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Isfahan University of Medical Sciences

Original Article

Anti-inflammatory and cytotoxic effects of *Jatropha podagrica* extracts on skin cancer

Reawfang Sriyom[1], Arunporn Itharat[2,3,*], Onmanee Prajuabjinda[2], Pakakrong Thongdeeying[2], Srisopa Ruangnoo[2], Sunita Makchuchit[3], Pranporn Kuropakornpong[3], Kanyarat Namphonsaen[4], Perika Monkanna[4], and Neal M. Davies[5]

[1]*Student of Doctor of Philosophy Program in Applied Thai Traditional Medicine, Faculty of Medicine, Thammasat University, Pathumthani, 12120, Thailand.*
[2]*Department of Applied Thai Traditional Medicine, Faculty of Medicine, Thammasat University, Pathumthani 12120, Thailand.*
[3]*Center of Excellence in Applied Thai Traditional Medicine Research (CEATMR), Thammasat University, Pathumthani, 12120, Thailand.*
[4]*Master Degree of Science program (Applied Thai Traditional Medicine), Faculty of Medicine, Thammasat University, Pathumthani, 12120, Thailand.*
[5]*Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, AB T6G 2P5, Canada.*

Abstract

Background and purpose: *Jatropha podagrica* Hook. belongs to the Euphorbiaceae family, which possesses anticancer activities and is traditionally applied to treat skin diseases. No reports of *J. podagrica* anti-neoplastic activity on an amelanotic melanoma and associated inflammatory mediators exist.
Experimental approach: The biological activities, including cytotoxic and anti-inflammatory effects of *J. podagrica* extracts, were evaluated. Key compounds in the extracts were identified using LC-MS/MS analysis.
Findings/Results: The hexane extract of the root (RMH) demonstrated the highest inhibition of NO production with an IC_{50} of 4.94 ± 0.25 µg/mL, followed by the ethanolic extracts of the root (RME) and stem (SME) with IC_{50} values of 24.90 ± 1.06 and 25.20 ± 0.10 µg/mL, respectively. However, RMH showed cellular toxicity at 50 µg/mL, while other extracts were non-toxic up to 100 µg/mL. None of the extracts affected the concentrations of inflammatory mediators PGE_2 or TNF-α. The cytotoxic activity of SME showed an IC_{50} of 5.62 ± 0.58 µg/mL, comparable to that of the anticancer drug 5-fluorouracil, with an IC_{50} of 0.59 ± 0.01 µg/mL. The selectivity index of SME was >17.79, significantly higher than that of 5-fluorouracil, which was 0.08. LC-MS/MS analysis identified two main compounds from the coumarin group: fraxetin at 5.357 min and its positional isomer tomentin at 5.943 min.
Conclusion and implications: The study indicates that SME exhibits good cytotoxic activity and inhibits key cancer hallmarks such as NO production. The presence of coumarins, identified through LC-MS/MS, suggests that these compounds may play a crucial role in the extract's anticancer effects, highlighting the potential for future development as cancer therapeutics.

Keywords: Anti-inflammatory activity; Coumarins; Cytotoxicity; *Jatropha podagrica*; Skin cancer.

INTRODUCTION

The skin serves as the first barrier protecting internal organs from external factors such as ultraviolet (UV) light, trauma, chronic inflammation, aging, and genetic predisposition. Prolonged exposure to these factors can lead to the transformation of normal cells into skin cancer cells (1). The incidence of skin cancer has been globally increasing annually, with an estimated 150,000 new cases reported in 2020 in Thailand. Melanoma is of significant concern due to its aggressive nature. There are three main types of skin cancer: basal cell carcinoma (BCC), squamous cell carcinoma (SCC), and malignant melanoma (MM). BCC and SCC are more common and typically grow slowly without metastasizing. In contrast, MM, though less common, metastasizes quickly and has a mortality rate exceeding 80% (2,3).

*Corresponding author: A. Itharat
Tel: +66-29269749, Fax: +66-29269705
Email: iarunporn@yahoo.com

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Natural plant-derived compounds are gaining attention worldwide as potential sources for new drug development, offering an alternative to conventional treatments and potentially reducing the side effects associated with synthetic chemical compounds (4). *Jatropha podagrica* Hook. (*J. podagrica*), a member of the Euphorbiaceae family, has been traditionally used to treat ulcers, abscesses, and as a monotherapy for cancer in Thai folk medicine (5). Literature reviews indicate that *Jatropha* species contain numerous active secondary metabolites, including coumarins, terpenes, alkaloids, phenolic acids, and lignans. These compounds exhibit a wide range of biological effects such as anti-bacterial, anti-inflammatory, anticancer, and wound-healing properties (6).

Fraxetin and tomentin are coumarin compounds previously identified in *J. podagrica*, known for their anticancer and anti-inflammatory activities, respectively. Fraxetin has been shown to suppress cancer cell proliferation by inactivating the PI3K/Akt and JAK2/STAT3 signaling pathways and promoting cell cycle arrest (7-11). Tomentin has demonstrated the ability to reduce inflammatory cytokines both *in vitro* and *in vivo* (12). Thus, coumarins may be the active compounds in this plant.

Previous studies have shown that ethanolic extracts from the stem of *J. podagrica* can inhibit nitric oxide (NO) production in lipopolysaccharide (LPS)-induced RAW264.7 macrophage cells, with half-maximal inhibitory concentration (IC_{50}) value of 13.44 ± 0.28 µmol/L, which is more effective than quercetin (IC_{50} = 17.00 ± 2.10 µmol/L) in reducing inflammation, a hallmark of predisposition to cancer (13). However, inflammation can also be driven by other pathways, such as prostaglandin E2 (PGE_2) and tumor necrosis factor-alpha (TNF-α), which have not been studied in the context of *J. podagrica* extracts. Additionally, the anti-inflammatory effects on these pathways and the cytotoxicity against melanoma skin cancer cells have not been explored.

The objective of this research was to investigate the cytotoxic effects of *J. podagrica* extracts, prepared using different extraction solvents, on skin cancer cells compared to normal skin cells. The study also aimed to identify the prominent compounds in the most active extract using liquid chromatography-mass spectrometry (LC-MS/MS) analysis.

MATERIALS AND METHODS

Chemicals and reagents

Commercial-grade solvents, including ethanol, dimethyl sulfoxide (DMSO), isopropanol, and methanol (HPLC grade), were purchased from RCI Labscan, Thailand. Trypsin-EDTA was purchased from Gibco, Canada. Fetal bovine serum (FBS) and phosphate-buffered saline (PBS) were purchased from Gibco, United Kingdom. Penicillin-streptomycin (P/S), Dulbecco's modified Eagle medium (DMEM), minimum essential medium (MEM), RPMI 1640 medium, sodium pyruvate (100 mM) 100X, and Trypan blue were purchased from Gibco, USA. LPS from *Escherichia coli*, N-(1-naphthyl) ethylenediamine dihydrochloride, sulforhodamine B sodium salt, and tris(hydroxymethyl)aminoethane were purchased from Sigma, USA. PGE_2 ELISA monoclonal and mouse TNF-α immunoassay ELISA kits were purchased from R&D Systems, USA. 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was from TCI, Japan. Trichloroacetic acid (TCA) was purchased from Merck, Germany. Sulfanilamide was from Sigma, China.

Herbal preparation and extractions

The plant *J. podagrica* Hook. was collected in September 2020 from Tak, Thailand. A voucher specimen (TTM No.0005450) was deposited at the Thai Traditional Medicine Herbarium. The leaves, stems, and roots of *J. podagrica* were boiled in water for 15 min and filtered using Whatman No. 1 filter paper. This process was repeated twice. The resulting filtrates were then concentrated using a lyophilizer.

Separately, each plant part was macerated with hexane and 95% ethanol at room temperature for three days, with the process repeated three times. The extracts were filtered through filter paper, and the remaining residue

from the hexane extraction was further macerated with 95% ethanol, following the same procedure. The filtrates were concentrated using a rotary evaporator at 45 °C. The percentage yield of all extracts was calculated, and the extracts were stored at -20 °C until further analysis (Fig. 1).

Determination of anti-inflammatory activity by inhibiting NO production

The anti-inflammatory effect, specifically the inhibition of NO production, was determined using a modified method from Makchuchit *et al*. (14). Murine macrophage leukemia cells (RAW 264.7) were cultured in RPMI 1640 medium supplemented with 10% FBS and 1% P/S at 37 °C in a 5% CO_2 atmosphere. The cells were subcultured using 0.25% trypsin-EDTA, followed by centrifugation with rotor NO. 1617 (rotor radius 13.8 cm) at 1,530 *g* for 5 min (Universal 320R, Hettich, Germany). The cell pellet was resuspended in complete medium, and the cell suspension was adjusted to a concentration of 1 × 10^5 cells/mL. One hundred µL of the cell suspension was added to each well of a 96-well plate and incubated at 37 °C with 5% CO_2 to allow the cells to adhere and form a monolayer. After 24 h, the medium was replaced with 100 µL of 10 ng/mL LPS and an equal volume of each sample at concentrations 1, 10, 50, and 100 µg/mL. The cells were incubated for an additional 24 h.

NO production was assessed by reacting 100 µL of the supernatant with 100 µL of Griess reagent, which consisted of 0.1% naphthyl ethylenediamine dihydrochloride, 1% sulfanilamide, and 5% phosphoric acid in distilled water. The optical density (OD) was measured at 570 nm to quantify NO levels using a microplate reader (Biotek Power Wave XS, USA). The percentage of NO inhibition was calculated using the following formula (14)

$$\text{Inhibition (\%)} = \left[\frac{(OD_{control} - OD_{sample})}{OD_{control}}\right] \times 100 \quad (1)$$

$OD_{control}$ = Mean of control medium (+LPS) - mean of control medium (-LPS)

OD_{sample} = Mean of sample (+LPS) - mean of sample (-LPS)

Additionally, cell viability was assessed using the MTT assay in LPS-free wells to ensure that the samples were not toxic to the cells. This is important because if the samples are toxic, they could reduce NO production due to cell death rather than a specific anti-inflammatory effect. In this study, the anti-inflammatory steroid prednisolone was used as a positive control.

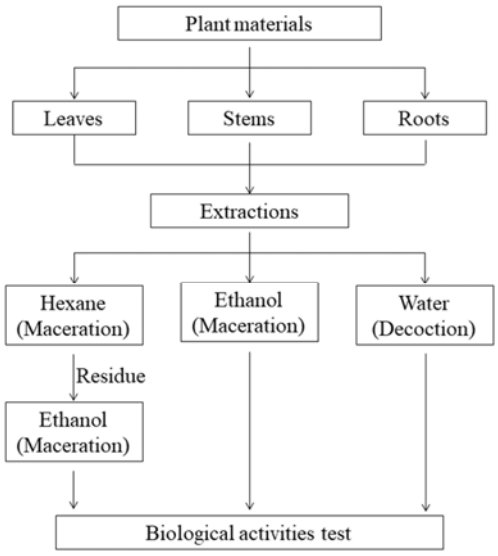

Fig. 1. Flow chart of materials preparation and extraction.

Determination of cell viability using the MTT assay

Cells were treated with all extracts of *J. podagrica* for 24 h. Viability was assessed using the MTT assay. After transferring the supernatant to each well, 10 µL of MTT solution (5 mg/mL) was added to each well, and the plates were incubated for 2 h. After incubation, the supernatant was removed, and the formazan crystals produced by viable cells were dissolved in 0.04 M HCl in isopropanol. The absorbance was then measured at 570 nm. A sample was considered toxic if the percentage of survival was less than 30% compared to the control group. The percentage of survival was calculated using the following equation (14-16).

$$\text{Survival (\%)} = \left(OD_{sample}/OD_{control}\right) \times 100 \qquad (2)$$

$OD_{control}$= Mean of control medium (-LPS)

OD_{sample}= Mean of sample (-LPS)

Measurement of anti-inflammatory activity through inhibition of PGE_2 production

The levels of PGE_2 were measured using a PGE_2 ELISA kit with monoclonal antibodies purchased from Cayman Chemical, following the manufacturer's instructions and the method described by Yun *et al*. (17), with slight modifications. RAW 264.7 murine macrophage cells were seeded at a density of 1×10^5 cells/mL in 96-well plates and incubated for 24 hours at 37 °C with 5% CO_2 to allow adherence. Cells were then treated with all *J. podagrica* extracts for 24 h. During the last 18-24 h of treatment, cells were stimulated with 10 ng/mL lipopolysaccharide (LPS) to induce PGE_2 and TNF-α production. After incubation, supernatants were collected for PGE_2 and TNF-α quantification via ELISA. Briefly, the supernatant from LPS-induced RAW264.7 cell cultures was transferred to the wells of a 96-well ELISA plate. The samples were then allowed to interact with the PGE_2 AChE tracer and PGE_2 monoclonal antibody.

The plate was incubated at 4 °C for 18 h. After incubation, each well was aspirated and washed five times with wash buffer. Then, 200 µL of Ellman's reagent was added to each well. The OD was measured at 412 nm. The percentage of inhibition was calculated using the following equation (17):

$$\text{Inhibition (\%)} = \left[\frac{\left(OD_{control} - OD_{sample}\right)}{OD_{control}}\right] \times 100 \qquad (3)$$

Measurement of anti-inflammatory activity through inhibition of TNF-α production

The inhibition of TNF-α production was measured using a method described by Makchuchit *et al.* with some modifications (14). This experiment utilized a mouse TNF-α Quantikine ELISA kit purchased from R&D Systems. Briefly, the supernatant from LPS-induced RAW264.7 cell cultures was transferred to a 96-well ELISA plate, where it interacted with the RD1-63 diluent.

After washing the wells with wash buffer, mouse TNF-α conjugate was added to each well, and the plate was covered with an adhesive strip. The substrate solution was then added, and the plate was protected from light. The reaction was stopped using a stop solution, and the OD was measured at 450 nm. The percentage of inhibition was calculated using equation 3 (14).

Determination of cytotoxic activity using the sulphorhodamine B assay

The sulphorhodamine B (SRB) assay (18) was conducted with slight modifications from the method described by Skehan *et al.* (19). Amelanotic melanoma cells (C32) were cultured in MEM medium supplemented with 10% FBS, 1% P/S, and 1% sodium pyruvate. The cells were incubated under appropriate conditions until they reached 80% confluency. Adherent cells were detached using 0.125% trypsin-EDTA and centrifuged at 380 *g* for 5 min (Universal 320R, Hettich, Germany). The cells were then resuspended in fresh medium to achieve a single-cell suspension.

Cells were seeded at a density of 2,000 cells/well in 96-well plates and incubated for 24 h. All extracts (a total of 12 samples) of *J. podagrica* were evaluated for cytotoxic activity at concentrations ranging from 1 to 100 µg/mL (100 µL/well) were added in triplicate, and the plates were incubated for 72 h. On the fifth day, the medium was replaced with fresh media (200 µL/well) to allow for a recovery period.

After 72 h, the cells were fixed with ice-cold 40% TCA for 1 h in the refrigerator. The plates were then washed with tap water five times, and excess water was removed. The plates were left to dry. Next, 50 µL/well of 0.4% SRB solution was added to stain the cells for 30 min. Unbound dye was washed off with 1% acetic acid five times. The OD was measured using a microplate reader at 492 nm.

Similarly, normal human keratinocyte cells (HaCaT) were cultured in DMEM medium and evaluated using the SRB assay to determine the selective index (SI). The percentage of inhibition was calculated using equation 3 (18,19):

The National Cancer Institute (NCI) provides guidelines indicating that a crude extract with an IC_{50} value of less than 20 µg/mL is considered to have strong cytotoxicity. The SI is used to assess the safety of herbal extracts by comparing their toxicity to cancer cells versus normal cells. SI is calculated as the ratio of the IC_{50} value for normal cells to the IC_{50} value for cancer cells. An SI greater than 1 indicates that the extract is more toxic to cancer cells than to normal cells, suggesting specificity in targeting cancer cells (4,20).

Identification of prominent compounds in J. podagrica extract using LC-MS/MS analysis

The prominent compounds in *J. podagrica* extract were analyzed using LC-MS/MS. The system included a UHPLC-DAD-Q-Orbitrap with Vanquish UHPLC system (Thermo Fisher Scientific Inc.) equipped with Thermo Scientific Vanquish- Binary Pump F, Thermo Scientific Vanquish- Split Sampler FT, Thermo Scientific Vanquish- Column Compartment H, and Thermo Scientific Vanquish- Diode Array Detector FG, coupled with Thermo Scientific Orbitrap Exploris™ 120 mass spectrometer. Separation of the main compounds was achieved using a Hypersil BDS C18 column (2.1 × 50 mm i.d., 2.4 µm) with a flow rate of 0.5 mL/min and a column temperature of 25 °C, controlled with still air. The ion source used was a heated ESI (electrospray ionization). The mobile phase consisted of A: 0.1% formic acid in water, B: 0.1% formic acid in methanol. The gradient elution program was as follows: 0% B for 2 min, a linear increase from 0% to 100% B over 8 min, and 100% B for an additional 2 min. The *J. podagrica* extract samples were prepared in methanol at a concentration of 1 mg/mL and detected at an absorbance of 305 nm. MS fragmentation was conducted in both positive and negative ESI modes with a scan range of 200-1000 m/z.

For the mass spectrometer, mass analysis was done in both positive and negative modes using internal mass calibration EASY-IC™. The ion source type was heated-ESI. Spray voltage setting was static mode with positive ion 3,500 V and negative ion 2,500 V. Nitrogen gas mode was static with flow setting: sheath gas 50 Arb, Aux gas 10 Arb, and sweep gas 1 Arb. The ion transfer tube temperature was 325 °C. Vaporizer temperature was 350 °C. Full scan mode range was 200-1000 m/z with resolution of 60,000 and RF Len 70%. The $ddMS^2$ mode was triggered with an intensity threshold of 5.0×10^6. The MS^2 parameters were isolation window: 1.5 m/z, collision energy type: normalized, orbitrap resolution: 15,000, and scan range mode: automatic. The instrument was controlled and analyzed by Chromeleon™ Chromatography Data System software. The recorded chromatogram was visualized and analyzed using FreeStyle software. Compounds were identified or tentatively identified by their mass spectral data.

Statistical analysis

All experiments were conducted in triplicate, and results are presented as the mean ± SEM. IC_{50} values were calculated using a standard statistical program. Data analysis was performed with SPSS 13.0 for Windows, employing one-way ANOVA followed by Dunnett's multiple comparison tests to compare the results with the positive control. A *P*-value less than 0.05 was considered statistically significant.

RESULTS

Percentage of yields

The percentage yields varied between 0.58% and 36.96%, depending on the solvent used. The aqueous extract yielded the highest percentage, followed by the ethanol and hexane extracts. The percentage yields for each part of *J. podagrica* Hook. are detailed in Table 1.

Table 1. Percentage of yield in each part of *Jatropha podagrica*.

Parts	Extracts	Solvents	CODE	Yield of extract (%)
Leaves	Maceration	Hexane	LMH	1.38
	Maceration (residue)	95% EtOH	LRE	15.02
	Maceration	95% EtOH	LME	12.78
	Decoction	Water	LDW	36.96
Stems	Maceration	Hexane	SMH	0.58
	Maceration (residue)	95% EtOH	SRE	6.80
	Maceration	95% EtOH	SME	4.27
	Decoction	Water	SDW	21.26
Roots	Maceration	Hexane	RMH	0.67
	Maceration (residue)	95% EtOH	RRE	9.26
	Maceration	95% EtOH	RME	9.69
	Decoction	Water	RDW	13.86

Table 2. Inhibitory effect of *Jatropha podagrica* extracts on NO, TNF-α, and PGE_2. All the values are shown as mean ± SEM (n = 3) with an F value of 135.921 for NO. **P* < 0.05 indicates significant differences compared to the positive control (prednisolone).

CODE	NO		IC_{50} value on TNF-α (µg/mL)	IC_{50} value on PGE_2 (µg/mL)
	IC_{50} ± SEM (µg/mL)	*P*-value		
LMH	75.27 ± 4.41*	< 0.001	> 100	> 100
SMH	45.63 ± 5.08*	< 0.001	> 100	> 100
RMH	4.94 ± 0.25	0.929	> 30	> 30
LME	31.37 ± 1.43*	< 0.001	> 100	> 100
SME	25.20 ± 0.10*	< 0.001	> 100	> 100
RME	24.90 ± 1.06*	< 0.001	> 100	> 70
LRE	50.40 ± 2.17*	< 0.001	> 100	> 100
SRE	70.50 ± 9.03*	< 0.001	> 100	> 100
RRE	30.52 ± 1.80*	< 0.001	> 100	> 100
LDW	> 100*	-	> 100	> 100
SDW	> 100*	-	> 100	> 100
RDW	> 100*	-	> 100	> 100
Prednisolone	0.20 ± 0.09		NT	NT

Abbreviations designated to the code column: first letter indicates part of materials: L= leaves, S = stems, R = roots; second letter indicates method of extraction: D = decoction, M = maceration, R = maceration residue of each part; last letter indicates the solvent used for the extraction: W = water, H = hexane, E = 95% ethanol; NO, nitric oxide; TNF-α, tumor necrosis factor-alpha; PGE_2, prostaglandin E2; NT,#ot tested.

Anti-inflammatory activity

The anti-inflammatory effects were evaluated by measuring the inhibition of NO, PGE_2, and TNF-α production in RAW 264.7 macrophage cell lines. The results indicated that the root macerated with hexane (RMH) was the most effective at inhibiting NO production (Fig. 2A and Table 2). This was followed by the ethanolic extracts of root (RME) and stem (SME) (Fig. 2B and Table 2). However, RMH exhibited toxicity to RAW264.7 cells at 50 µg/mL, whereas RME and SME did not show toxicity up to 100 µg/mL (data not shown). None of the extracts significantly inhibited the production of PGE_2 or TNF-α (Table 2).

Cytotoxic activity

Cytotoxicity was assessed in amelanotic melanoma (C32) cells and human keratinocytes (HaCaT) cells. The ethanolic extract of the stem (SME) demonstrated the strongest cytotoxic activity, followed by the ethanolic extracts of leaves (LME) and root (RME) (Fig. 3, Table 3).

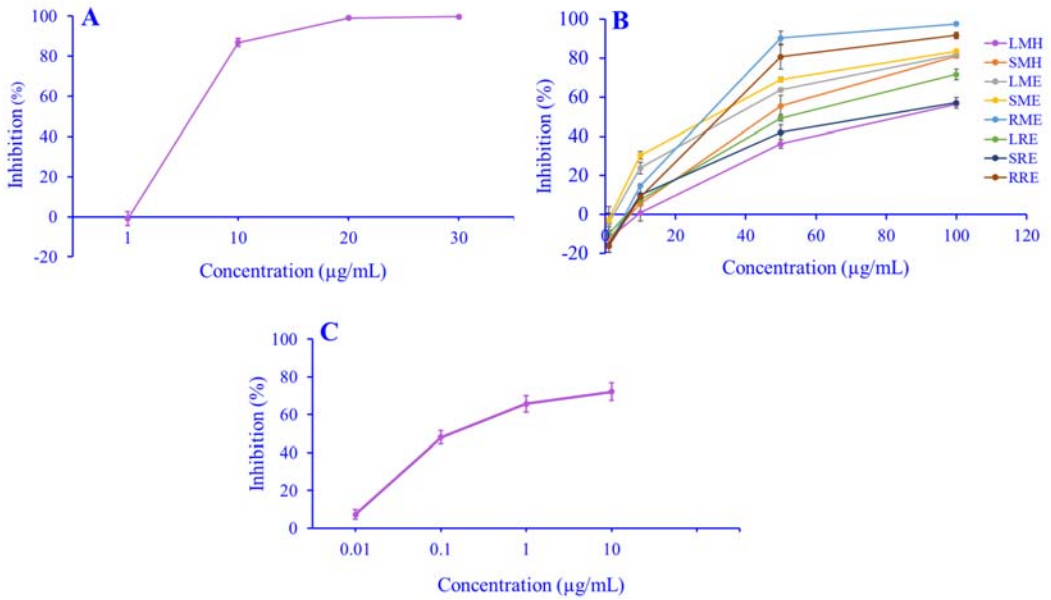

Fig. 2. Inhibition of LPS-induced NO production by various extracts of *Jatropha podagrica* and positive control. Graphs used for IC_{50} value determinations of *Jatropha podagrica* extracts and prednisolone on RAW264.7 cells. (A) Inhibition of NO production by (A) RMH; (B) by LMH, SMH, LME, SME, RME, LRE, SRE, and RRE; and (C) by prednisolone. Data are expressed as mean ± SEM. LPS, Lipopolysaccharide; NO, nitric oxide; in the abbreviations stand for the extracts: first letter indicates part of materials: L= leaves, S = stems, R = roots; second letter indicates method of extraction: D = decoction, M = maceration, R = maceration residue of each part; last letter indicates the solvent used for the extraction: W = water, H = hexane, E = 95% ethanol.

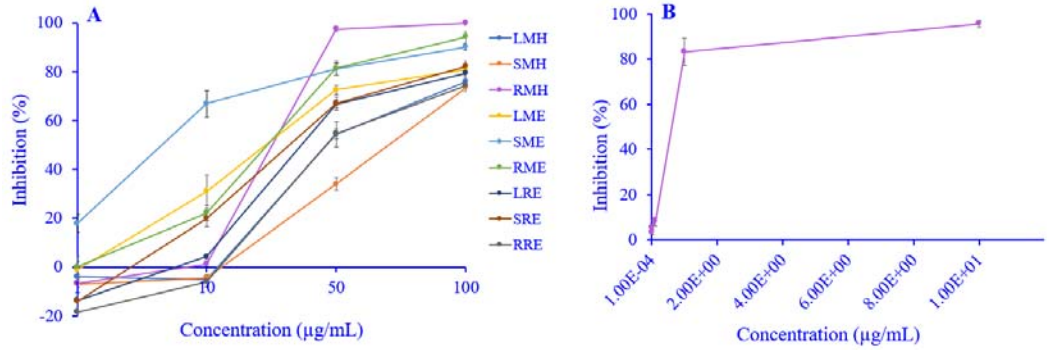

Fig. 3. The effect of *Jatropha podagrica* extracts and 5-FU on the growth of C32 cells using the SRB assay. Percent of cell growth inhibition (A) by various extracts of *Jatropha podagrica*, and (B) by 5-FU (positive control). All extracts except aqueous extracts affected the growth of C32 cells in a concentration-dependent manner. Data are expressed as mean ± SEM; n = 3. 5-FU, 5-fluorouracil; in the abbreviations stand for the extracts: first letter indicates part of materials: L= leaves, S = stems, R = roots; second letter indicates method of extraction: D = decoction, M = maceration, R = maceration residue of each part; last letter indicates the solvent used for the extraction: W = water, H = hexane, E = 95% ethanol.

According to NCI criteria, a crude extract with an IC_{50} value ≤ 20 µg/mL is considered to have *in vitro* cytotoxic activity. SME met this criterion with its IC_{50} value of 5.62 ± 0.58 µg/mL, whereas the other extracts did not achieve IC_{50} values lower than 20 µg/mL. Additionally, the SI indicates that SME is both safe and specific in targeting cancer cells without significant toxicity to normal skin cells. The SI value for SME was higher compared to the positive control 5-FU, although the difference was not statistically significant (Table 3). Consequently, SME was selected for further analysis of its main components using LC-MS/MS.

Table 3. IC_{50} values of cytotoxic activity of *Jatropha podagrica* extract on C32 and HaCaT cell lines using SRB assay. All the values are shown as mean ± SEM (n = 3) with F values of 302.042 and 493.307 for C32 and HaCaT, respectively. *P < 0.05 indicates significant differences compared to the positive control (prednisolone).

CODE	C32		HaCaT		Selective index
	IC_{50} (µg/mL)	*P*-value	IC_{50} (µg/mL)	*P*-value	
LMH	47.39 ± 3.78*	< 0.001	> 100*	-	> 2.11
SMH	69.22 ± 3.31*	< 0.001	> 100*	-	> 1.44
RMH	30.24 ± 0.03*	< 0.001	24.73 ± 0.89*	< 0.001	0.82
LME	22.69 ± 3.52*	< 0.001	> 100*	-	> 4.41
SME	5.62 ± 0.58	0.459	> 100*	-	> 17.79
RME	26.32 ± 2.98*	< 0.001	58.28 ± 4.66*	<0.001	2.21
LRE	36.91 ± 0.82*	< 0.001	> 100*	-	> 2.71
SRE	30.43 ± 1.28*	< 0.001	> 100*	-	> 3.29
RRE	46.01 ± 1.65*	< 0.001	> 100*	-	> 2.17
LDW	> 100*	-	> 100*	-	-
SDW	> 100*	-	> 100*	-	-
RDW	> 100*	-	> 100*	-	-
5-Fluorouracil	0.59 ± 0.01	(Ref)	0.05 ± 0.00	(Ref)	0.08

Abbreviations designated to the code column: first letter indicates part of materials: L= leaves, S = stems, R = roots; second letter indicates method of extraction: D = decoction, M = maceration, R = maceration residue of each part; last letter indicates the solvent used for the extraction: W = water, H = hexane, E = 95% ethanol.

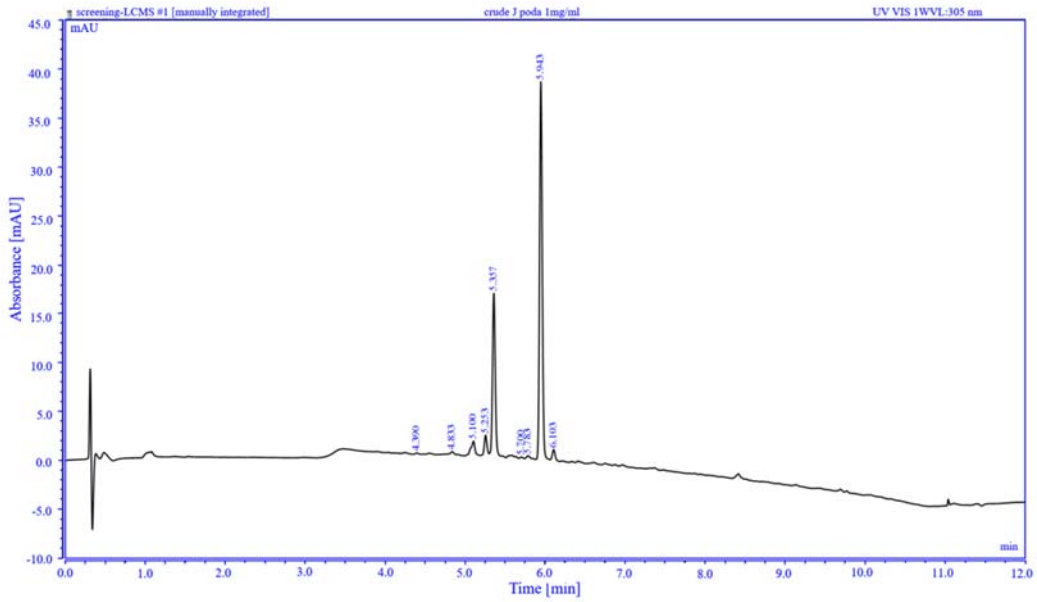

Fig. 4. UPLC-UV chromatogram of ethanolic stem extract of *Jatropha podagrica* (SME) detected with UV 305 nm. LC-MS/MS analysis revealed two prominent peaks in the SME extract: compound 1 and compound 2 at retention times of 5.357 and 5.943 min, respectively. Compound 1 was identified as fraxetin, and compound 2 was identified as tomentin or its positional isomer.

Main compounds analysis

LC-MS/MS analysis revealed two prominent peaks in the SME extract, as shown in Fig. 4. Compound 1, with a retention time of 5.357 min, exhibited a $[M-H]^-$ ion at m/z 207.02990, confirmed by its daughter ions and $[M+H]^+$ at m/z 209.04445, and $[M+Na]^+$ at m/z 231.02639 (Fig. 5). This compound was identified as fraxetin. Compound 2, with a retention time of 5.943 min, did not match any spectra in the National Institute of Standards and Technology (NIST) or mzCloud databases but had a $[M+H]^+$ ion at m/z 223.06010. This compound was tentatively identified as tomentin or its positional isomer, pending confirmation with a standard (Fig. 6). The chemical structures of fraxetin and tomentin are shown in Fig. 7.

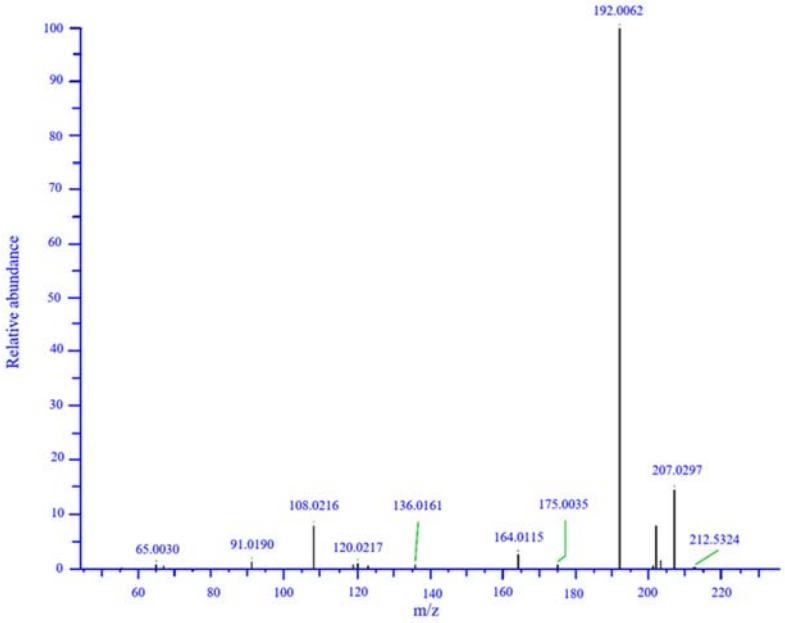

Fig. 5. Product ion mass spectra of [M-H]- of fraxetin at m/z = 207.0294.

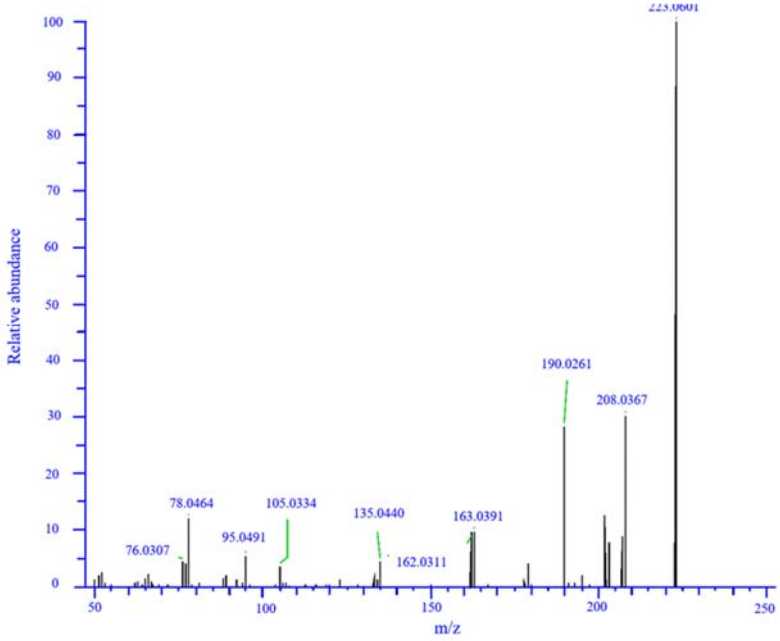

Fig. 6. Product ion mass spectra of [M-H]+ of tomentin at m/z = 223.0601.

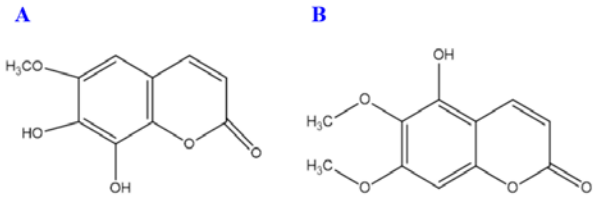

Fig. 7. Structure of main compounds that were detected in ethanolic stem extract of *Jatropha podagrica* (SME). (A) structure of compound 1 as fraxetin ($C_{10}H_8O_5$) and (B) compound 2 as tomentin ($C_{11}H_{10}O_5$). Both substances are coumarin compounds with a wide range of bioactivity.

DISCUSSION

Jatropha podagrica Hook. has been traditionally used in Thai folk medicine for treating skin diseases, such as ulcers and abscesses, and as a monotherapy for cancer (5). This study evaluates the biological effects of *J. podagrica* extracts, focusing on their anti-cancer properties and their impact on inflammatory mediators.

The yield percentage of the extracts varied between 0.58% and 36.96%, depending on the solvent used. The aqueous extract yielded the highest percentage, followed by ethanol and hexane extracts. The variation in yield is influenced by factors such as solvent type, extraction method, time, temperature, and the polarity of secondary metabolites in the plant material (21). The hexane extract yielded the lowest percentage, likely due to the high polarity of the plant's secondary metabolites, which are better solubilized in water than in nonpolar solvents like hexane. Water's high dielectric constant and polar protic nature make it more effective at dissolving polar substances compared to ethanol or hexane (22). Additionally, decoction, which involves higher temperatures than maceration, reduces viscosity and surface tension, facilitating better solvent penetration and dissolution of the plant's components (21).

Inflammation is a critical factor in cancer development, with mediators such as NO, PGE_2, and TNF-α playing significant roles in inflammation, cell proliferation, differentiation, and apoptosis (23-25). Elevated levels of these mediators can cause tissue damage, necrotic cell death, stimulate cell division, and promote tumor growth (26). Conversely, persistently low levels of these mediators are also linked to carcinogenesis. Natural compounds with anti-inflammatory and cytotoxic activities have been reported to influence these pathways (26-28). In this study, the stem macerated with ethanol (SME) showed the most effective inhibition of NO production, consistent with previous findings that the stem of *J. podagrica* effectively inhibits NO production in LPS-induced RAW264.7 macrophages with an IC_{50} value of 13.44 ± 0.28 µmol/L, which is more effective than the flavonoid quercetin (IC_{50} 17.00 ± 2.10 µmol/L) (13). While RMH and RME also exhibited anti-inflammatory effects, they were toxic to RAW264.7 cells at higher concentrations. Previous research identified several bioactive compounds in the stem of *J. podagrica*, including methyl gallate, gallic acid, fraxetin, tomentin, fraxidin, scoparone, 3-acetylaleuritolic acid, acetylaleuritolic acid, and sitosterol, all known for their antioxidant, antibacterial, anti-inflammatory, and anti-tumor activities (7, 10, 29-31).

According to NCI criteria, a crude extract with an IC_{50} value ≤ 20 µg/mL is considered to have *in vitro* cytotoxic activity, while purified compounds with an IC_{50} ≤ 4 µg/mL are highly active. In this study, SME exhibited strong cytotoxicity towards C32 cells with an IC_{50} value of 5.62 ± 0.58 µg/mL, while other extracts did not achieve an IC_{50} below 20 µg/mL. The LME showed an IC_{50} value greater than 20 µg/mL; it fell within the range of 19.17–26.21 µg/mL, suggesting potential cancer-inhibitory effects. Previous studies indicated that hydro-alcoholic extract from leaves and seeds of *J. podagrica* showed cytotoxic activity against two cancer cell lines: PC12 (rat pheochromocytoma) and A549 (human lung adenocarcinoma cells). Interestingly, it did not show cytotoxicity in normal cells, consistent with our study (32). In addition, it exhibits antioxidant effects and protects cells from oxidative stress that leads to DNA damage and cell mutations. However, previous studies have found that japodagricanones A and B from *J. podagrica* leaves and twigs are not active compounds because they had no significant effects against various human cancer cell lines (33). Moreover, stem extract has been reported to cause apoptosis of two human osteosarcoma cells (Saos-2 and MG-63) in a dose-dependent manner (34). While fraxidin, acetylaleuritolic acid, and γ-sitosterol, that isolated from the stem and root of *J. podagrica,* showed activity against HeLa (cervical carcinoma) with IC_{50} values of 39.9, 35.7, and 15.9 µg/mL, respectively (35). The SI indicated that SME is both safe and effective, targeting cancer cells specifically without significant toxicity to normal cells. The SI for SME was higher than

that for 5-FU, a drug used widely in cancer chemotherapy. Additionally, the % survival of cells generally decreased with increasing concentration, but the extract showed an increased % survival at 100 µg/mL. This increase may be due to the presence of lectins, which are carbohydrate-binding proteins that can non-specifically bind SRB dye (36).

LC-MS/MS analysis identified two prominent compounds in the SME extract. Compound 1, with a retention time of 5.357 minutes, was identified as fraxetin based on its $[M-H]^-$ ion at m/z 207.02990 and other characteristic ions (Fig. 5). Compound 2, with a retention time of 5.943 minutes, did not match any known spectra but had a $[M+H]^+$ ion at m/z 223.06010, suggesting it could be the positional isomer tomentin (Fig. 6). Fraxetin and tomentin are known to possess various biological activities, including antioxidant, anti-bacterial, and anti-tumor effects (7-11,29,37-40). Fraxetin, a coumarin compound, affects several signaling pathways involved in cancer progression, including inducing G2/M cell cycle arrest in melanoma cell proliferation and inducing S-phase cell cycle in colon adenocarcinoma (COAD) cell proliferation (8,29). In addition, fraxetin inhibits the proliferation, migration, and invasion of DU145 prostate cancer cells by inducing apoptosis (9). Several studies about fraxetin showed anti-proliferation and inhibited tumor growth, such as non-small-cell lung cancer (NSCLC), breast cancer (MCF-7), lung cancer, and liver cancer (Huh7 and Hep3B) (10,29,37-38). Moreover, fraxetin inhibits the proliferation and metastasis of glioma cells by inactivating JAK2/STAT3 signaling and induces cell cycle arrest in the G0/G1 phase. Additionally, fraxetin increases the expression of pro-apoptosis members like Bax and decreases the expression of anti-apoptotic members such as Bcl-2 and Bcl-XL (11,39). Furthermore, fraxetin treatment of MG-63 cells prevented the synergistic effect of anti-Fas IgM with TNF-alpha or IL-1beta on cell death, in addition to inhibiting anti-Fas IgM-induced apoptosis. Inhibition of TNF-alpha and IL-1beta-mediated Fas expression and increased FLIP expression are linked to fraxetin's apoptotic inhibition, which suppresses caspase-8 and caspase-3 activation (40). Tomentin showed *in vivo* anti-inflammatory activities by reducing the formation of λ-carrageenan footpad edema and auricular edema by 58% and 57%, respectively. at 45 mg/kg (12).

CONCLUSION

Jatropha podagrica extracts demonstrated notable biological activity, with SME showing significant anti-inflammatory and anticancer effects. While RMH effectively inhibited NO production, it is toxic to cells. Aqueous extracts, in contrast, showed no significant effect. All extracts failed to inhibit inflammation through the PGE_2 and TNF-α pathways. SME emerged as the most promising extract, demonstrating strong cytotoxicity against skin cancer cells while maintaining safety towards normal non-cancerous cells. Fraxetin and tomentin, detected in SME, may be key active compounds. This study supports the traditional use of *J. podagrica* for treating skin diseases and cancer; however, further investigative research, including product development and clinical trials, is needed to fully evaluate its therapeutic potential.

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Conflicts of interest statement

The authors disclosed no conflicts of interest in this study.

Authors' contributions

This project was conceived and supervised by A. Itharat. R. Sriyom was the principal researcher, summarized data, and wrote the manuscripts with support and edited by A. Itharat and O. Prajuabjinda. P. Thongdeeying advised on the analysis of LC-MS results. S. Ruangnoo and P. Monkanna carried out the cytotoxic experiments. S. Makchuchit and K. Namphonsaen conducted the anti-inflammatory experiments.

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