Original Article

Mahidol Univ J Pharm Sci 2015; 42 (3), 110-117

Hemostatic effect of n-hexane extracts of *Jatropha curcas* Linn leaf

P. Wongkrajang[1], Nurlely[2,7], S. Phornchirasilp[2], R. Temsiririrkkul[3], S. Thongpraditchote[4], N. Ruangwises[5], A. Mitrevej[6], R. Khammanit[2], R. Hanyongyuth[1].*

[1] Department of Clinical Pathology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, 10700 Thailand

[2] Department of Pharmacology,

[3] Department of Pharmaceutical Botany,

[4] Department of Physiology,

[5] Department of Pharmaceutical Chemistry,

[6] Department of Manufacturing, Faculty of Pharmacy, Mahidol University, Bangkok, 10400 Thailand

[7] Department of Pharmacy, Faculty of Mathematics and Natural Sciences, Lambung Mangkurat University, Banjarbaru, South Kalimantan, Indonesia

Abstract

The leaf extract of *Jatropha curcas* Linn has been widely investigated for its chemical and pharmacological properties. Methanolic extract and hexane extract of *Jatropha curcas* leaf has been demonstrated to exhibit antimicrobial, antiviral, antioxidant, anticancer, analgesic, anti-inflammatory, and wound healing properties. In this study, the hemostatic effect and the possible mechanism of *n*-hexane extract of *Jatropha curcas* Linn leaf was revealed through bleeding time, platelet function test, and coagulation test. It was found that *n*-hexane *Jatropha curcas* Linn leaf extract exhibited a significant decrease in bleeding time ($P<0.05$) Our study suggests the possible mechanism of *n*-hexane extract of *Jatropha curcus* Linn as an enhancer of platelet aggregation due to the activity of phorbol esters or phorbol ester derivatives. The safety and efficacy of this extract should be further investigated and verified prior to the development of tropical hemostasis products for inhibition of bleeding.

Keyword: *Jatropha curcas* Linn, hemostasis, bleeding time, coagulation study, platelet function test, phorbol esters

1. INTRODUCTION

Jatropha curcas (*J. curcas*) Linn (Euphorbiaceae) or "saboo dum" in Thai language is a multipurpose herbal plant that is commonly found in tropical areas, such as Central and South America, Africa, and South-East Asia. High quality oil from seeds of *J. curcas* is commonly used for biodiesel. However, toxic compounds, namely phorbol esters, were found in this plant, especially in the seed[1]. There have been many studies on the effect of *J. curcas* extract with polar and non-polar solvents. The studies on anti-inflammatory and analgesic properties were performed using aqueous extract of *J. curcas* root, stem bark, and leaf[2]. The leaf, most notably, has been used to treat many diseases effectively. The leaf of *J. curcas* has been reported to possess many chemical compounds, such as flavonoids, apigenin, vitexin, isovitexin sterols, and triterpenes[3]. Methanolic extract of *J. curcas* leaf has been demonstrated to exhibit antimicrobial, antiviral, antioxidant, anticancer, analgesic, anti-inflammatory, and

***Corresponding author:** Email address: Srichan.pho@mahidol.ac.th

wound healing properties[4-6], while hexane extract of *J. curcas* leaf has demonstrated only antimicrobial activity[7,8].

One of the traditional uses of *J. curcas* latex is for its hemostatic activity[9].
However, no scientific study of the hemostatic activity of leaf extract has been undertaken. As such, the objective of this study was to investigate the hemostatic effect and the possible mechanism of *n*-hexane extract of *J. curcas* Linn leaf.

2. MATERIALS AND METHODS

2.1. Materials

J. curcas Linn leaves were collected Kasetsart University, Kamphaeng Saen campus, Nakhon Pathom Province, Thailand. Male *ICR* mice (15-20 g) were purchased from National Laboratory Center, Mahidol University (Bangkok, Thailand). The reagents used in this study were *n*-hexane, dimetyl sulfoxide (DMSO), phorbol-12-myristate-13-acetate (PMA), adenosine diphosphate (ADP) (reagents purchased from Sigma Aldrich, Munich, Germany), Thromborel® S Reagent, and Dade® Actin® Activated PTT Reagent (reagents purchased from Siemens Healthcare Diagnostics, Marburg, Germany).

2.2. Plant materials

J. curcas Linn leaf was taxonomically authenticated by Assoc. Prof. Rungravi Temsiririrkkul. A voucher specimen was deposited at Department of Pharmaceutical Botany, Faculty of Pharmacy, Mahidol University, Thailand.

2.3. Preparation of plant extract

A Plant materials were washed, shade dried, and pulverized. The dried powder of leaves (1.5 kg) was percolated with *n*-hexane solvent and the filtrate was concentrated at 50°C by rotary vacuum evaporator. The filtrate was then evaporated in a fume hood to obtain the crude extract (37.97 g). The extract was then dissolved in 0.1% DMSO.

2.4. Experimental animals

Male *ICR* mice were acclimatized for one week before the experiment in a standard laboratory animal house at 25±2ºC with constant humidity (65%) and a 12 h light/dark cycle. The animals were fed a standard animal diet and water *ad libitum* during the experiment. The animal handling protocol was approved by the Animal Care and Use Committee, Faculty of Pharmacy, Mahidol University (No. PYT009/2555).

2.5. Experiments on healthy volunteers

This study was performed in the Central Laboratory of the Department of Clinical Pathology, Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand. The study protocol followed Declaration of Helsinki (Tokyo 1975) guidelines for humans and was approved by the Siriraj Institutional Review Board (EC 723/2554(EC2)). Written informed consent was obtained from all subjects.

Inclusion criteria were healthy men and women, 20-60 years old. Exclusion criteria were liver diseases, kidney diseases, hematologic diseases, or intake of any medications for at least 1 week before specimen blood was collected. Blood samples were collected from 40 adult volunteers by venipuncture from an antecubital vein into a one-tenth volume 0.105 M (3.2%) sodium citrate vacutainer tube.

Blood samples from 40 adult volunteers were used for coagulation studies; prothrombin time (PT), activated partial thromboplastin time (aPTT), and platelet aggregationassay.

2.6. Hemostatic activity

2.6.1. Bleeding time This activity was assessed by determining the bleeding time[10] with a slight modification that involved the use of 24 male ICR mice, divided into 4 groups of 6 mice each. Briefly, isoflurane inhalation was administered to anesthetize the animals before incising the paw. A disposable No. 11 surgical blade and artery forceps were used to create an incision, 1 cm long by 1 mm deep. Immediately, 10 µl of the tested extracts (5%, 7.5%, or 10% *n*-hexane extracts of *J. curcas* Linn leaf) or control group (0.1% DMSO) were dropped onto the wound, with a stopwatch being started

simultaneously. Filter paper was used to absorb the blood flowing out of the severed blood vessels until blood flow stopped. Bleeding time was measured at noon time to eliminate the possible effects circadian fluctuations. The percentage of bleeding time reduction in several concentrations of *n*-hexane extract of *J. curcas* Linn leaf were calculated using the following equation

$$\text{Percentage of bleeding time reduction (BT)} = \frac{\text{BT of control group-BT of treated group}}{\text{BT of control group}} \times 100$$

For additional hemostatic investigations, which included prothrombin time (PT), activated partial thromboplastin time (aPTT), and platelet aggregation assay, we selected only the most potent concentration of *n*-hexane extract of *J. curcas* Linn leaf.

2.6.2. Assay for prothrombin time (PT) and activated partial thromboplastin time (aPTT)

The method for the determination of PT was Quick method[11]. Plasma was obtained by centrifuging citrated blood for 15 minutes at 3500 rpm. Thromborel® S (Siemens Healthcare Diagnostics, Marburg, Germany) was then pre-warmed in a 37°C water bath for at least 10 minutes before commencement of the test. One hundred microliters of plasma was placed in a test tube and incubated in the water bath for 60 seconds. For the control, 10 microliters of pre-warmed 0.1% DMSO, followed by 200 microliters of the prewarmed Thromborel® S reagent was rapidly pipetted into the plasma while simultaneously starting a timer. The tube was then gently tilted back and forth until a clot formed, at which time the timer was stopped and the clotting time was recorded. For the tests, 10 microliters of pre-warmed sample solution (*n*-hexane extracts of *J. curcas* Linn leaf) was mixed with the plasma, just before adding 200 microliters of Thromborel® S reagent.

For the determination of aPTT, the Langdell method[12] was used. Dade® Actin® FS Activated PTT Reagent, which is the partial thromboplastin with activator (purified soy phosphatides and ellagic acid) and calcium chloride (0.02 M) were separately pre-warmed to 37 °C in a water bath. One hundred microliters of plasma was placed in a test tube. For the control, 10 microliters of pre-warmed 0.1% DMSO followed by 100 microliters of Dade® Actin® FS Activated PTT Reagent were combined and mixed rapidly. For the tests, 10 microliters of pre-warmed sample solution (*n*-hexane extracts of *J. curcas* Linn leaf) followed by 100 microliters of Dade® Actin® FS Activated PTT Reagent were combined and mixed rapidly. The mixture was then incubated for 3 minutes in the water bath, after which 100 microliters of pre-warmed calcium chloride solution was added while simultaneously starting a timer. The test tube remained in the water bath where it was gently tilted back and forth until a clot was observed and the time was recorded.

2.5.3. Assay for platelet aggregation assay

Platelet aggregation was assessed by light transmission aggregometry according to a standard protocol[13]. Platelet-rich plasma (PRP) was obtained by centrifuging citrated blood at 1000 x g for 15 minutes. The PRP was further centrifuged at 3000 x g for 15 minutes and the supernatant was collected as platelet-poor plasma (PPP). The PRP was adjusted to a concentration of approximately 3×10^8 platelets/ml by adding PPP, as needed. Platelet aggregation was assessed at 37 °C using an AggRAM aggregometer (Helena Laboratories Corp., Beaumont, TX, USA). Light transmission was adjusted to 0% for PRP and 100% for PPP for each measurement. The platelet function was measured after adding the agonist and curves were recorded for 7 minutes. Platelet aggregation was then measured. Aggregation was initiated by adding 50 microliters of agonists to the PRP, which are *n*-hexane extracts of *J. curcas* Linn leaf, 1% phorbol-12-myristate-13-acetate (PMA) (Sigma Aldrich, Munich, Germany), and ADP (Sigma Aldrich, Munich, Germany) at a concentration of 5 micromole/L. DMSO 0.1% was used as a control. After the addition of agonists, kinetic reading of the plate was started immediately and percent transmission was noted.

2.8. Phorbol ester determination by high performance liquid chromatography (HPLC)

Phorbol ester analysis in the samples was performed by the modified method of Donlaporn and Suntornsuk[14]. The number of phorbol ester extractions (1-5 replications) was investigated. *J. curcas* Linn leaf extract (0.5 g) was poured into a flask containing 20 ml of methanol (Fisher Scientific, Loughborough, U.K.). The mixture was stirred using a magnetic stirrer for 5 minutes. The mixture was then further centrifuged at 4000 g for 5 minutes and the supernatant (methanol) was collected and dried under vacuum at 40°C using a vacuum oven. The dried extract was dissolved in 10 ml of methanol and passed through a 0.22 µm membrane filter (ChroMex, Derbyshire, U.K.). Twenty microliters of extract solution was analyzed for phorbol esters by HPLC using a BDS Hypersil C18 column (250×4.6 mm, ID 5 µm) (Thermo Scientific, Waltham, MA, USA). The column was thermally controlled at room temperature. A mixture of acetonitrile (HPLC grade) (Fisher Scientific, Waltham, MA, USA) and deionized water at a ratio of 95:5 (v:v) was used for the mobile phase at a flow rate of 1 ml/min. The detection wavelength was set at 280 nm. The results were expressed as equivalent to phorbol-12-myristate-13-acetate (PMA), which was used as an external standard. The PMA was dissolved in methanol (HPLC grade) (Fisher Scientific, Waltham, MA, USA).

2.9. Statistical analysis

Statistical analyses were performed using PASW Statistics for Windows, Version 18.0. (SPSS Inc., Chicago, USA). All data are presented as mean ± standard error of mean (SEM). Control tubes and sample tubes were compared to standard specimens which contained no solution using Student's *t-test*. *P*-value < 0.05 was considered statistically significant.

3. RESULTS

3.1. Bleeding time

The bleeding time of all extract-treated groups reduced significantly in comparison with the vehicle-treated group ($p<0.05$). The percentage of bleeding time reduction at concentrations of 5, 7.5, and 10% *n*-hexane extract of *J. curcas* Linn leaf were 25.13, 40.36, and 53.56, respectively. *J. curcas* Linn leaf extract demonstrated hemostatic activity in a dose-dependent manner, as illustrated in Table 1.

Table 1. Bleeding time and the percentage of bleeding time reduction in all groups

Group	N	Bleeding time (min)	% Bleeding time reduction
Control	6	4.720 ± 0.224	-
5% n-hexane extract of J. curcas Linn leaf	6	3.534 ± 0.381*	25.13
7.5% n-hexane extract of J. curcas Linn leaf	4	2.815 ± 0.257*#	40.36
10% n-hexane extract of J. curcas Linn leaf	5	2.192 ± 0.264*#	53.56

*$p<0.05$ significant difference for all groups compared with control group
#$p<0.05$ significant difference for 7.5% and 10% extract groups compared with 5% extract group

3.2. Prothrombin time (PT) and activated partial thromboplastin time (APTT)

Given that 10% *n*-hexane extract of *J. curcas* Linn leaf demonstrated the most potency for reducing bleeding time, we chose this concentration to evaluate prothrombin time (PT) and activated partial thromboplastin time (APTT). As described in Table 2, 10% *n*-hexane extract of *J. curcas* Linn leaf showed statistical significance ($p<0.05$) for both PT and APTT, when compared to control specimen

3.3. Platelet aggregation assay

Percent transmission of platelet aggrega tion is described in Table 3 and Figure 1. Ten microliters of 10% *n*-hexane extract of *J. curcas* Linn leaf induced platelet aggregation as shown by the increase in percent transmission. The noted characteristic for platelet aggregation was the same as the pattern that was induced by phorbol-12-myristate-13-acetate.

Table 2. Effect of 10% n-hexane extract of *J. curcas* Linn leaf on PT and APTT

Solution	APTT (seconds)	PT (seconds)
Control	42 + 2.45	14.72 + 0.44
10% *n*-hexane extract of *J. curcas* leaf	40.32 + 2.55*	15.32 + 0.73*

*$p<0.05$

Table 3. Effect of 10% n-hexane extract of *J. curcas* Linn leaf on platelet aggregation assay

Solution	% Transmission
Adenosine diphosphate (ADP)	90 + 2.5
1% phorbol-12-myristate-13-acetate (PMA)	65.625 + 14.76
10% *n*-hexane extract of *J. curcas* Linn leaf	41.28 + 9.36
Control (0.1% DMSO)	2.24 + 1.3

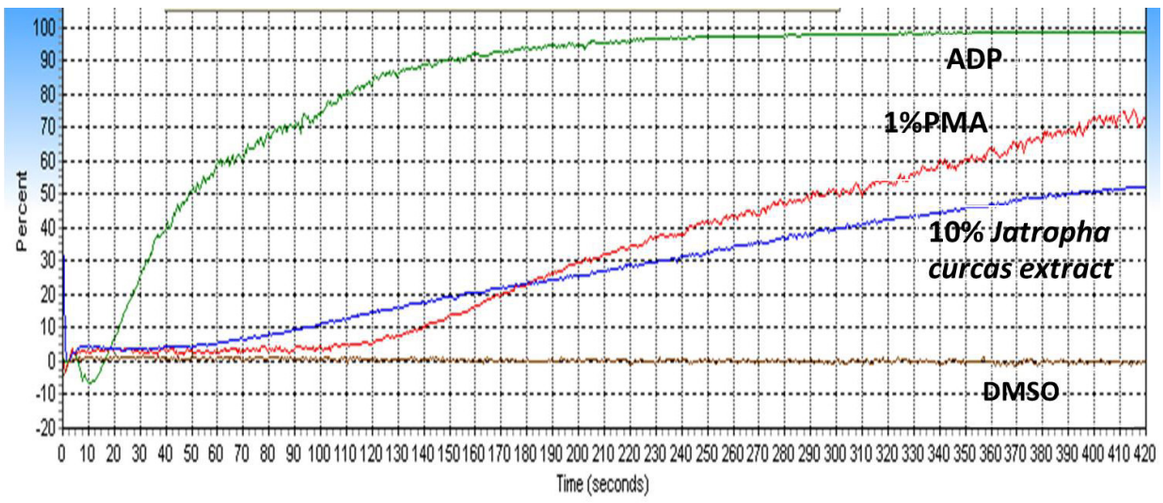

Figure 1. Effect of 10% *n*-hexane extract of *J. curcas* Linn leaf on platelet aggregation.

3.4. Phorbol ester determination by high performance liquid chromatography (HPLC)

HPLC chromatogram (Figure 2) showed four major peaks phorbol esters presenting at 5.0-6.5 min, closely related to those reported by Donlaporn and Suntornsuk[14].

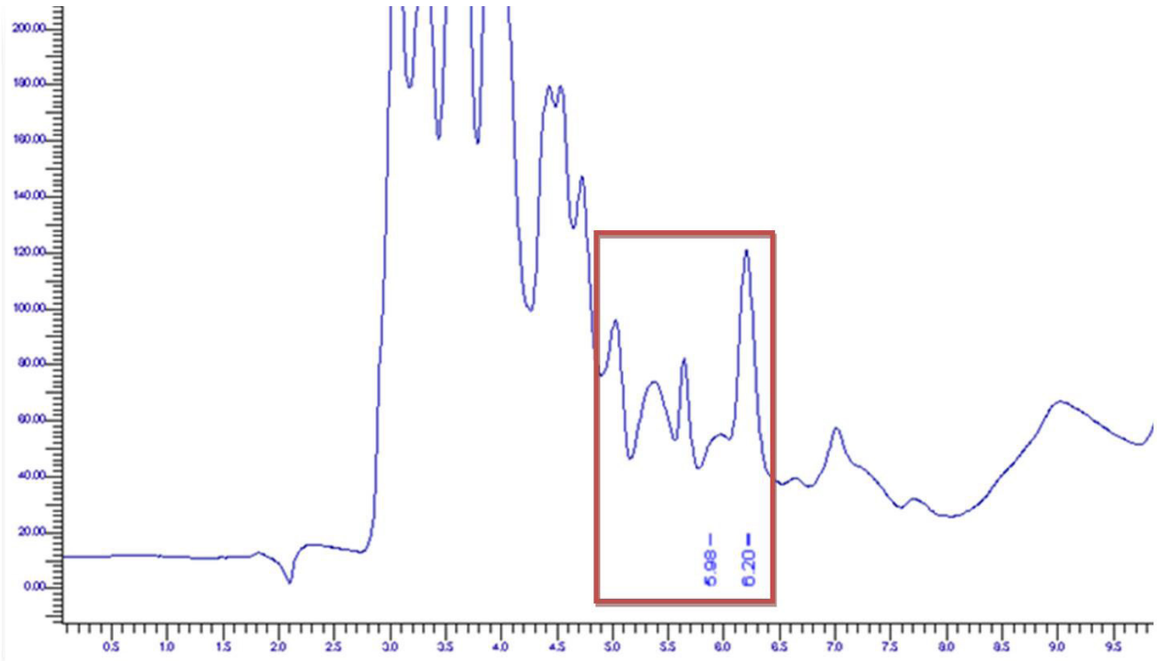

Figure 2. HPLC chromatogram of 10% *n*-hexane extract of *J. curcas* Linn leaf.

4. DISCUSSION

In this study, the hemostatic effect of *n*-hexane leaf extract of *J. curcas* Linn leaf was tested by measuring bleeding time, coagulation, and platelet aggregation. Our results revealed that reduction of bleeding time increased proportionally with the increase in extract concentration. Ten percent *n*-hexane extract demonstrated the highest level of activity, as shown in Table 1. We therefore selected this extract concentration for the coagulation study and the platelet aggregation assay. It was found that 10% *n*-hexane extract of *J. curcas* Linn leaf had only statistically significant effect on coagulation but no effect on clinical significant effect due to the difference value results of PT and APTT from 10% n-hexane extract of J. curcas Linn leaf and control were 4.1% and 4%, respectively which were less than allowable total error (TEa) of PT and APTT. TEa is the combination of imprecision and bias of a single test measurement thus, it fits the desired form of a tolerance limit. Based on the Clinical Laboratory Improvement Amendments (CLIA) regulations TEa of PT and APTT are within 15%[15]. But it had the effect of activating platelet aggregation. The activation of platelet aggregation may result from phorbol esters or phorbol ester derivatives in *J. curcas* Linn leaf extract, which are the main active components in the *n*-hexane extract, as indicated by the HPLC chromatogram results described in Figure 2. Furthermore, the pattern of platelet aggregation activity induced by phorbol-12-myristate-13-acetate (PMA) in our experiment showed the same pattern was the *n*-hexane extract, but the potency of the *n*-hexane extract was lower. Therefore, the mechanism of hemostatic activity of *n*-hexane extract of *J. curcas* Linn leaf may be the activation of platelet aggregation.

This is the first study that reveals the presence of phorbol ester in *n*-hexane extract of *J. curcas* Linn leaf by HPLC analysis. In previous study, *J. curcas* leaf extract from hexane revealed the presence of beta sitosterol[8], trans-phytol, squalene, phytol and nonadecanone as major components from GC-MS analysis[16]. Phorbol esters was found only in the kernel seed extract from hexane by HPLC analysis[8]. The phorbol ester that we found in our study may be due to the difference specie or variety of *J. curcas* from other studies.

Phorbol esters are characterized by many biological activities, such as tumor promotion, platelet aggregation, and apoptosis[17]. Phorbol esters can induce platelet aggregation and secretion by stimulation of Protein Kinase C (PKC)[18]. PKC plays an essential role in platelet aggregation and adhesion, to include actin rearrangements, adhesion through integrins, and secretion of granule contents[19]. The pattern of platelet aggregation induced by ADP was shown to be different from PMA, because ADP-induced shape change of platelets leads to aggregation[20].

Phorbol esters can interact with PKC, leading to activities that affect various enzymes, biosynthesis of proteins, cell differentiation processes, and gene expression[17]. There have been many studies regarding the toxicity of phorbol esters in the seed of *J. curcas* in different animal models, such as rats and mice. Multiple organ toxicity in animals was observed, including gastrointestinal disturbance, hemolytic activity, skin irritation and necrosis, and tumor-promoting activity[21, 22]. By way of example, topical application in guinea pigs was shown to induce inflammation and epidermal hyper-proliferation by inducing DNA synthesis through prostaglandin activation, especially prostaglandin E [23]. Therefore, mutagenic propensities of this extract should be investigated.

However from our previous study found that *n*-hexane extract of *J. curcus* leaf at the concentration up to 10% w/v had local anesthetic in *Hartley* guinea pigs , with no irritation in *Sprague-Dawley* rats and cytotoxicity in fibroblast cell in L929 cell line[24].

In addition, at this point, we could not confirm that phorbol esters have mainly responsible for hemostatic activities. Further experiments of the extract with phorbol ester removal should be carried out to proof this speculation.

5. CONCLUSION

Our study suggests the possible mechanism of *n*-hexane extract of *Jatropha curcus* Linn as an enhancer of platelet aggregation due to the activity of phorbol ester or phorbol ester derivative. However, the development of this extract as a topical hemostasis product requires further investigation, because phorbol ester can interact with PKC, potentially affecting various enzymes, biosynthesis of proteins, cell differentiation processes, and gene expression. The safety and efficacy of this extract should be verified prior to the development of tropical hemostasis products that stop bleeding.

6. ACKNOWLEDGEMENT

This study was funded by a scholarship grant from Mahidol University (grant number 70/2555). The research was partial supported by the “Chalermphrakiat” Grant Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand.

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