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ORIGINAL ARTICLE

In Vitro Studies of *Jatropha curcas* L. Latex Spray Formulation for Wound Healing Applications

Yara İyileşmesine Yönelik *İn Vitro Jatropha curcas* L. Lateks Sprey Formülasyonu Çalışmaları

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ABSTRACT

Objectives: There is an increasing demand for wound healing products of natural origin. Our objective was to develop a spray formulation from *Jatropha curcas* (*J. curcas*) L. latex extracts for wound healing applications.

Materials and Methods: *J. curcas* L. latex was subjected to solvent extraction. The phytochemical structure was elucidated by ^{1}H-NMR and confirmed by liquid chromatography-mass spectrometer spectrometry. A topical spray formulation prepared from *J. curcas* latex extracts was evaluated in terms of its antimicrobial activity and radical scavenging activity. The toxicity of the formulation on fibroblast cell lines, collagen production, and wound healing activities were tested.

Results: The ^{1}H-NMR and mass spectrometric analyses revealed the pure compound as curcacycline A. The *J. curcas* latex extract formulation had radical scavenging and antibacterial activities. Moreover, the formulation was not toxic to the human fibroblast cells and it stimulated collagen production and healed cell injury in 24 h.

Conclusion: The *J. curcas* latex extract promoted wound healing after cell injury. Our findings indicate the possibility of utilizing the *J. curcas* latex extract spray formulation as a potential antibacterial, antioxidant, and wound healing product from nature.

Key words: *Jatropha curcas* L. latex, wound healing, spray formulation, collagen production, antioxidant

ÖZ

Amaç: Doğal kaynaklı yara iyileştirici ürünlere olan talep giderek artmaktadır. Amacımız, yara iyileşmesinde etkili *Jatropha curcas* (*J. curcas*) L. lateks ekstrelerinden bir sprey formülasyonu geliştirmektir.

Gereç ve Yöntemler: *J. curcas* L. lateks solvan ekstraksiyonuna tabi tutuldu. Fitokimyasal yapı, ^{1}H-NMR ile aydınlatıldı ve LC-MS spektrometrisi ile doğrulandı. *J. curcas* lateks ekstrelerinden hazırlanan topikal bir sprey formülasyonu, antimikrobiyal aktivitesi ve radikal süpürücü aktivitesi açısından değerlendirildi. Formülasyonun, fibroblast hücre hatları üzerindeki toksisitesi, kollajen üretimine etkisi ve yara iyileştirme aktivitesi test edildi.

Bulgular: ^{1}H-NMR ve kütle spektrometrisi analizleri, saf bileşiğin kurkasiklin A olduğunu ortaya koydu. *J. curcas* lateks ekstraktı formülasyonunun radikal temizleyici ve antibakteriyel aktiviteleri vardı. Ayrıca, formülasyonun insan fibroblast hücrelerinde toksik etki göstermediği ve 24 saat içinde kollajen üretimini uyardığı *ve* hücre hasarını iyileştirdiği tespit edildi.

Sonuç: *J. curcas* lateks özütünün, hücre hasarından sonra yara iyileşmesini desteklediği gösterildi. Bulgularımız, *J. curcas* lateks ekstraktının sprey formülasyonunun doğal potansiyel bir antibakteriyel, antioksidan ve yara iyileştirici ürün olarak kullanılma olasılığı olduğunu göstermiştir.

Anahtar kelimeler: *Jatropha curcas* L. lateks, yara iyileşmesi, sprey formülasyonu, kollajen üretimi, antioksidan

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INTRODUCTION

A wound is the result of an injury that damages the dermis of the skin. It may vary from a simple, acute wound to a chronic wound. Naturally, the human body possesses the potential to initiate wound healing to replace the damaged cellular structures and tissue layers. Wound healing is a complex process orchestrated by sequential events arising from homeostasis, inflammation, and proliferation/granulation to remodeling/maturation.[1] Wound care is a million-dollar industry and encompasses simple topical treatments for deep seated tissue regeneration using stem cell therapy.[2] Wound care determines the appropriate treatment to promote wound healing with minimal infections. Despite medical advancements, there is a mounting demand for alternative treatments from the clinical and economic perspective to treat wounds. In ancient times, tribal healers used plant parts to cure wounds. Even now, plants are considered an enormous repository of novel bioactive agents. It has been determined that at present there are more than 450 plant species being exploited for their wound healing ability,[3] yet the search for novel wound healing agents from natural resources with minimal scar formation is incessant. In this context, *Jatropha curcas* (*J. curcas*) L., a plant used for wound healing in folk medicine, was evaluated for its wound healing ability *in vitro* to substantiate its traditional use. In addition, the present study attempted to utilize the Thai traditional knowledge by formulating the *J. curcas* extract as a spray suitable for modern day use. It has been used in folk medicine to treat burns, dermatitis, syphilis, inflammation, rash, rheumatism, scabies, and sores, and its latex is known to possess wound healing activity.[4,5] In addition to its enormous applications in folk medicine, the natural binding ability of latex powder in tablets has also been demonstrated.[6] Fagbenro Beyioku et al.[7] and coworkers reported the antiparasitic activity of *J. curcas* sap and proposed it as an effective malaria vector control agent. Preliminary evaluation of the anti-HIV activity of *J. curcas* leaf extract was also reported.[8] *J. curcas* latex reduced the blood clotting time[9] and *in vitro* studies of latex extracts have clearly demonstrated wound healing activity.[10] Although *J. curcas* latex has been used in traditional wound healing, studies have revealed that pure undiluted latex caused caustic lesions in mouse models.[11] The sap was also found to be highly toxic to mice when administered orally or intraperitoneally.[7] Against this backdrop, the present study aimed to evaluate the bioactivity of a spray formulation containing pure compound from *J. curcas* latex and evaluate its *in vitro* antimicrobial, antioxidant, and wound healing potential.

MATERIALS AND METHODS

Apparatus, cell lines, microbial species, and reagents

The normal phase and reversed phase for thin layer chromatography (TLC) and silica gel for column chromatography were purchased from Merck (Merck, Darmstadt, Germany). The ^{1}H-NMR spectrum was characterized by Varian fourier transform-NMR spectrometer (Varian, Palo Alto, CA, USA). The molecular weight and fragmentation pattern of purified samples were further analyzed by electrospray ionization (ESI)-liquid chromatograph mass spectrometer (Micromass LCT, Altrincham, UK). The solvent content in the formulation after spraying was detected by gas chromatograph-mass spectrometer (MS) (Trace GC Ultra, Thermo Scientific, Inc., TX, USA) using an AT-WasMS capillary column (30 mmx0.25 mm; 0.25-µm film thickness). High performance liquid chromatography [(HPLC), Waters, Milford, MA, USA] was used to investigate the physical stability of the spray formulation. Scanning electron microscopy [(SEM)-Quanta; FEI Quanta 400, model: 1450 EP, Carl Zeiss Micro-Imaging, Inc., Thornwood, NY, USA] was used to obtain information about the morphology and film thickness of the spray formulation. The antibacterial activity was evaluated using gram-positive bacteria including *Staphylococcus aureus* ATCC 25925 (*S. aureus*) and *Staphylococcus epidermidis* ATCC35983 (*S. epidermidis*) and gram-negative bacteria including *Escherichia coli* ATCC 25922 (*E. coli*) and *Pseudomonas aeruginosa* ATCC 27853 (*P. aeruginosa*) that were compared with clinical isolates of *S. aureus*, *S. epidermidis*, *E. coli*, and *P. aeruginosa* (derived from clinical infections from Songkla Nagarind Hospital, Hat Yai, Thailand). The bacteria were maintained in brain heart infusion (BHI) broth (Becton, Dickinson and Company, NJ, USA). The cytotoxicity test was evaluated by a cell proliferation and viability assay using the human keratinocyte cell line (HaCaT) (Cell Lines Service GmbH, Eppelheim, Germany) and the human fibroblast cell line (BJ) (ATCC, Manassas, VA, USA). Dulbecco's modified eagle medium (DMEM), Eagle's minimum essential medium (EMEM), fetal bovine serum (FBS), and antibiotics (penicillin and streptomycin) were purchased from Gibco (Grand Island, NY, USA). The Sircol® collagen assay kit was purchased from Biocolor Life Science Assays (Belfast, Northern Ireland, UK). The epidermal growth factor Proteoglycan positive control (IPC) standard was bought from Ichimaru Pharcos (Ichimaru Pharcos Co. Ltd., Tokyo, Japan). A phase-contrast microscope (Olympus CK2, Tokyo, Japan) was used to photograph the monolayer of cells and the distance between the scratches was analyzed using an image processing program, Image J1.42q (Wayne Rasband, National Institute of Health, Bethesda, MD, USA). All other reagents were of analytical grade and used without further purification.

Collection of Jatropha latex

J. curcas L. is a shrub belonging to the family Euphorbiaceae. It is originally native to the tropical areas of the Americas from Mexico to Argentina, and has spread throughout the world in tropical and subtropical regions. *J. curcas* is 2-5 m tall and produces a watery latex.[12] In the present work, the latex was collected from *J. curcas* (specimen voucher number: SKP 071100301) plants at the botanical garden maintained by the Department of Pharmaceutical Botany and Pharmacognosy, Prince of Songkla University, Hat Yai, Thailand. The latex was obtained by cutting the leaf stalk and the collected latex was immediately stored at 4 ˚C until further use.

Extraction, isolation, and purification

Latex (100 mL, 107.8 g) was diluted with 20 mL of distilled

water and the mixture was extracted with *n*-hexane (200 mLx3 replications). The solvent phase was separated and the aqueous phase was further extracted with ethyl acetate (200 mLx3 replications) followed by butanol (200 mLx3 replications). The remaining aqueous phase was allowed to dry and the other solvent extracts were evaporated using a rotary evaporator. All the test extracts were primarily isolated by TLC using normal and reverse phase precoated silica plates. The mobile phase was optimized using various solvents such as hexane, ethyl acetate, acetone, dichloromethane, chloroform, acetonitrile, and methanol for the normal phase, whereas different ratios of methanol and water were used to optimize the mobile phase for the reverse phase TLC. The TLC plates were sprayed with 20% H_2SO_4 and developed on a hot plate (100 ˚C for 90 s). The solvent mixture that yielded better separation in TLC was used as a mobile phase for column chromatography using silica gel (230-400 mesh). Dried butanol extract (3 g) was loaded on the silica column (6x32 cm) and sequentially eluted with various ratios of chloroform:ethyl acetate:methanol (7:2:1, 6:3:1, and 5:4:1). Fractions were collected, concentrated, and tested for purity by TLC. Purified fractions were subjected to ^{1}H-NMR in deuterated dimethyl sulfoxide (DMSO)-d_7 and the spectra were recorded at 500 MHz. The spectrum was processed using vnmr software running on the Solaris operating system. The molecular weight and fragmentation patterns were analyzed by ESI-liquid chromatography- (LC) MS. The ESI-LC-MS instrument was operated using an ESI source (positive ion mode), with a source voltage of 4.0 kV, spray current of 100.0 µA, and a desolvation temperature of 130 ˚C (source temperature: 120 ˚C; acquisition mode: scan 100-1500 m/z). Nitrogen gas was used for desolvation and as tube lens gas.

Preparation of the spray formulation

Half a percent *J. curcas* latex extract (butanol extract) or curcacycline A was dissolved in ethanol and acetone as a co-solvent (80:20 v/v). One percent sorbitan monooleate (Span® 80) was used as a wetting and lubricating agent. The mixture was stirred overnight until a clear solution formed. The clear solution (4 g) was put into a spray canister and 8 g of hydrofluoroalkane propellant (HFA134a) was added and then it was sealed. These formulations are henceforth referred to as *J. curcas* latex extract formulation (JcF) and curcacycline A formulation (CAF).

Stability tests

The physical stability of JcF was observed for 3 months during storage by monitoring the pH, weight, color change, and appearance of the sediment. The curcacycline A was resolved by HPLC in a C18 reverse phase analytical column (150x4.6 mm; 5 µm) using a methanol and water isocratic system (50:50). The sample (15 µL) was automatically injected and elution was performed for 15 min. The results were detected at the optimum wavelength of optical density $(OD)_{266}$ nm. The LC was performed using the Waters Alliance 2690 liquid chromatography system C_{18} column (2.1x100 mm; 3.5 µm).

Gas chromatography (GC)-mass spectrometry (MS) of the spray formulation

GC-MS was employed to detect acetone in case it was left as a residue after spraying. The JcF was analyzed by GC-MS to trace the solvent level of the final product after application. JcF was sprayed 10 times in a glass beaker from a distance of 10 cm and briefly dried at ambient temperature. The dried content was reconstituted in acetonitrile and subjected to GC-MS analysis with an AT-WaxMS capillary column (30 m×0.25 mm; 0.25-µm film thickness) using the following conditions: column oven temperature: initial 50 ˚C, hold for 9 min; ramp to 120 ˚C at 2 ˚C min^{-1}; ramp to 200 ˚C at 25 ˚C min^{-1}, hold for 5 min; injection temperature: 150 ˚C; injection mode: splitless; sampling time: 1.00 min; constant flow: 1 mL min^{-1}; ionization mode: electron ionization; acquisition mode: scan, 30-500 amu; ion source temperature: 230 ˚C. The chromatogram obtained was identified by comparison with the mass spectral database (NIST and Wiley Library, 2005).

The scanning electron microscopy analysis

SEM-Quanta was used to obtain information on the morphology and film thickness of the spray formulation. The spray formulation was sprayed on stubs and coated with gold/palladium (20 nm). The specimens were viewed under a Zeiss EVO LS10 microscope using high-vacuum mode at 10 kV.

Antioxidant activity

The antioxidant activity was quantitatively investigated. In the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity test, various concentrations (7.75, 15, 31, 62, and 124 µg/mL) of *J. curcas* latex extract, JcF, or CAF were tested for their free radical scavenging activity using DPPH.[13] A blank sample (ethanol:acetone (80:20 v/v), 1% sorbitan monooleate Span® 80) was used as a negative control. The DPPH solution (0.2 mM) was freshly prepared by dissolving 7.89 µg of DPPH in 100 mL of methanol. Test extracts (1 mL) were mixed with 2 mL of DPPH solution and incubated in the dark for 30 min. After incubation, the OD was read at 517 nm. The DPPH solution was mixed with 1 mL of DMSO as a negative control and ascorbic acid as a positive control. The antioxidant activity was calculated using the following formula:

$$\text{Antioxidant activity (\%)} = \frac{OD_{517} \text{ of negative control} - OD_{517} \text{ of sample}}{OD_{517} \text{ of negative control}} \times 100$$

Antibacterial activity

Antibacterial activity was tested using two strains of gram-positive bacteria, *S. aureus* and *S. epidermidis*, and gram-negative bacteria, *E. coli* and *P. aeruginosa*. The bacteria were maintained in BHI broth at 37 ˚C by the cylinder cup diffusion method and the clear zone of *J. curcas* latex extract, JcF, and CAF was screened. The stock solution of all extract was prepared at 500 mg/mL and then the extract solution was loaded into the cylinder cup to obtain a final concentration of 10 mg/cup. After incubation at 35 ˚C for 24 h, the inhibition zones (mm) were measured by vernier caliper, recorded, and considered an indication of antibacterial activity.

Broth microdilution was employed to obtain the minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs). ATCC strains and clinical isolate strains of *S. aureus*, *S. epidermidis*, *E. coli*, and *P. aeruginosa* were used in this assay. The assay was performed in 96 well polystyrene plates. Wells containing 100 µL of BHI broth with formulation were inoculated with 10 µL of bacterial suspension containing 10^5 CFU/mL. The plate was incubated at 37 ˚C for 18 h. After incubation, 30 µL of resazurin (0.02% w/v) was added to each well and the plate was further incubated for 5 h. The MIC was determined as the lowest concentration of test extract in which pink coloration was not observed. Vancomycin and gentamicin were used as positive controls. For the determination of the MBC, the agar dilution method was employed.[14] A blank sample (ethanol:acetone (80:20 v/v), 1% sorbitan monooleate Span® 80) was used as a negative control.

Evaluation of cytotoxicity

The HaCaT and the human fibroblast cell line (BJ) were maintained in DMEM and EMEM, respectively, supplemented with 10% FBS and antibiotics (100 U penicillin and 100 U/mL streptomycin) at 37 ˚C with 5% CO_2. The cytotoxicity test was evaluated by a cell proliferation and viability assay using the HaCaT and BJ cell lines.[15] The cells ($2x10^4$ cells/mL) were seeded into the wells of a 96 well plate with their respective medium. Various concentrations (7.75, 15, 31, 62, and 124 µg/mL) of *J. curcas* latex extract, JcF, and CAF were added to the wells, followed by incubation at 37 ˚C for 24 h with 5% CO_2. Cells without tested samples served as a control. In addition, a blank sample (ethanol:acetone (80:20 v/v), 1% sorbitan monooleate Span® 80) was used as a negative control. After incubation, the media were removed and the cells were rinsed with sterile phosphate buffered saline (PBS). The wells were supplemented with 100 µL of fresh media and 10 µL of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide (MTT, 5 mg/mL) and incubated at 37 ˚C for 4 h. After incubation, the contents of the wells were removed and the formazan crystals formed were dissolved by adding 200 µL of DMSO and measured at 540 nm. The percentage of cell viability was calculated using the following formula:

$$\text{Viability (\%)} = \frac{\text{Control OD}_{540} - \text{Treated OD}_{540}}{\text{Treated OD}_{540}} \times 100$$

Estimation of the soluble collagen produced by human fibroblast cells

For estimation of the soluble collagen produced by human fibroblast cells, the soluble collagen produced by the BJ cell line was determined.[16] BJ cells were seeded into the wells of a 96 well plate at an initial concentration of $2x10^4$ cells/mL in EMEM supplemented with 10% FBS. Various concentrations (7.75, 15, 31, 62, and 124 µg/mL) of extracts, *J. curcas* latex extract, JcF, and CAF, were added to the cells and incubated at 37 ˚C for 24 h in a 5% CO_2 atmosphere. Proteoglycan IPC was extracted from nasal cartilage of *Oncorhynchus keta* (salmon) having a property like epidermal growth factor. It was used as a positive control. BJ cells alone served as a control. After incubation, 100 µL of supernatants were collected separately and the total soluble collagen (type 1) was quantified using 1 mL of a Sircol® collagen assay kit. The mixture was incubated at room temperature for 30 min and then subjected to centrifugation at 15.000 rpm for 10 min. The collagen was obtained as a pellet and dissolved in 1 mL of an alkaline reagent (0.5 M NaOH). The solution was transferred to a 96 well plate and the optical density was measured at 540 nm. A standard curve was prepared using standard bovine skin collagen type 1 obtained from American disease-free animals. The soluble collagen produced by BJ cells in the presence and absence of JcF was calculated based on the standard curve. A blank sample (ethanol:acetone (80:20 v/v), 1% sorbitan monooleate Span® 80) was used as a negative control.

Assay for the cell culture wound closure

The wound closure assay in cultured cells was performed to assess the *in vitro* wound healing ability of JcF in the HaCat and BJ cell lines.[16] The wells of a 6 well plate were seeded with $5x10^4$ cells/mL with appropriate culture medium and allowed to grow as a confluent monolayer. A linear scratch was then created using a sterile tip and the wells were washed with sterile PBS to remove cell debris. Culture medium (2 mL) was added to the wells with (treated, 31 µg/mL) or without JcF (blank sample) as a negative control. This was considered day 0 and photographs of the monolayer were acquired at 100x magnification using a phase-contrast microscope. The plates were incubated at 37 ˚C for 24 h in a 5% CO_2 atmosphere. After incubation, the cells were again photographed on day 1. These images were examined using an image processing program and the distance between the scratches was measured and the cell migration rate was calculated using the following formula:

$$\text{Migration rate (\%)} = \frac{\text{Distance between scratches (day 0)-Distance on day 1}}{\text{Distance on day 0}} \times 100$$

Statistical analysis

Assays were performed in triplicate and the values were expressed as mean ± standard deviation. One-way ANOVA was carried out using SPSS version 17.0. The significance was set at $p \leq 0.05$.

Ethics Committee approval and patient informed consent were not required as the experiment did not involve animal or human studies.

RESULTS

Extraction, isolation, and purification

Solvent extraction (hexane, ethyl acetate, butanol, and water extracts) of the *J. curcas* latex (107.8 g) yielded a total of 10.81 g that comprised the extracts from hexane (5 g; 19%), ethyl acetate (5 g; 19%) butanol (4.81 g; 30%) and water (5.36 g; 33%). All the test extracts were subjected to TLC using normal and reverse phase precoated silica plates and it was found that the hexane, ethyl acetate, butanol, and water fractions had similar TLC profiles (data not shown), but a few additional bands were observed in the butanol fraction. Thus, the butanol fraction was chosen for further purification with the silica

gel open column chromatography. Hence, the butanol fraction (3 g) was subjected to further fractionation by open column chromatography. Among the 200 fractions collected, we found that fractions 9 and 10 yielded a single abundant band in the TLC with a retention factor of 0.29. Then the pure compound from fractions 9 and 10 was further identified by ^{1}H-NMR and found to be curcacycline A (pure compound), which is a major constituent of *J. curcas* latex (Table 1). Curcacycline A was further confirmed by mass spectrometric analysis corresponding to $C_{37}H_{67}N_8O_9^+Na^+$ (Figure 1).

Spray formulation

J. curcas JcF was prepared from the butanol extract fraction employing various compositions (Table 2) and it was found that the formulation with *J. curcas* extract (0.5%), ethanol (78.5%), acetone (20%), and Span® 80 (1%) had preserved its bioactivity. JcF (pH: 7) exhibited a clear solution (Figure 2). In addition, CAF was also prepared in the same conditions with JcF and it was seen that the formulation with curcacycline A (0.5%), ethanol (78.5%), acetone (20%), and Span® 80 (1%) showed a clear solution without precipitation (data not shown). We collected both formulations for further investigation of bioactivity.

Table 1. Comparison of ^{1}H-NMR of pure compound with reported curcacycline A[17]

	Pure compound	Curcacycline A
	δ (ppm), *J* coupling constants (Hz), Integration proton, (500 MHz, DMSO-d_6)	δ (ppm), *J* coupling constants (Hz), Integration proton (400 MHz, DMSO-d_6
1.	8.521 (brs., 1H)	8.67 (t, *J*=5.7 Hz, 1H)
2.	8.46-8.32 (brs., 1H, 1H)	8.63-8.54 (m, 1H)
3.	-	8.51 (s, 1H)
4.	8.16 (d, *J*=7.5 Hz, 1H)	8.17 (d, *J*=8.5 Hz, 1H)
5.	8.04 (brs., 1H)	8.03 (s, 1H)
6.	7.80 (d, *J*=8 Hz, 1H)	7.73 (d, *J*=7.5 Hz, 1H)
7.	7.59 (brs., 1H, 1H)	7.53 (d, *J*=8.9 Hz, 1H)
8.	-	7.39 (d, *J*=9.9 Hz, 1H)
9.	5.30 (d, *J*=10.5 Hz, 1H)	5.34 (d, *J*=10.8 Hz, 1H)
10.	4.67 (d, *J*=7.5 Hz, 1H)	4.76 (dd, *J*=9.9, 3.7 Hz, 1H)
11.	4.49 (brs., 1H)	4.60 (dd, *J*=14.2, 8.3 Hz, 1H)
12.	4.30-4.26 (m, 2H)	4.37-4.22 (m, 2H)
13.	4.11-4.09 (dd, *J*=8 Hz, 4.5 Hz, 1H)	4.12-4.05 (m, 1H)
14.	3.96-3.86 (dd, dd, (*J*=14 Hz, 7.5 Hz), (*J*=17 Hz, 7 Hz), 4H)	3.96-3.79 (m, 4H)
15.	3.46 (d, *J*=17 Hz, 1H)	3.45 (d, *J*=5.2 Hz, 1H)
16.	3.43 (dd, *J*=11.5 Hz, 5.5 Hz, 1H)	3.40 (d, *J*=4.9 Hz, 1H)
17.	2.32-2.26 (m, 1H)	3.15 (d, *J*=5.2 Hz, 1H)
18.	1.96-1.89 (m, 1H)	2.50-2.46 (m, 1H)
19.	1.79 (d, *J*=13.5, 1H)	2.32 (td, *J*=13.8, 6.9 Hz, 1H)
20.	1.64-1.40 (m, 13H)	1.69-1.18 (m, 13H)
21.	0.90-0.80 (m, 32H)	1.08-0.69 (m, 32H)

NH

NH

s: Singlet, d: Doublet, dd: Doublet of doublets, m: Multiplet, brs: Broad singlet, DMSO: Dimethyl sulfoxide

Chemical and physical stability tests

Storage did not affect the stability, pH, weight, color, or homogeneity of JcF. HPLC analysis of JcF revealed a major peak eluted at 1.98 min with 81% recovery. LC-MS analysis and the ESI-MS spectra fragmentation pattern revealed the major constituent as curcacycline A. The GC-MS analysis revealed the presence of 7.4% ethanol (data not shown) but acetone residue was not detected due to quick evaporation. The 10.000x micrographs of the spray formulation from SEM are shown in Figure 3. Figure 3A displayed the film on the stub with interspersed droplets and small particles observed on the surface. Furthermore, cross-section SEM revealed that the formulation formed a thin film after spraying on the stub (Figure 3B). Furthermore, curcacycline A was used to formulate the CAF using the same excipient, which revealed similar physical properties to JcF (data not shown).

Antioxidant activity and antibacterial activity

The antioxidant activity of *J. curcas* latex extract, JcF, and CAF is illustrated in Figure 4. The results showed that *J. curcas* latex extract and JcF significantly increased antioxidant activity in a concentration-dependent manner, but CAF did not. In addition, the inhibition zone of antimicrobial activity is given in Table 3. The results revealed that both *J. curcas* latex extract and JcF possessed antibacterial activity with MIC and MBC at 5 mg/mL against both gram-negative and gram-positive bacteria and their clinical isolates, whereas vancomycin and gentamicin exhibited MIC at 1 and 4 µg/mL against gram-positive and gram-negative bacteria, respectively. Notably, an inhibition zone of antimicrobial activity of CAF was not observed against either type of bacteria.

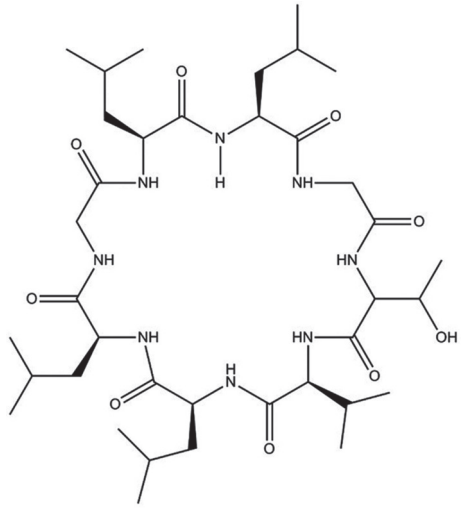

Figure 1. The molecular structure of curcacycline A

Table 2. *Jatropha curcas* extract spray formulation design

Ingredients	Compositions % (w/w)								
	F1	F2	F3	F4	F5	F6	F7	F8	F9
Jatropha curcas extract	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Ethanol	78.5	78.5	77.5	75.5	76.9	78	76.5	75.4	78.5
Acetone	20	20	20	20	20	20	20	20	20
PEG 400	-	0.5	1	2	0.5	0.5	1	2	-
Span 80	1	0.5	0.2	1	0.5	0.1	1	0.5	-
Eudragit® E100	-	0.1	0.5	1	0.1	0.5	1	0.1	0.5
Kollidon® VA 64	-	0.1	0.5	1	0.1	0.5	1	0.1	0.5
Kolliphor P	-	1	0.5	0.2	1	0.5	0.2	1	0.5
Test results	✓	x	x	x	x	x	x	x	x

✓: No precipitation, x: Precipitation

Figure 2. Formulation of *Jatropha curcas* latex extract filled in metered dose spray canister

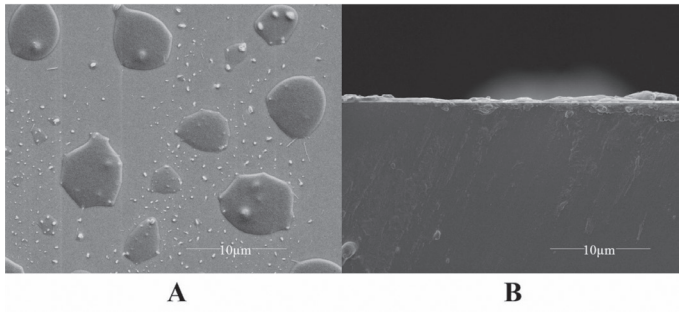

Figure 3. The surface morphology of JcF spray droplets (A) and thin film micrographs of JcF (B)
JcF: *Jatropha curcas* latex extract formulation

Cytotoxicity

The cytotoxicity of *J. curcas* latex extract, JcF, and CAF in HaCat and BJ cells was evaluated by MTT assay. The results revealed that cell viability of HaCat cells was higher than 80% at all concentrations of all tested samples (Figure 5A). However,

Table 3. Antibacterial activity of *Jatropha curcas* extract and JcF against reference and clinical isolates of gram-positive and -negative bacteria. Notably, antibacterial activity of CAF is not shown in this table due to inability to measure its inhibition zone

Pathogen	Strain	Zone of inhibition (mm)	
		J. curcas latex extract	JcF
S. aureus	ATCC25925	23±0.2	25±0.5
	Clinical isolate	15±0.3	14±0.5
S. epidermidis	ATCC35983	23±0.2	27±0.2
	Clinical isolate	20±0.2	20±0.1
E. coli	ATCC25922	23±0.2	21±0.1
	Clinical isolate	12±0.5	12±0.2
P. aeruginosa	ATCC27853	22±0.1	20±0.2
	Clinical isolate	14±0.1	12±0.2

JcF: *Jatropha curcas curcas* latex extract formulation, CAF: Curcacycline A formulation

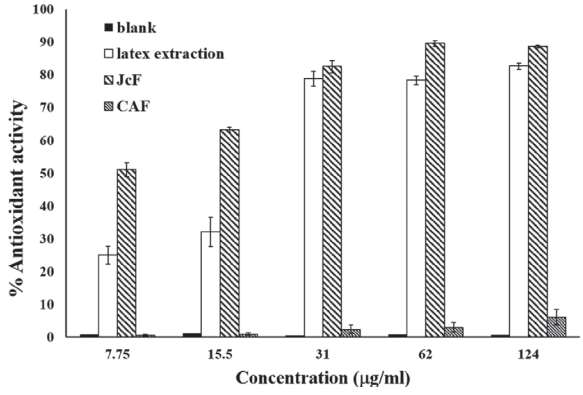

Figure 4. Antioxidant activity of *Jatropha curcas* latex extract, JcF, and CAF. Various concentrations of tested samples were tested for their free radical scavenging activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH). A blank sample (ethanol:acetone (80: 20 v/v), 1% sorbitan monooleate Span® 80) was used as a negative control
JcF: *Jatropha curcas* latex extract formulation, CAF: Curcacycline A formulation

the concentration of 62 and 124 µg/mL *J. curcas* latex extract significantly decreased cell viability of BJ cells while JcF- and CAF-treated BJ cells were not toxic (>80%) (Figure 5B). Notably, JcF- and CAF-treated HaCat and BJ cells displayed more than 80% cell viability at all concentrations (Figure 5).

Collagen production and wound healing

The collagen production after treatment with *J. curcas* latex extract, JcF, and CAF in human fibroblast BJ cells was determined by Sircol assay. The results showed that *J. curcas* latex extract and JcF promoted collagen production in BJ cells in a concentration-dependent manner, whereas collagen production was not observed in CAF-treated cells (Figure 6). In fact, *J. curcas* latex extract slightly promoted collagen production (47-198 pg/mL), while JcF prominently promoted it (179-335 pg/mL). These results revealed that JcF exhibited good collagen production activity and was nontoxic to both cells, but this was not the case for *J. curcas* latex extract or CAF. Thus, we selected JcF for further investigation of wound healing activity. The wound healing in HaCat and BJ cells was also determined using a scratch assay, and the proteoglycan IPC (1 µg/mL) was used as a positive control. In the present study, we used a concentration of 31 µg/mL JcF for wound healing. The results after treatment for 24 h showed that JcF completely promoted wound healing in HaCat and BJ cells (Figures 7A and 7B). Indeed, the wound healing of JcF-treated HaCat cells was more rapid than that of proteoglycan IPC, but was similar in BJ cells.

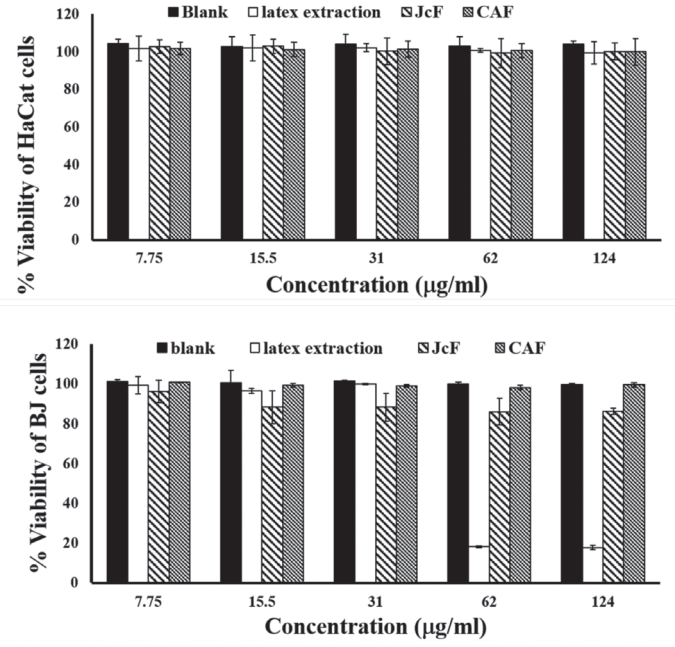

Figure 5. Cytotoxicity evaluation of *Jatropha curcas* latex extract, JcF, and CAF using HaCaT cells (A) and BJ cells (B). A blank sample (ethanol:acetone (80:20 v/v), 1% sorbitan monooleate Span® 80) was used as a negative control

JcF: *Jatropha curcas* latex extract formulation, CAF: Curcacycline A formulation, HaCaT: human keratinocyte cell line, BJ: Human fibroblast cell line

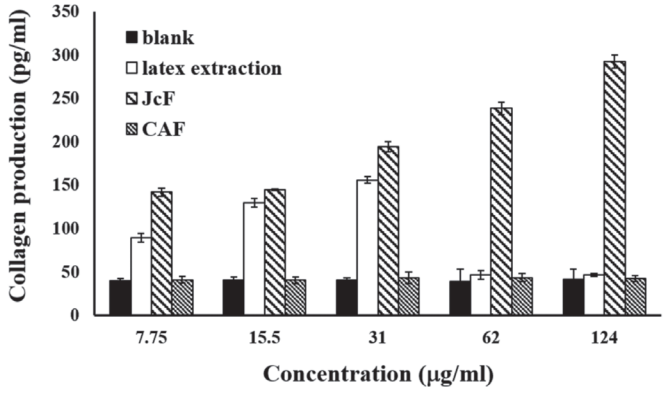

Figure 6. Collagen production by BJ cells treated with various concentrations of *Jatropha curcas* latex extract, JcF, and CAF. A blank sample (ethanol:acetone (80:20 v/v), 1% sorbitan monooleate Span® 80) was used as a negative control

BJ: Human fibroblast cell line, CAF: Curcacycline A formulation, JcF: *Jatropha curcas* latex extract formulation

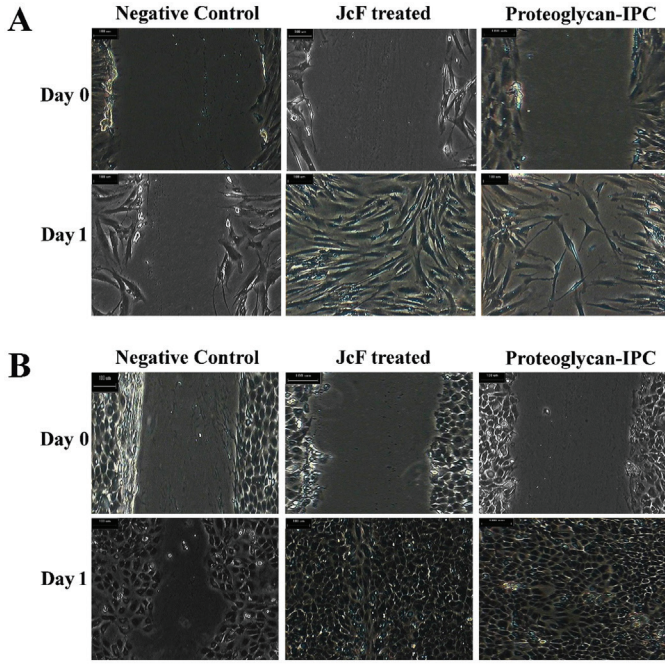

Figure 7. (A) *In vitro* wound closure assay of BJ cells with (JcF and proteoglycan IPC) and without (negative control) treatment. (B) *In vitro* wound closure assay of HaCaT cells with (JcF and proteoglycan IPC) and without (negative control) treatment. Scale bar indicates 100 µm

BJ: Human fibroblast cell line, JcF: *Jatropha curcas* latex extract formulation, IPC: Positive control, HaCaT: Human keratinocyte cell line

DISCUSSION

In the structure elucidation of pure compound from *J. curcas* latex extracts, 1H-NMR revealed eight positions of chemical shifts at 8.52, 8.46, 8.32, 8.16, 8.04, 7.80, and 7.59 ppm (Table 1). This characteristic of ^{1}H-NMR indicates the protons of amino acid residues in curcacycline A, a cyclic octapeptide, which has been reported in various parts of *J. curcas*.[17] In addition, the ESI-MS displayed 789.5 m/z corresponding to $C_{37}H_{67}N_8O_9^+Na^+$ and a

similarity search in the mass spectral database revealed a close match as curcacycline A.[18] The data from ^{1}H-NMR and ESI-MS together supported that pure compound was curcacycline A.

Next, *J. curcas* latex extract and curcacycline A were used to formulate the JcF and CAF using ethanol, acetone, Span® 80, and propellants as excipient. The results revealed that formulations retained properties including pH, weight, color, and homogeneity of the formulation, suggesting this formulation was stable at room temperature. In addition, LC-MS analysis of the JcF revealed that curcacycline A was stable in this formulation. Furthermore, the GC-MS results detected a low amount of ethanol residue due to quick evaporation. The concentration of ethanol in JcF is also less than that of typical antiseptic alcohol, which contains >70% ethanol. The absence of acetone guaranteed the safety of JcF for wounds. In addition, the film thickness of JcF from SEM data suggested that the spray formulation formed a uniform film containing some particles on a film surface. Moreover, cross-section SEM showed that JcF formed a thin layer of film after spraying onto a stub with a thickness of 1-2 µm. For CAF, the physical and chemical properties of this formulation were similar to those of JcF.

In the biological activity studies, *J. curcas* latex extract and JcF had high antioxidant potency, but CAF did not. A similar phenomenon was observed for antimicrobial activity in the microbial tests on gram-positive and -negative bacteria in which *J. curcas* latex extract and JcF showed significant antimicrobial activities, but CAF did not. These suggested that curcacycline A is not an active compound for that biological activity. Most antioxidants are likely to promote wound healing.[19] The *J. curcas* extract illustrated its potential to possess antimicrobial and antioxidant activities. Thus, JcF with antioxidant activity could promote wound healing while preventing reactive oxygen species mediated further cellular damage at the wound site. Most wound infections are colonized by polymicrobial infections consisting of both gram-positive and gram-negative bacteria but many classes of antibiotics are effective against them. Although broad spectrum antibiotics can be administered to control wound infections, this could cause an increase in the incidence of antibiotic resistance in clinical use.[20] In addition, there will be a requirement for the administration of wound healing products. The ability of *J. curcas* extract to control both gram-positive and gram-negative bacteria and its wound healing potential assure its perpetual applications in wound care products. The ability of *J. curcas* latex ointments in controlling pathogens was also proven, which is consistent with the present study.[21,22]

The *in vitro* cytotoxic activity of *J. curcas* latex extract, JcF, and CAF in human keratinocyte and human fibroblast cell lines was investigated. All concentrations of JcF and CAF were not toxic and stimulated cell growth in both cells, but this was not the case for *J. curcas* latex extract in BJ cells, suggesting that JcF and CAF are safer than *J. curcas* latex crude extract. Furthermore, JcF increased collagen production and so will have a positive impact on improving wound healing, but CAF did not. This suggested that curcacycline A is not an active compound for collagen production. Collagen is known to play a major role in wound healing and re-epithelialization, which is crucial for wound closure. It acts as a reinforcing factor by increasing the strength of the wound, which helps to restore the functionality of the skin.[1] Previous studies with *J. curcas* latex revealed its toxicity towards fibroblasts.[23] However, in our studies JcF promoted wound healing potential without inducing any toxicity to fibroblast cells. In the scratch assay, 'expanding' mode of human keratinocytes was enhanced in the presence of JcF and the results were significantly better than the positive control of proteoglycan IPC, suggesting a potentially high wound healing property. This expanding mode may activate wound closure and switch back to a 'balanced' mode that will maintain homeostasis.[24] JcF reduced the time required for wound re-epithelialization, which also hastened the wound healing process, which is already evident from studies carried out with *J. curcas* plant exudates,[25] leaf extract,[26] and ointment prepared from it.[27] The ointment prepared from *J. curcas* was proven to be safe for albino rats as histopathology of liver and kidney were found to be unaltered.[28] The rate of re-epithelialization is important to avoid scarring, which is considered a social stigma. The efficiency of the spray formulation in increasing the natural healing process will aid in the development of better wound care products. Wound healing is a complex process orchestrated by various cellular events from cell migration and proliferation to remodeling. Several studies have shown that the accumulation of oxidative stress hampers the healing process and advised the use of locally applied antioxidants to reduce hypoxia and to promote wound healing.[29,30] Previous research has revealed the active ingredient responsible for wound healing as curcain.[31,32] However, in our study we did not detect curcain in the extraction process.

CONCLUSION

The present study for the first time reports the formulation of a spray from *J. curcas* latex extract with potential applications in wound healing. The formulation of a spray from curcacycline A had no such applications. The study also found that the latex extract formulation preserved bioactive potentials such as antimicrobial, antioxidant, and wound healing without any loss in function. Development of a spray product for treating wounds has advantages over other formulations like ointment or creams that alter the healing process. The ability to induce collagen and prevent microbial infections, and the antioxidant property of JcF greatly enhance its wound healing potentials.

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