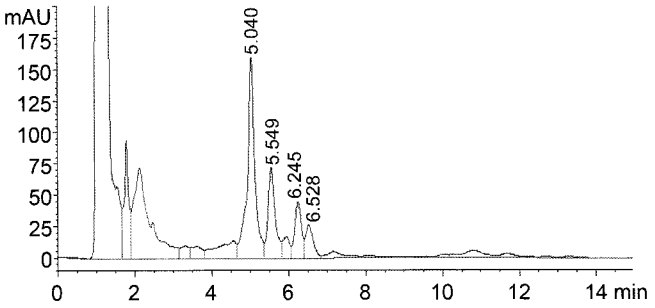

Fig. 2. HPLC chromatogram of the extract from *J. curcas* seed cake.

exhibited antimycobacterial activity against *Mycobacterium tuberculosis* [10]. In addition, the results showed the crude extract was less effective than all the commercial fungicides, as indicated by the EC_{50} values in Table 4. These may be because the crude extract still had impurities whereas each commercial fungicide used was in a pure form.

In conclusion, the extract of *J. curcus* seed cake would serve as a natural fungicide against fungal phytopathogens for agricultural applications at a low cost and safe practice. *J. curcas* seed cake, a by-product generated in large quantities by the biodiesel fuel industry, could thus be utilized as a source of the antifungal agent.

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