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Research article

HPLC analysis and *in vitro* antioxidant mediated through cell migration effect of *C.hystrix* water extract on human keratinocytes and fibroblasts

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ABSTRACT

Citrus hystrix or kaffir lime is a native tropical plant containing a high level of phenolic and flavonoid compounds. Its fruits are used as a food ingredient to enhance the sour-sweet scent and flavor in many dishes. Due to its polyphenol-containing, it has also been used as traditional medicine for health benefits including oral and gum health, stress relief, hair care, and skincare. In this study, we demonstrated the antioxidant activity of *C. hystrix* water extract and its effect on human keratinocyte and fibroblast migration. The extract showed a high amount of phenolic and flavonoid contents. The HPLC analysis indicated the presence of gallic acid, catechin, caffeic acid, rutin, and quercetin. We showed that *C. hystrix* water extract exhibited free radical scavenging capacity, determined by DPPH assay, with IC_{50} of 14.91 mg/mL, and nitrite radical scavenging capacity, determined by NO assay, with IC_{50} of 4.46 mg/mL. The *C. hystrix* water extract displayed unnoticeable toxicity at all tested doses. We showed that the treatment of water extracts as low as 50 μg/mL decreased the reactive oxygen species (ROS) from H_2O_2-induced ROS formation in both cell lines. Besides, *C. hystrix* water extract promoted cell migration in a dose-dependent manner. Together, these results demonstrated the positive benefit of *C. hystrix* water extract as a wound-healing accelerator. Its health benefits may be due to the antioxidant capability of its phytochemical compounds contained in *C. hystrix* water extract that enhances the migration of two major cell types: fibroblast and keratinocytes, responsible for the proliferation and remodeling phase of wound healing.

1. Introduction

Citrus fruits are abundant in multiple phytoconstituents and have increased attention for studying to improve health outcomes. Their leaf, peel, and juice have been reported to contain high levels of phenolic, flavonoid, tannin, alkaloid, tocopherol, glycerol glycolipid, furanocoumarin, and ascorbic acid [1–3]. The essential oil is abundant with many volatile compounds such as limonene,

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citral, terpinen-4-ol, α-Pinene, citronellal, camphene, nerol, *trans*-geraniol, and β-pinene [4,5]. Multiple evidence has suggested the pharmacological properties of citrus. For example, the flavonoid-rich fraction of bergamot (*Citrus bergamia*) juice exerted antioxidant and anti-inflammatory activities against an intestinal ischemia/reperfusion (I/R) injury in mouse models [6]. The chloroform extract of bergamot fruit exhibited an inhibitory effect on IL-8 expression which could reduce the inflammation of cystic fibrosis [1]. Besides bergamot, kaffir lime is another citrus fruit that is widely used in Traditional Ayurvedic and Thai medicine.

Citrus hystrix, also known as kaffir lime, is a citrus fruit in the Rutaceae family. It is a native plant in Southern China and Tropical Southeast Asia [3]. Kaffir lime is generally grown worldwide as a garden shrub. In Thailand, kaffir lime is extensively used as an ingredient in several Thai curry dishes to enhance flavor and aroma of food. Raw kaffir limes are green at their harvesting stage. The whole fruit and its juice are used in various food recipes. In addition to its versatile cooking usage, kaffir lime is also used in traditional medicine. Its fruit is used to alleviate various health conditions including inflammatory illness, antimicrobial infection, neurodegenerative disorders, cardiovascular health, fever, headache, flu, and sore throats, and skin disorders [3,7–9]. The ethanol extract of kaffir rinds and leaves have a neuroprotective effect, which prevented SH-SY5Y, human neuroblastoma cell's senescence upon high-glucose induction [10]. And the presence of anti-tyrosinase, acetylcholinesterase, and β-glucuronidase activities was observed in *C. hystrix* juice, which has been suggested to be used as nutraceuticals [11].

A wound is an injury that breaks the integrity of skin. Wound healing is fascinating. It comprises of dynamic overlapping processes including homeostasis, inflammation, proliferation, and remodeling [12]. It occurs through the crosstalk of cell communication and cell-cell interaction among macrophages, neutrophils, endothelial, keratinocytes, and fibroblasts [12,13]. During the inflammation phase, macrophages and neutrophils secrete reactive oxygen species (ROS) to defend against the invading pathogens [14]. Next, in the proliferative phase, the different blood cells arrive including platelets, macrophages, neutrophils release various growth factors and cytokines to initiate the keratinocyte and fibroblast proliferation and migration toward the wound area [12]. Fibroblasts undergo the phenotypic transition to myofibroblast which contributes to the collagen synthesis, which initiates the tissue granulation and provokes wound contraction and remodeling [12,15].

The impairment of wound healing is clinically problematic as this results in a chronic wound. It may be caused by the overproduction of ROS from uncontrolled inflammatory responses or microbial infection [16]. Thereby, the phytochemical with antioxidant properties has received increasing attention as it could be the effective remedy for alleviating chronic wounds [17]. Despite accumulating data showing its pharmacological effect, it had no scientific evidence exploring the effect of *C. hystrix* on wound healing enhancers. Therefore, the present study aimed to determine *in vitro* antioxidant activity of whole *C. hystrix* fruit extract and the potential reaction on the human cell-based assay.

2. Materials and methods

2.1. Plant material and preparation

The *Citrus hystrix* DC specimen was collected from Lamtakhong Research Station, Pak Chong District, Nakhon Ratchasima Province, Thailand. The plant specimen has been identified by Siri Ruckhachati: Thai Medicinal Plants Information Center, Mahidol University, Thailand as herbarium No. PBM 005823. For material preparation, *C. hystrix* fruits were washed with clean water, blended whole using an electronic blender, and then dried using a freeze dryer. After that, the fine powder was further extracted in either water or methanol solvent.

2.2. Preparation of the extracts

A water extraction process has followed the same steps as described previously [18]. In brief, 500 μL of sterile water was heated to a boil on a hotplate. 50 g of fine *C. hystrix* powder was added, mixed thoroughly, and boiled for 5 min. The extract was cooled down and filtered through Whatman No.1 filter paper. The filtrate was dried using a freeze dryer for 24 h.

A methanol (MeOH) extraction process has followed the same steps as described previously [19]. In brief, 100 g of fine powder was mixed with 500 mL of 95% methanol and kept in an amber bottle. The solvent was left on a shaker at room temperature for three days. The methanol extract was filtered through Whatman No.1 filter paper. The residue from filtration was repeatedly extracted in methanol two more times. The filtrates were evaporated in a rotary evaporator to remove methanol.

A stock solution of both extracts was dissolved in DMSO to get a final concentration of 200 mg/mL for further study. The final concentration of vehicle control DMSO was restricted at 0.1% in the entire study.

2.3. Phenolic and flavonoid contents

The phenolic and flavonoid contents in water extract and methanol extract were determined as previously described [20,21]. The polyphenols were determined by the colorimetric method. 10 μL of the sample dissolved in deionized water (790 μL) and mixed with Folin-Ciocalteu's phenol reagent (50) was incubated at room temperature for 5 min. Then, the sample was added to 150 μL of saturated sodium carbonate solution and further incubated at room temperature for 50 min. The data absorbance was measured at 765 nm using a UV–Vis-spectrophotometer (Jasco Corporation). The concentration of phenolic contents was calculated from the standard curve of gallic acid and expressed as mg of gallic acid equivalents per gram of extract (GAE/g extract). The flavonoid contents of the extract were determined by the aluminum chloride colorimetric method. 60 μL of 1 M potassium acetate was added to 300 μL of the 5 mg/mL C. *hyrax* water and methanol extracts (dissolved in deionized water) and further added with 60 μL of 10% of aluminum chloride

solution followed by the incubation of mixture sample at room temperature for 30 min. Quercetin was used as a standard and the absorbance was spectrophotometrically measured at 415 nm. The data can be represented as mg quercetin equivalent per g of water extract (mg QE/g extract).

2.4. High performance liquid chromatography (HPLC) analysis

The determination of phenolic compound in the extract was investigated by HPLC (Waters® 2695 Separations Module, USA) as previously described with some modification [22]. The HPLC column was carried out using the Reliant C18 column at 4.6 × 250 mm and 5 μm of particle size. Phenolic compounds present in the water extract were identified using phenolic compounds from Sigma-Aldrich (Sigma-Aldrich, St Louis, USA). The mobile phase A and B were acetonitrile (solvent A) and 0.5% TFA (solvent B). The mobile phase was run in the following gradient elution program: 0 min, 100%B; 5 min, 5%A/95%B; 10 min, 10%A/90%B; 20 min, 15%A/85%B 30 min 25%A/75%B and 51min, 100%B. The flow rate was 1 mL per minute for a 60-min duration. The injection volume was 10 μL of each sample. The signal detection was determined using a UV–Vis detector at a wavelength of 280 nm. The data was analyzed by Empower 3 Chromatography Data Software.

2.5. In vitro antioxidant determination of the water extract

2.5.1. DPPH free radical scavenging capability

The antioxidant activity was determined by DPPH free radical scavenging as previously described [23,24]. In brief, the water extract with various concentrations dissolved in methanol was mixed with DPPH solution. The reaction sample was incubated in the dark for 15 s and immediately measured at 515 nm using a microplate reader (Bio-Tek Instruments, Winooski, VT, USA). The percentage of inhibition was calculated using the following formula:

$$\text{DPPH free radical scavenging activity } (\%) = \frac{\text{Abs of control–Abs of extract or standard} * 100}{\text{Abs of control}}$$

$$\text{Abs} = \text{Absorbance}$$

2.5.2. Nitric oxide (NO) free radical scavenging capability

The NO free radical scavenging assay was determined by the Griess test. The Griess reagent can be reacted with nitrite, a stable metabolite product of NO. The water extract dissolved in 100 μL of 10% DMSO were mixed with 1 M of sodium nitroprusside in phosphate buffer solution then incubated under ultraviolet-B radiation chamber at a dose of 20 mJ/cm^2 for 10 min (BIO-LINK®, Vilber Lourmat UV-Crosslinker, France). 10% DMSO was used as vehicle control, while the gallic acid solution was used as a positive control. After that, the sample mixture was mixed with 500 μL of Griess reagent. The absorbance of nitrite generation was measured at 546 nm using a microplate reader (Bio-Tek Instruments, Winooski, VT, USA). The percentage of nitrite inhibition was calculated using the following formula:

$$\text{NO free scavenging capacity } (\%) = \frac{\text{Abs of control–Abs of extract or standard} * 100}{\text{Abs of control}}$$

$$\text{Abs} = \text{Absorbance}$$

2.6. Cell culture

Human keratinocyte (HaCaT) and Human 68 fibroblast (HFB) cells were purchased from Cell Lines Service (Germany) and ATCC (USA). The cells grew in Dulbecco's Modified Eagle Medium; DMEM with sodium pyruvate and L-glutamine (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (Gibco BRL, Gaithersburg, MD, USA) and incubated at 37 °C, 5% CO_2 incubator. The cells were sub-cultured by trypsinization when they reached 80% confluency.

2.7. Cell viability

The sublethal dose of *C. hystrix* water extract was assessed by using a resazurin cell viability kit (Sigma-Aldrich, St. Louis, MO, USA). Cells were plated on a 96-well plate at a seeding density of 2 × 10^4 cells per wells and treated at various concentrations of the extract (25, 50, 100, and 200 μg/mL). Upon 24 h of treatment, the viability of cells was measured by using a fluorescent microplate reader at 530/590 nm (Bio-Tek Instruments, Winooski, VT, USA). All experiments were carried out in four replicates. The percentage of cell viability was calculated using the following formula:

$$\text{Cell viability } (\%) = \frac{\text{Fluorescence intensity of sample} * 100}{\text{Fluorescence intensity of control}}$$

2.8. DCFH-DA intracellular ROS assay

The antioxidant activity of the extract was determined by (2′-7′dichlorofluorescin diacetate) DCFH-DA intracellular ROS assay as previously described [20]. Both keratinocytes and fibroblasts were seeded into 24 well plates at 5 × 10^4 cells/well and incubated at

37 °C in 5% CO_2 incubator overnight. The cells were pre-treated with various sublethal concentrations of extract (50, 100, and 200 μg/mL) for 2 h. The cells were then stained with 20 μM of DCFH-DA for 20 min and followed by one-time PBS washing. After then, keratinocytes and fibroblasts were treated with hydrogen peroxide (H_2O_2) as previously described in Ref. [20]. Briefly, keratinocytes were treated with 2 mM H_2O_2 and fibroblasts were treated with 1 mM H_2O_2 for 2 h at 37 °C in 5% CO_2 incubator. After 45 min of cell incubation, the DCF fluorescent intensity was immediately measured at 485/530 nm using a fluorescent microplate reader (Bio-Tek Instruments, Winooski, VT, USA). The antioxidant activity of the extract was calculated from the fluorescence intensity and showed as relative fold changes to control.

2.9. In vitro scratch assay

The cell migration capacity of the extract was estimated by the scratch assay as previously described by Fronza, M. et al. [25]. The keratinocytes and fibroblasts were seeded into 6 well plates at a concentration of 4×10^4 cells/well and incubated for 24 h at 37 °C in 5% CO_2 incubator overnight. The next day, a sterile yellow pipette tip (size 200 μl) was used to scratch the adherent cell. The detached cells were removed with one-time PBS rinsing, followed by cell culture medium replacement. The sublethal dose of water extract (50, 100 and, 200 μg/mL) was added to the cell culture plate and further incubated for 15 h at 37 °C in 5% CO_2 incubator. 0.1% DMSO (Sigma Aldrich, St. Louis, MO, USA) was used as vehicle control, while allantoin, a commercial wound dressing was used as a positive control [26]. The total area was visualized and captured under the inverted brightfield microscope. The images were analyzed by using ImageJ. The wound coverage area, from three separated experiments, at the initial time point and the endpoint was determined. The rate of cell migration was calculated by using the following formula:

$$\text{Cell migration } (\%) = \frac{\text{Area at 0 h} - \text{Area at 15 h} * 100}{\text{Area at 0 h}}$$

2.10. Statistical analysis

All experiments were repeated at least three replicates. *In vitro* studies of phenolic and flavonoid contents and antioxidants were presented with mean ± SD. Other results were expressed as mean ± SEM. The statistical analysis was determined by using One-Way ANOVA followed by Tukey's test (GraphPad Prism program version 8). The *p-value* less than 0.05 was considered statistically significant.

3. Results

3.1. The presence of polyphenolic and flavonoid contents in the C. hystrix extracts

Polyphenolic and flavonoid compounds are the chemical structure containing repeated phenolic moieties. Based on the multiple hydroxyl substituents on an aromatic ring, they are good electron and proton donors. Thus, polyphenolic and flavonoid compounds can act as free radical scavengers. Several previous studies have revealed the correlation between polyphenol contents in many plants and its antioxidant activity [27,28]. It has been suggested that the higher polyphenol contents indicate the powerful antioxidant property [28]. In this study, we elucidated the presence of polyphenolic and flavonoid compounds in both water and MeOH extract. We showed that the water extract had phenolic and flavonoid contents equal to 24.77 ± 0.97 mg GAE/g extract and 15.43 ± 2.03 mg QE/g extract, respectively. Whereas the MeOH extract showed the contents of the phenolic and flavonoid contents equal to 20.52 ± 0.35 mg GAE/g extract and 6.95 ± 0.43 mg QE/g extract (Table 1), the calibration curves shown in Fig. 1A and B. The results suggested that the *C. hystrix* water extract had higher polyphenols and flavonoid contents than the MeOH extract. These results are correlated with the previous study described by Heim, K.E. et al. [29]. Thus, the water extract was used in the subsequent experiments of the entire study.

3.2. HPLC analysis

In comparison with the peaks of standard compounds (Fig. 2A), the HPLC chromatogram of *C. hystrix* water extract revealed the presence of multiple polyphenols at various retention times; 9.571, 19.972, 23.461, 30.005, and 41.738. These were identified as gallic acid, catechin, caffeic acid, rutin, and quercetin, respectively (Fig. 2B). Moreover, the polyphenols in *C. hystrix* water extract were quantified from the calibration curve of standard compounds. The highest active compound in the *C. hystrix* water extract was quercetin. The amount of other active compounds displayed in Table 2.

Table 1
The contents of total phenolics and flavonoids in the *C. hystrix* extracts.

C. hystrix	Phenolic contents (mg GAE/g extract)	Flavonoid contents (mg QE/g crude extract)
Water extract	24.77 ± 0.97	15.43 ± 2.03
MeOH extract	20.52 ± 0.35	6.95 ± 0.43

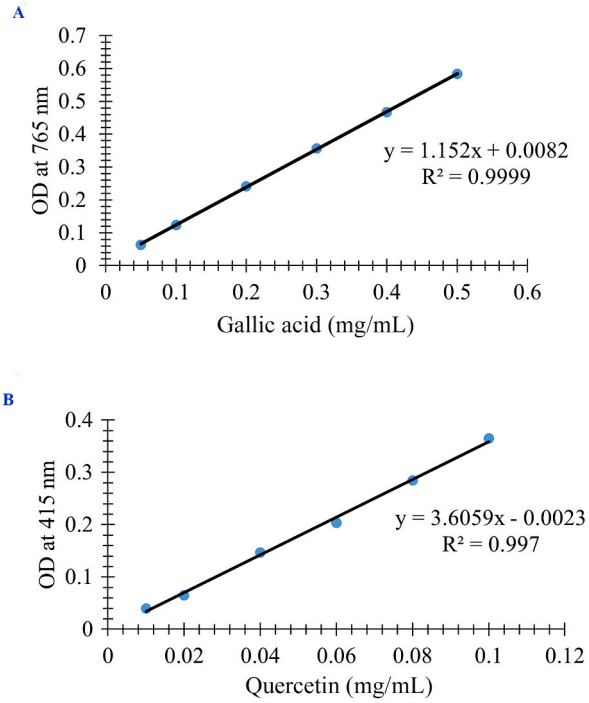

Fig. 1. **A** is graph plotted for gallic acid standard calibration curve with R-squared to quantify the content of phenolics. **B** is graph plotted for quercetin standard calibration curve with R-squared to quantify the content of flavonoids.

3.3. The presence of polyphenolic compounds correlated with antioxidant and nitric oxide scavenging properties

Reactive oxygen species (ROS) and nitric oxide (NO) act as a secondary messenger for multiple extracellular and intracellular mechanisms including wound healing. Due to their oxidative instability, the presence of ROS and NO has to be tightly controlled. The overproduction of ROS and NO may worsen wound healing and result in a chronic wound. In this study, we demonstrated the capability of the *C. hystrix* water extract to scavenge the ROS by using DPPH assay. The percentage of DPPH free radical inhibition shows in Table 3 and Fig. 3A (positive control; Fig. 3B). The IC_{50} of *C. hystrix* water extract was 14.91 mg/mL compared to positive control Trolox of 0.12 mg/mL. The capacity of NO radical scavenging was determined (Table 4 and Fig. 3C, positive control; Fig. 3D). The results showed that *C. hystrix* water extract exhibited NO scavenging capacity with IC_{50} of 4.46 mg/mL compared to positive control gallic acid of 30.19 μg/mL. Along with the ROS, NO is one of the secondary messengers involved in the inflammation and remodeling phase of wound healing. The uncontrolled NO production may result in a chronic wound as similar as ROS [30]. Although the ROS and NO scavenging capacity of the *C. hystrix* water extract was lower than the standard antioxidants; Trolox and gallic acid, it appeared that the inhibitory activity increased in a dose-dependent manner. Together, these results suggested the ability of the extract to donate electrons and protons to free radicals *in vitro*. This property may result by the presence of polyphenolic and flavonoid contents in the extract.

3.4. C. hystrix water extract had minimal effect on keratinocytes and fibroblast cell viability

We performed a resazurin cell viability assay to determine the toxicity of *C. hystrix* water extract of two major cell types involved in wound healing; keratinocytes and fibroblast. We found that all tested doses (25, 50, 100, and 200 μg/mL) have minimal effect on the keratinocyte viability compared to vehicle control after 24 h of treatment (Fig. 4A). Besides, the treatment of *C. hystrix* water extract at 50 μg/mL and higher exhibited the significantly increased viability of fibroblast cell line (Fig. 4B). This may result from the increase of metabolic activity from *C. hystrix* water extract or the induction of fibroblast cell proliferation. However, the proliferative capacity of *C. hystrix* extract has been yet determined in this study.

3.5. The protective effect of C. hystrix water extract against H_2O_2-induced oxidative stress

Besides the capability to scavenge free radicals, *C. hystrix* water extract also decreased the H_2O_2-induced oxidative stress in cell lines; determined by DCFH-DA staining assay [31]. The concentration of H_2O_2 to induce oxidative stress in two cell lines was determined in our previous study [20]. We showed that fibroblasts were more sensitive to H_2O_2 than keratinocytes. After 2 h of H_2O_2 treatment, fibroblast increased the presence of intracellular ROS up to 9.12 ± 0.45 folds, while keratinocyte showed about 3.12 ± 0.18

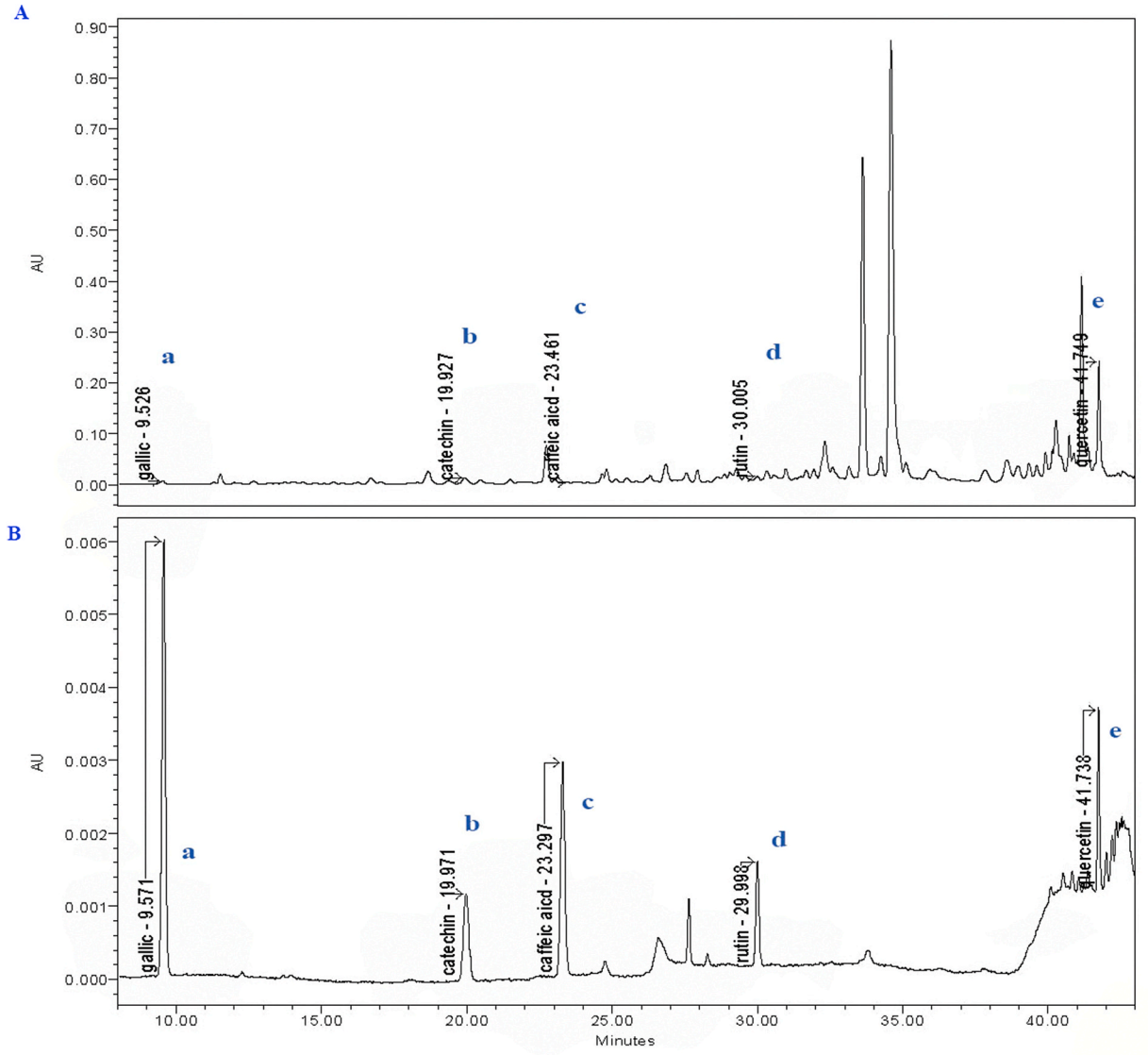

Fig. 2. HPLC chromatogram at 280 nm. **A** shows retention times of standard compounds, while **B** shows the presence of polyphenols content in the *C. hystrix* water extract.

Table 2
The quantification of the active compounds contained in the *C. hystrix* water extract.

Compounds	*C. hystrix* water extract (μg/mL)
Gallic acid	3.76
Catechin	116.83
Caffeic acid	322.18
Rutin	577.43
Quercetin	1874.12

folds in comparison to the condition without H_2O_2 treatment of each cell line (Fig. 5A and B). The treatment with the maximum concentration of water extract (200 μg/mL) did not induce the intracellular ROS in both cell lines. Interestingly, the pre-incubation of water extract for 2 h before H_2O_2 treatment resulted in the decrease of intracellular ROS in both cell lines. The reduction of intracellular ROS from *C. hystrix* water extract was displayed in a dose-dependent manner.

Table 3
IC_{50} values of DPPH radical scavenging activity of *C. hystrix* water extract.

% Inhibition on DPPH free radical	Concentrations (mg/mL)					IC_{50} (mg/mL)
	5	10	15	20	25	
The water extract	22.79 ± 0.72	39.69 ± 0.80	51.45 ± 0.34	62.25 ± 0.29	74.96 ± 0.66	14.91
Trolox	–	–	–	–	–	0.12

Trolox was used as a standard compound varying concentrations from 0.05 to 0.2 mg/mL.

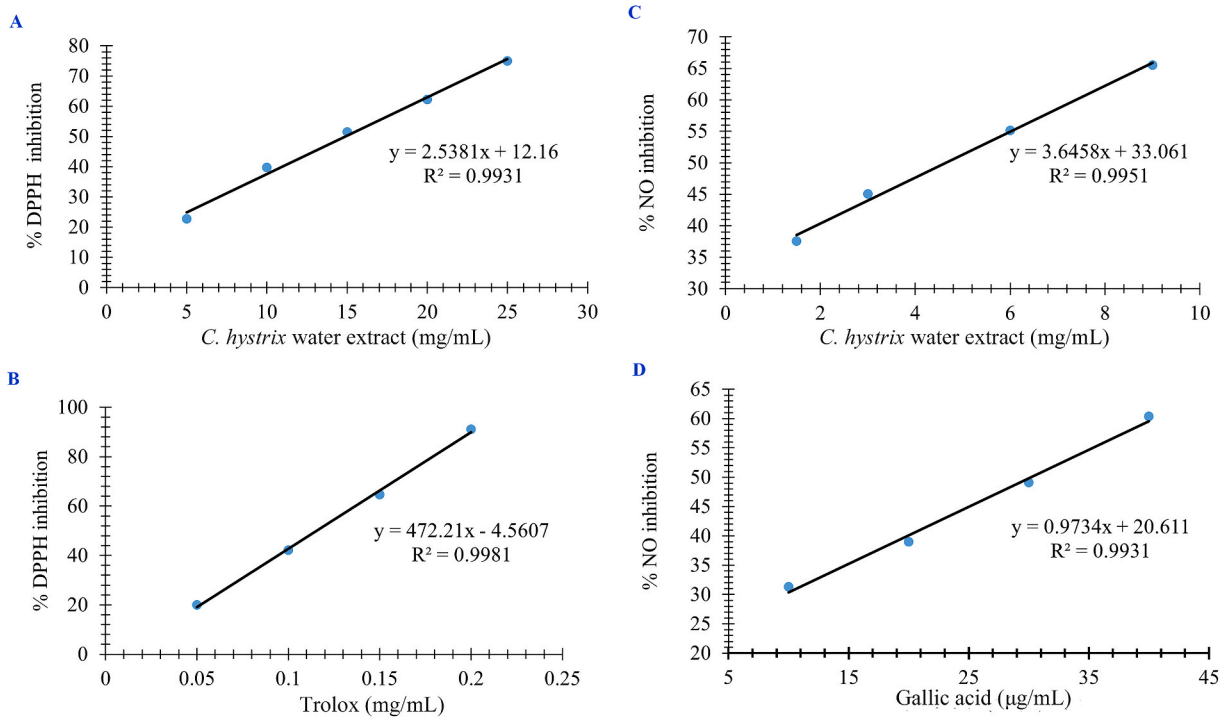

Fig. 3. Showing graphs for the assessment of the antioxidant activity in *in vitro* study. Graphs were plotted for the concentration of the *C.hystrix* water extract and standard compounds in mg/mL versus the percentage of DPPH and NO inhibition. The percentage of DPPH scavenging activity can be shown for the extract in graph (A) and for Trolox in graph (B). The percentage of NO scavenging activity can be shown for the extract in graph (C) and for gallic acid in graph (D).

Table 4
IC_{50} values of NO radical scavenging activity of *C. hystrix* water extract.

% Inhibition on NO free radical	Concentrations (mg/mL)				IC_{50}
	1.25	2.5	5	7.5	
The water extract	22.79 ± 0.72	39.69 ± 0.80	51.45 ± 0.34	62.25 ± 0.29	4.46 mg/mL
Gallic acid	–	–	–	–	30.19 µg/mL

Gallic acid was used as a standard compound varying concentrations from 5 to 45 µg/mL.

3.6. *Effect of the extract on the cell migration of keratinocytes and fibroblasts*

We showed that the highest treated dose of the water extract, 200 µg/mL has minimal cytotoxicity in both cell lines. In this experiment, the extract of 50, 100, and 200 µg/mL were considerably chosen to assess the cell migratory activity. Following the water extract exposure for 15 h, the wound coverage area was determined relative to the initial time point (time zero). The wound coverage area of time zero was expressed as 100% as shown in Fig. 6B, and Fig. 7B. The vehicle control had wound coverage of 74.42 ± 3.04% in keratinocytes and 53.09 ± 3.87% in fibroblasts compared with its time zero. In the treatment of commercial wound dressing, allantoin gave 50.22 ± 2.08% and 33 ± 1.71% in keratinocyte and fibroblast, respectively. We found that the *C. hystrix* water extract was capable to induce keratinocyte and fibroblast migration and was exhibited in a dose-dependent manner (Figs. 6A and 7A). The increasing dose from 50, 100–200 µg/mL reduced the wound coverage area of keratinocyte from 56.10 ± 2.04%, 45.05 ± 3.18%, and

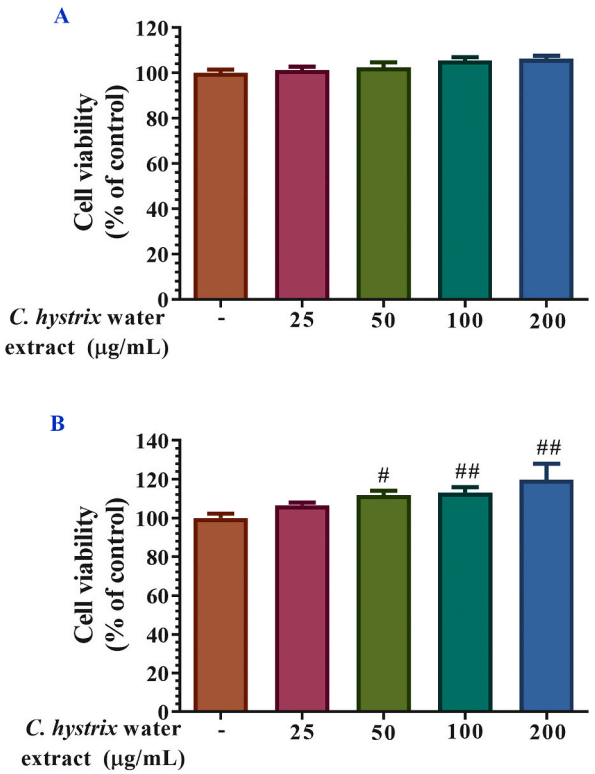

Fig. 4. The cell viability of the *C.hystrix* water extract-treated keratinocytes (**A**) and fibroblasts cells (**B**) for 24 h. The percentage of cell survival was determined using resazurin cell viability assay. The data was showed as mean ± SEM of six experiment with performed in triplicate. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ compared with control.

34.21 ± 2.68%, respectively (Fig. 6B). Whereas gave rise to 41.14 ± 2.01%, 32.84 ± 1.34%, and 24.05 ± 2.16%, respectively in fibroblast (Fig. 7B). We showed that the treatment of 200 μg/mL C. *hystrix* water extract in both cell lines was not statistically different from allantoin, a commercial wound-healing accelerator. These results indicated that *C. hystrix* water extract might be a potential candidate to promote wound healing.

4. Discussion

Nowadays, medicinal plants are considered safe and effective for accelerating the wound healing process. Due to the free-radical scavenging property of their phytoconstituents, medicinal plants such as *C. hystrix* have been widely used in skincare and wound care products [17,32]. The enrichment of several polyphenol and flavonoid contents in *C. hystrix* play a role as an electron and proton donor to the free-radical and transition metals [1,3]. Thus, it serves as an antioxidant compound and metal chelator [33,34]. In this study, the presence of phenolics and flavonoids in both water extract and methanol extract was evaluated, in which the polyphenol content in the latter extract is less than the former. This observation agreed with the result reported by previous studies showing that *C. hystrix* juice has a high amount of phenolic and flavonoid contents [35,36]. Together with the presence of polyphenol and flavonoid contents, the results showed the antioxidant property of *C. hystrix* water extract using DPPH and NO scavenging assays. The IC_{50} of DPPH free-radical scavenging was 14.91 mg/mL, while IC_{50} of nitrite radical scavenging was of 4.46 mg/mL. Besides the ability to reduce ROS and NO in a test tube, *C. hystrix* water extract showed the protective property against H_2O_2-induced ROS in keratinocytes and fibroblasts. The H_2O_2 exposure to keratinocytes and fibroblasts resulted in the increase of the ROS generation, determined by DCFH-DA staining. The pre-treatment of *C. hystrix* water extract for 2 h, before H_2O_2 treatment, decreased the ROS generation in both cell lines in a dose-dependent manner. We found the active antioxidant compounds including gallic acid, catechin, caffeic acid, rutin, and quercetin in the *C. hystrix* extract, which quercetin was found the most in the water extract. As described by previous studies, the combination of multiple chemical constituents of herbal extract have exerted their synergistic effect [37,38]. Thus, they were frequently use as a whole crude extract instead of using purified single compound. In thus study, we believed that the potential effects of *C. hystrix* water extract on wound-healing may result by multiple chemical constituents. Some compound may act on the similar target, while the of combination of multiple compounds caused synergistic effect.

Besides, this study firstly demonstrated the enhanced migratory capacity of *C. hystrix* water extract on keratinocytes and fibroblasts, the two major cell types involved in the wound-healing process. Previous studies reported that the citrus extracts contained antioxidant, anti-inflammatory, and anti-infective properties [10,35,36,39]. They found that the essential oil extract of *Citrus*

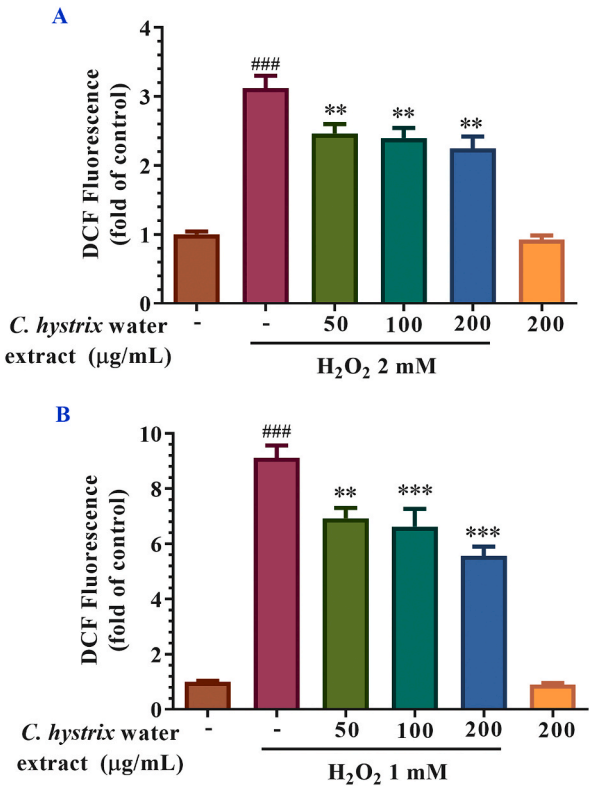

Fig. 5. The antioxidant effect of the *C.hystrix* water extract against H_2O_2-induced oxidative stress in keratinocytes and fibroblasts using DCFH-DA assay. The extracts were pre-incubated for 2 h on keratinocytes (**A**) and fibroblasts cells (**B**) before H_2O_2 treatment for 24 h. The detected ROS (DCF) intensities were quantified as folds of control. The data was showed as mean ± SEM (n = 6). $^{###}P < 0.001$ compared with control, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with H_2O_2 treated cells.

aurantium containing limonene (48.7%), linalyl acetate (23.6%), and linalool (5.5%) increased the ROS production in polymorphonuclear leukocytes [39]. This property contributed to the defense mechanisms against fungal and bacterial infection in the wound-healing process. Although the essential oil extract of *C.aurantium* induced the ROS generation in a concentration-dependent manner, the cytotoxicity is unlikely correlated with oxidative stress. In addition, the induction of ROS in polymorphonuclear leukocytes was lower than the cytotoxic value but attributed to the disinfection process [39]. The anti-infective property of citrus oil extract is likely correlated with its major constituent; limonene, a compound of terpenes family has been found in the rind of several citrus fruits. In this study, several volatile compounds including terpenes, aldehydes, lipids, alkanes, and psoralens were found in the *C. hystrix* water extract. This observation suggested the benefit of water extract in wound healing cascades, especially the inflammatory phase.

There is strong evidence reported that the damage from free radicals, including hydrogen peroxide, hydroxyl radical, nitric oxide, and superoxide anion prolonged the wound healing process [40–44], while plant enriched antioxidants could accelerate wound healing [22]. Thereby, the *C. hystrix* water extract which is composed of gallic acid, catechin, caffeic acid, rutin, and quercetin might alleviate the inflammation process. The treatment of *C. hystrix* water extract (25–200 μg/mL) had negligible cytotoxicity. In addition, the *C. hystrix* water extract significantly induced viability of fibroblast, determined by resazurin assay. This result might be due to the increase of cell number according to cell proliferation or the stimulation of mitochondrial activity. Together with the result from *in vitro* scratch assay that showed the decrease of wound closure as fibroblast migration and proliferation [45]. This result can conclude that *C. hystrix* water extract promoted fibroblast migration and somewhat cell proliferation capacity. Besides, the migratory ability of fibroblasts, *C. hystrix* water extract also promoted the migratory ability of keratinocytes. The highest dose of 200 μg/mL could induce the *in vitro* scratch wound closure in both cell lines and was not statistically different from Allantoin, the wound dressing drug.

Fibroblasts and keratinocytes are two major cell types involved in the wound-healing process. The migration and proliferation of fibroblast to the wounded site are mainly responsible for collagen deposit, wound contraction, and ECM synthesis [15], while keratinocyte is responsible for the re-epithelialization process which accelerates the wound-healing process from the proliferative phase toward the remodeling phase. Together, these finding concluded that *C. hystrix* water extract could be a promising therapeutic candidate for wound dressing products. However, further studies need to investigate the wound-healing in animal models. In addition, the molecular mechanisms associated with the wound-healing process have not been yet understood and need further study before manipulating the *C. hystrix* water extract as an ingredient in wound care materials.

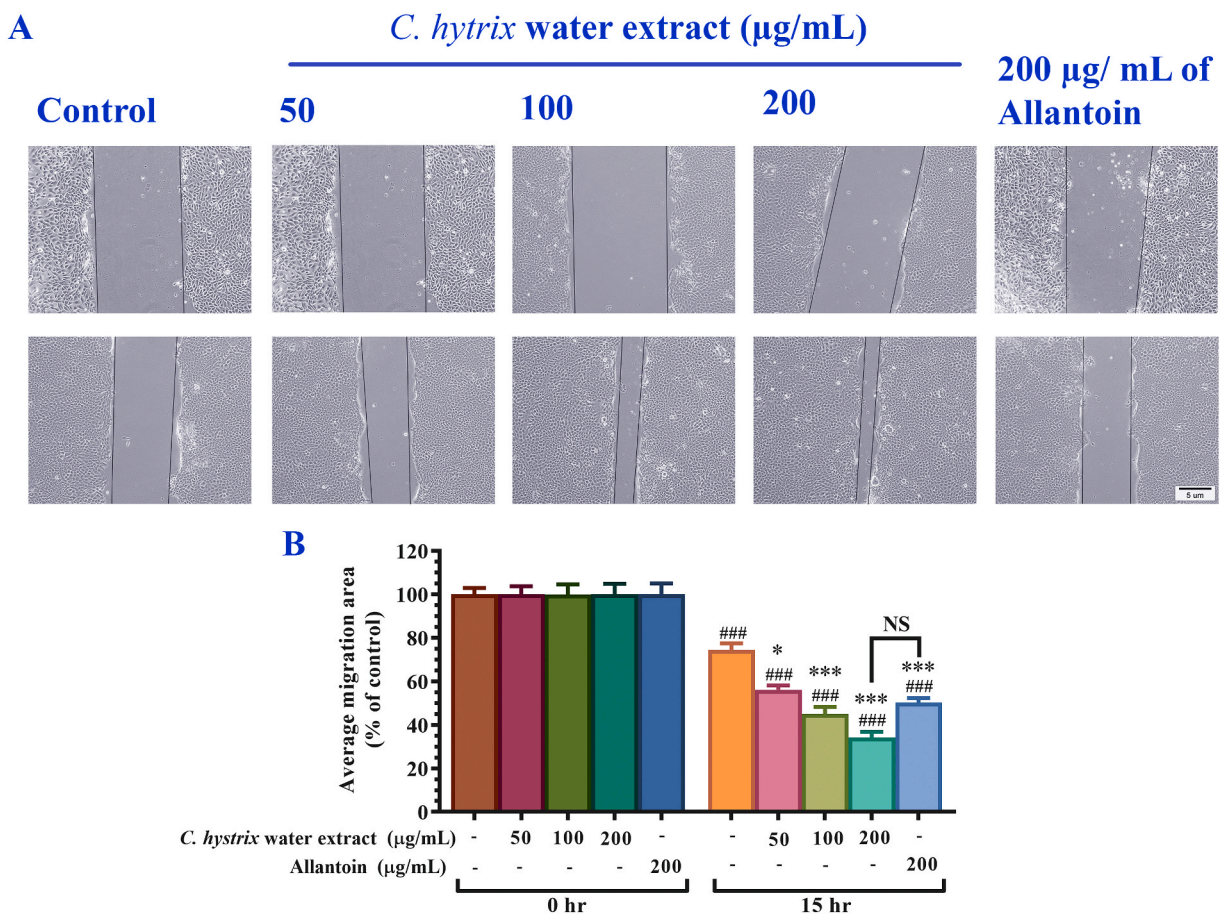

Fig. 6. Bright-field images representing the cell migration effect of keratinocytes-treated the *C.hystrix* water extract at 0 and 15 h using scratch assay (**A**). The cells were treated with 50,100, and 200 µg/mL of the extract and 200 µg/mL of allantoin for 15 h. The percentage of cell migration expressed as mean ± SEM of ten separate experiment was showed in figure **B**. $^{###}P < 0.001$ at 15 h of cell treatment versus the same experiment condition at 0 h of cell treatment. $^{*}P < 0.05$ and $^{***}P < 0.001$ against control group (0.1% DMSO). NS indicates non-significantly statistic between the extract and allantoin at 200 µg/mL.

Declarations

Author contribution statement

Yamaratee Jaisin: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Piyanee Ratanachamnong: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Poommaree Namchaiw: Contributed reagents, materials, analysis tools or data; Wrote the paper.

Cholticha Niwaspragrit: Punyabhorn Rattanacheeworn, Ph.D: Contributed reagents, materials, analysis tools or data.

Yotsayut Chunchaowarit: Performed the experiments; Analyzed and interpreted the data.

Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of interest's statement

The authors declare no conflict of interest.

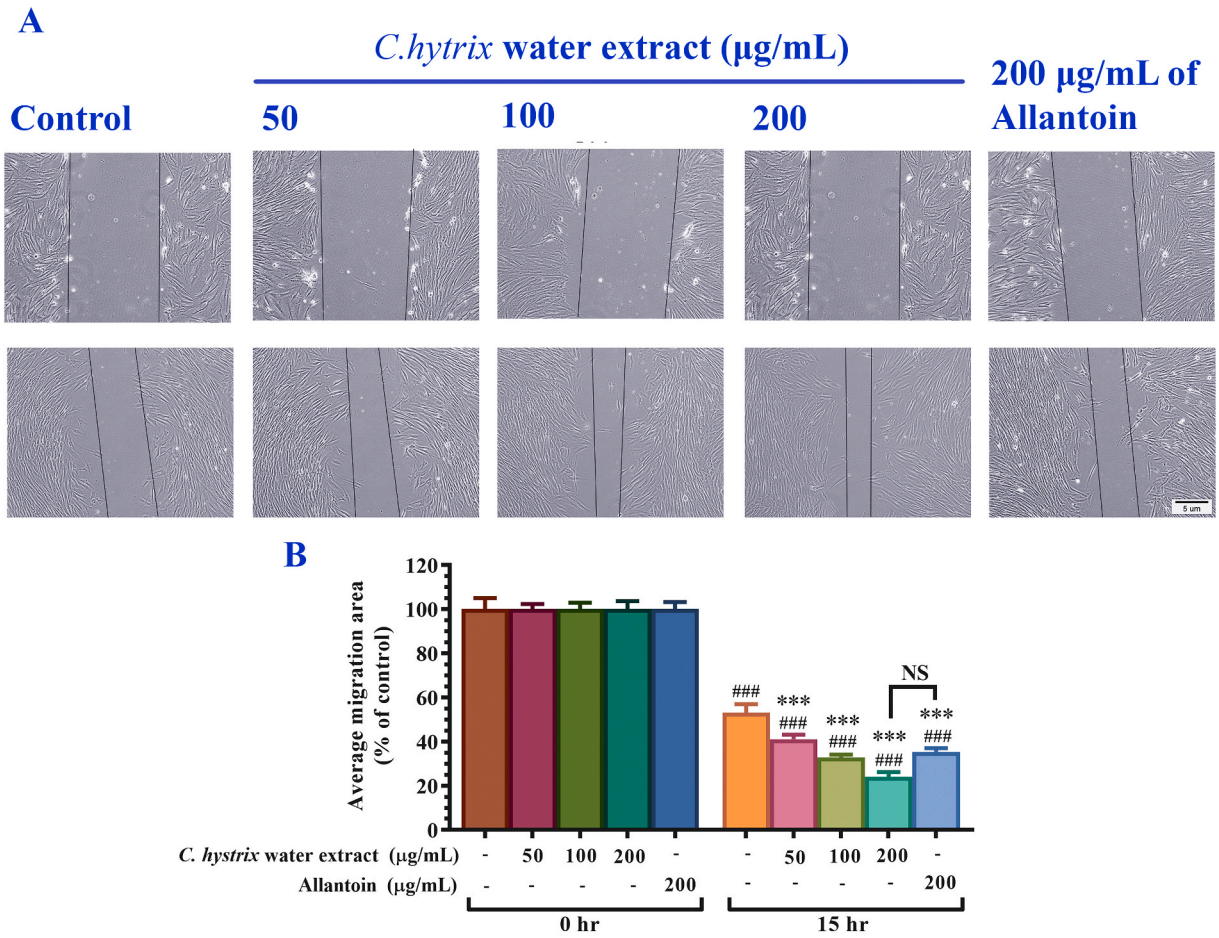

Fig. 7. Bright-field images representing the cell migration effect of fibroblasts-treated the *C.hystrix* water extract at 0 and 15 h using scratch assay (**A**). The cells were treated with 50,100, and 200 µg/mL of the extract and 200 µg/mL of allantoin for 15 h. The percentage of cell migration expressed as mean ± SEM of ten separate experiment was showed in figure **B**. $^{###}P < 0.001$ at 15 h of cell treatment versus the same experiment condition at 0 h of cell treatment. ***P < 0.001 against control group (0.1% DMSO). NS indicates non-significantly statistic between the extract and allantoin at 200 µg/mL.

Additional information

No additional information is available for this paper.

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