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Original article

Piper crocatum Ruiz & Pav. ameliorates wound healing through p53, E-cadherin and SOD1 pathways on wounded hyperglycemia fibroblasts

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ABSTRACT

Introduction: *Piper crocatum* Ruiz & Pav *(P. crocatum)* has been reported to accelerate the diabetic wound healing process empirically. Some studies showed the benefits of *P. crocatum* in treating various diseases but its mechanisms in diabetic wound healing have never been reported. In the present study we investigated the diabetic wound healing activity of the active fraction of *P. crocatum* on wounded hyperglycemia fibroblasts (wHFs).
Methods: Bioassay-guided fractionation was performed to get the most active fraction. The selected active fraction was applied to wHFs within 72 h incubation. Mimicking a diabetic condition was done using basal glucose media containing an additional 17 mMol/L D-glucose. A wound was simulated via the scratch assay. The collagen deposition was measured using Picro-Sirius Red and wound closure was measured using scratch wound assay. Underlying mechanisms through p53, αSMA, SOD1 and E-cadherin were measured using western blotting.
Results: We reported that F_{IV} is the most active fraction of *P. crocatum*. We confirmed that F_{IV}|(7.81 μg/ml, 15.62 μg/ml, 31.25 μg/ml, 62.5 μg/ml, and 125 μg/ml) induced the collagen deposition and wound closure of wHFs. Furthermore, F_{IV} treatment (7.81 μg/ml, 15.62 μg/ml, 31.25 μg/ml) down-regulated the protein expression level of p53 and up-regulated the protein expression levels of αSMA, E-cadherin, and SOD1.
Discussion/conclusions: Our findings suggest that ameliorating collagen deposition and wound closure through protein regulation of p53, αSMA, E-cadherin, and SOD1 are some of the mechanisms by which F_{IV} of *P. crocatum* is involved in diabetic wound healing therapy.
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Abbreviations: wHFs, wounded hyperglycemia fibroblasts; HFs, Hyperglycemia fibroblasts; NFs, Normal fibroblasts; MTT, 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenylte trazolium bromide; p53, tumor suppressor protein; αSMA, alpha smooth muscle actin; SOD1, superoxide dismutase 1; MeOH, Methanol; $CHCl_3$, Chloroform; DMEM, Dulbecco's Modified Eagle's Medium; TLC, Thin layer chromatography; WB, Washed benzene; ETOAc, Ethyl acetate; ROS, Reactive oxygen species.

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1. Introduction

Diabetic wounds remain a challenging health care problem. They frequently recur and cause considerable suffering, and health care costs (Jeffcoate et al., 2018). Several limitations of current diabetic wound therapies were observed in some studies. For example hyperbaric oxygen therapy has side effects such as oxygen toxicity (Han and Ceilley, 2017), while growth factor therapy and skin substitutes have excellent potential to improve wound healing but are limited due to their cost (Enriquez-Ochoa et al., 2020). Because of the economic burden in some developing countries that impacts in wound healing modalities, there is a need for discovering an alternative diabetic wound therapy that can alleviate any safety and be cost-effective (Cavanagh et al., 2012). To overcome these issues, diabetic wound healing can be facilitated by natural products of medicinal plants. The medicinal plants might be contributed in wound healing because of their bioactive compounds such as alkaloids, essential oils, flavonoids, tannins, terpenoids, saponins, and phenolic compounds (Ibrahim et al., 2018). These bioactive compounds can modulate one or more phases of the wound healing process. Furthermore, they are easily absorbed by the superficial layers of the skin. In comparison with chemical drugs and other synthetic drugs, herbal therapies were reported to be harmless, cheaper, and have more permanent curative potential (Verma et al., 2018).

Piper crocatum Ruiz & Pav (*P. crocatum*) is one of the popular traditional herbal medicines from Indonesia. This species is easily obtained, and has been used as herbal medicine empirically for generations in Asian countries to treat diabetic wounds (Sharma et al., 2013). Many studies have conducted the characterization of its compounds. *P. crocatum* leaves have been confirmed to have a total polyphenol content of 142.56 mg GAE/g in methanol (MeOH) extract (Prayitno et al., 2018) and 41.29 ± 0.52 GAE/g in the fresh leaves (Saputra et al., 2018). Megastigmane glucoside isomer, monoterpenes, sesquiterpenes, a phenolic amide glycoside, a neolignane, and a flavonoid C-glycoside were also found in recent chemical studies of *P. crocatum* MeOH extract (Li et al., 2019).

Extracts of *P. crocatum* with many of these ingredients have benefits in various therapeutic activities, including anti-microbial (Puspita et al., 2019; Rinanda et al., 2012), anti-diabetic (Nasi et al., 2015; Safithri and Fahma, 2008; Shinta and Sudyanto, 2016), anti-inflammatory (Fitriyani et al., 2011; Laksmitawati et al., 2017), and anti-oxidant (Hendryani et al., 2015; Tonahi et al., 2014). *P. crocatum* extracts have also been found to have proliferative activity in Baby Hamster Kidney Cell Line 21 (BHK-21) fibroblasts (Permadi et al., 2016). In contrast, other studies confirmed that *P. crocatum* extracts suppressed proliferation in both cancer cells and cancer wounds (Mulia et al., 2016; Wulandari et al., 2018; Zulharini et al., 2017). Thus, the compound of the extract needs to be simplified and standardized in the future with the intention of gaining compounds with consistent effects in treating diseases. In that case, the bioassay-guided fractionation is necessary to obtain the bio-selective compounds that are responsible for wound healing of the diabetic wounds (Weller, 2012).

Delayed wound healing in diabetic patients occurs due to vascular, immunity and bio-mechanic disorders. Hyperglycemia is an intrinsic factor that prolongs the proliferation and inflammation phase in diabetic wounds by interfering with fibroblasts' functions (Goulding, 2015). Diabetic fibroblasts are suspected of having mitochondrial damage which disrupts tissue cellularity through excessive apoptosis (Berlanga-acosta et al., 2013). Fibroblasts as the main architect cell of wound healing are contractile and have a significant function in the synthesis and deposition of the extra cellular matrix (ECM) (Putte et al., 2016).

Fibroblasts delay diabetic wound through several ways. Some of these delays are decreased production of collagen, transforming growth factor-β (TGF-β) (Gasca-lozano et al., 2017), E-cadherin (Keshri et al., 2016), over expression of senescence-associated secretory phenotype (SASP) (Wang and Shi, 2020) and accumulation of reactive oxygen species (ROS) (Cheng et al., 2018). Declines in the production of TGF-β decreases the expressions of alpha smooth muscle actin (αSMA) and further slows the differentiation of fibroblasts into myofibroblasts, resulting in wound contraction frailty and collagen deposition disruption (Martinelli-Kläy et al., 2014). The formation of αSMA and collagen is also influenced by E-cadherin level, a trans-membrane protein that mediates intercellular adhesion (Gasca-lozano et al., 2017).

E-cadherin deficiency in chronic wounds causes weakening of cells' bonding, thus inhibiting cell migration to the wound surface. The barrier of migration is exacerbated by SASP secretion which plays a role in increasing tissue destruction and stopping the cell cycle at G_0 (Deursen & van Deursen, 2014; Frykberg & Banks, 2015). This condition happens due to p53 activation as an effect of the decline of superoxide dismutase (SOD) 1. The decrease of SOD1 affects the secretion of collagen, αSMA and TGF-β because nuclear related factor 2 (Nrf2) needs to binds to SOD1 in stimulating the ECM production of targeted genes (Li et al., 2019). Accordingly, wounded hyperglycemia fibroblasts (wHFs) as an *in vitro* model for diabetic ulcers are important targets in the diabetic wound therapy. Our study aimed to identify the most active fraction of *P. crocatum* and to gain further knowledge concerning its effects in collagen deposition and wound closure via regulation of αSMA, p53, SOD1 and E-cadherin of wHFs.

2. Materials and methods

2.1. Plant materials

The *P. crocatum* leaves were collected from Magelang, Central Java, a district of Indonesia, on February 2017, identified at the plant systematic laboratory (voucher specimen no: 5284/UN/FFA/BF/PT/2018), and deposited in the Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta, Indonesia.

2.2. Bioassay-guided fractionation

2.2.1. Extraction

We performed extraction according to the procedures of Shamley and Wright (2017). Dried leaves were cut into pieces, dried, and ground into powder. 537 g of powder were extracted by maceration using methanol (MeOH) and the residue was extracted with chloroform ($CHCl_3$). Filtering was done using a Buchner funnel. The filtrate of each extract was then combined and evaporated. The success of extraction was monitored by thin layer chromatography (TLC) and further tested for its pro-healing activity.

2.2.2. Partition

Partition was done by the centrifuge method as performed in previous study (Rabiu and Haque, 2020). Seventy grams (70 g) of active extract were partitioned using wash-benzene (WB) to get wash benzene-soluble and wash benzene-insoluble compounds. The extract was dissolved in WB, stirred vigorously, put into the tube, and then centrifuged at 10,000 rpm for 10 min. WB-soluble and WB-insoluble sub-extracts then were stored in different porcelains and evaporated. The success of partition was monitored by TLC and further tested for its pro-healing activity.

2.2.3. Fractionation

The active sub-extract (41 g) was then fractionated by liquid chromatography that was modified using vacuum with a stationary phase of silica gel GF_{254} (Merck, 0.25 mm). The mobile phase used were wash benzene (100%), wash benzene: ethyl acetate (9:1; 8:2; 7:3; 6:4; 5:5; 4:6; 3:7; 2:8; 1:9 v/v), ethyl acetate (100%), and $CHCl_3$: MeOH (1:1 v/v). TLC identification of liquid chromatography yielded 12 fractions that were combined into four fractions, i.e., fraction I (F_I), fraction II (F_{II}), fraction III (F_{III}), and fraction IV (F_{IV}) based on spot similarity, then used for the pro-healing tests. The active fraction that had the highest percentage of proliferation from the MTT assay was then used for further testing (Yulianti et al., 2021).

2.2.4. Pro-healing activity assay

The pro-healing activity was observed with 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Bio basic, Ontario, Canada) assay, triplicate (Kumar et al., 2018). Briefly, wHFs (1×10^4) were seeded into 96-well plates and allowed to grow for 24 h. Cells then were treated with samples and incubated in 5% CO_2 at 37 °C for 24 h. Cells were washed and MTT solubilized with media was added to each well and incubated for four hours in incubator (5% CO_2, 37 °C). The MTT was then removed and 200 µl dimethyl sulfoxide (DMSO) were added as a stop solution. Absorbance was recorded at 570 nm using microplate absorbance reader (Imark™, Bio-Rad). The proliferation rates were counted using the following formula:

$$\%\ \text{living cells} = \frac{(\text{treated cell absorbance} - \text{media absorbance})}{(\text{controlled cell absorbance} - \text{media absorbance})} \times 100\%$$

2.3. Preparation of wHFs

Fibroblasts were grown from the circumcised human prepuce tissue purchased from dermatology laboratory and isolated by separating the dermal tissue from epidermal tissue. Normal prepuce tissues were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, New York, NY, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, New York, NY, USA), 1% penicillin–streptomycin (Gibco, New York, NY, USA), and 1% fungizone (Gibco, New York, NY, USA) until the 3rd passage. Subcultures were made by the warm trypsinization method (Ueck et al., 2017). A diabetic wound model was achieved by continuously growing cells under hyperglycemic conditions; cells were cultured in complete media (basal glucose concentration of 5.6 mMol/L) containing an additional 17 mMol/L D-glucose (Sigma-Aldrich, Cat. no. 110187-42-2). The wHFs were ready for treatment when they showed a significant decrease in numbers. Cell counting was performed using automatic cell counter Millipore Scepter 2.0 (Scepter™ Sensors, 60 um, Mexico City, Mexico). A wound was simulated *in vitro* via the scratch assays, whereby a sterile 1 mL disposable pipette was used to scrape the confluent monolayer in a diagonal line, creating a cell-free zone in the center with cells on either side of the 'wound' (Houreld et al., 2018).

2.4. Collagen assay

Collagen assay was performed in triplicate by Picro-Sirius Red method (Chen et al., 2013; Wirohadidjojo et al., 2011). Briefly, 96-well plates were seeded with 1×10^4 of hyperglycemia fibroblasts (HFs) in 100 µl complete high glucose DMEM (Gibco, New York, NY, USA). The HFs were grown for 24 h. Then, HFs were scratched (wHFs) and treated with samples and incubated in 5% CO_2 at 37 °C for 72 h. wHFs were washed and fixed with 100 µl Bouin's fixation solution (Sigma-Aldrich, Cat no. MFCD00146169) in the darkroom at room temperature for an hour. wHFs were washed carefully with distilled water and then dried overnight. wHFs were stained with 1% Picro-Sirius Red (Abcam, Cat. no. ab150681) for an hour at room temperature. wHFs were then washed with 0.1 hydrogen chloride (HCl), treated with 0.5 sodium hydroxide (NaOH) and incubated for 30 min at room temperature. Image acquisition was conducted using inverted microscope (Nikon's Eclipse TE2000-U, USA). Absorbance levels were recorded at 570 nm using microplate absorbance reader (Imark™, Bio-Rad). The collagen deposition was counted using the formula below:

$$\%\ \text{Collagen} = \frac{(\text{treated, cell, absorbance-media, absorbance})}{(\text{controlled, cell, absorbance-media, absorbance})} \times 100\%$$

2.5. Wound closure assessment

Wound closure assessment was performed in triplicate by scratch wound assay (Venter and Niesler, 2019). HFs (5×10^4) were seeded into 24-well plates and allowed to grow in 500 µl complete high glucose DMEM for 24 h. HFs were scrapped in a diagonal line (wHFs) and then treated with F_{IV} and incubated in 5% CO_2 at 37 °C for 72 h. wHFs were washed carefully, stained with hematoxylin solution and incubated at room temperature for an hour, then washed again. The images were taken with an inverted microscope (Nikon's Eclipse TE2000-U, USA) and imaged with Optilab^{pro} software for Windows. The percentage of wound closure was analyzed using Image J software for Windows with the guidance of Venter and Niesler (2019). Wound closure was calculated as the percentage of newly formed cells by fibroblasts covered area (5 images per sample) (Baranyi et al., 2019).

2.6. Protein extraction and western blotting of α-SMA, P53, SOD-1 and E-cadherin

The measurement of αSMA, p53, SOD1 and E-cadherin was done using western blotting, in triplicate. HFs (30×10^4) were seeded into 6-well plates and allowed to grow in 2 mL complete high glucose DMEM for 24 h and then scratched (wHFs). wHFs were then treated with F_{IV} (7.81; 15.62; 31.25) µg/ml and incubated in 5% CO_2 at 37 °C for 72 h.

Protein cells were extracted using M-PER® (Thermo; Cat. No. 87785) according to the manufacturer instructions. Cells were rinsed using PBS 2×10 minutes, scraped using scrapper, homogenized using 200 µl M-PER®, and centrifuged on 12,000 rpm for 20 min at 4 °C. Supernatant were stored at −80 °C. Protein quantification was conducted using Pierce™ 660 nm protein assay reagent (Thermo; Cat. No. 22660). 15 µg/µl protein was separated onto 10% SDS-PAGE and transferred to a polyvinylidene fluoride membrane (PVDF) (Immun-Blot®, Bio-Rad) and incubated with anti-p53 (Cat. No. A5761, anti-rabbit, 1:500 dilution), anti-αSMA (Cat. No. ab5694, anti-rabbit, 1:1000 dilution), anti SOD1 (Cat. No. ab51254, anti-rabbit, 1:1000 dilution), anti E-cadherin (Cat. No. ab1416, anti-rabbit, 1:1000 dilution), and anti-βActin (Cat. No. ab8227, anti-rabbit, 1:1000 dilution). A total of 5% skim milk in TBST was used for blocking followed by incubation with the appropriate secondary antibody. Proteins were visualized using ECL Prime Western Blotting Detection Reagents (GE Healthcare, RPN2232). Blots were photographed with a Gel doc machine (Geldoc Syngene Gbox Seri Chemi xrq) (Arfian et al., 2019; Jara et al., 2019).

This study received approval from Medical and Health Research Ethics Committee of the Medicine, Public Health and Nursing Faculty, Universitas Gadjah Mada (KE/FK/1238/EC/2018).

2.7. Statistical analysis

We used the ANOVA, Kruskal-Wallis, Tukey's test, Dunnett's test, Mann-Whitney *U*, and independent *T*-test for analysis of independent variable groups. Pearson correlation was used to analyze the underlying mechanism. Values are presented as mean ± standard deviation (SD) of the mean of the number of determinations. The degree of significance was set at $p < 0.05$, and all analyses were performed using SPSS (IBM, Corp, Armonk, NY) (Pallant, 2011).

3. Results

We performed bioassay-guided fractionation to select the active fraction. Prior to this process, we identified the passage of wHFs that showed a significant decrease in cell numbers. We found that wHFs at the 10th passage showed a significant decrease in cells numbers compare to wHFs at the first passage ($p = 0.016$; Independent *T*-test) (Fig. 1). Therefore, the wHFs of 10th passage were ready for treatment and were used in further testing.

3.1. The finding of the most active fraction

The bioassay-guided fractionation workflow is presented in Fig. 2**.** We first examined the proliferation activity of eight serial concentrations (3.91, 7.81, 15.62, 31.25, 62.5, 125, 250, and 500 μg/ml) in the wHFs. Methanol (MeOH) extract of *P. crocatum* had a higher proliferation activity compared to chloroform ($CHCl_3$) extract ($p = 0.003$; Kruskal-Wallis). As shown in Fig. 3**,** the proliferation of wHFs was induced by MeOH extract in a concentration-dependent manner. At concentrations of 3.91; 7.81; 15.62; 31.25; 62.5; 125; and 250 μg/ml, the MeOH extract of *P. crocatum* was able to increase proliferation rate of wHFs in the presence of seven different concentrations followed by MTT assay for cell survival after 24 h. Therefore, the MeOH extract was used for further separation (partition) using wash benzene (WB).

Applying in the wHFs, WB-soluble sub-extract of *P. crocatum* had a higher proliferation activity compared to WB-insoluble sub-extract ($p < 0.0001$; ANOVA). As shown in Fig. 4, the proliferation of wHFs was induced by WB-soluble sub-extract in a dose-dependent manner. At concentrations of 3.91; 7.81; 15.62; 31.25; 62.5; 125; and 250 μg/ml, the WB-soluble sub-extract of *P. crocatum* was able to increase proliferation rate of wHFs in the presence of seven different concentrations followed by MTT assay for cell survival after 24 h. Therefore, the WB-soluble sub-extract was used for further separation (fractionation).

Twelve fractions were obtained and were yielded for TLC identification. The fractions then were combined into four fractions: fraction I (F_I), fraction II (F_{II}), fraction III (F_{III}), and fraction IV (F_{IV}) based on spot similarity (Fig. 5). Furthermore, F_{IV} of *P. crocatum* had the highest proliferation activity as shown in Fig. 6. The proliferation of wHFs was induced by F_{IV} in a dose-dependent manner. At concentrations of 3.91; 7.81; 15.62; 31.25; and 62.5 μg/ml, F_{IV} of *P. crocatum* was able to increase proliferation rate of wHFs in the presence of five different concentrations followed by MTT assay for cell survival after 24 h. Therefore, F_{IV} was defined as the most active fraction.

Moreover, $\mathbf{F_{IV}}$ was yielded to TLC on silica gel using mobile phase of MeOH/$CHCl_3$ (1:2, v/v) and two drops of glacial acetic acid and observed at UV λ 366 nm. Glycoside polyphenol profile characterized by purple fluorescence (Lata and Mittal, 2017) was detected after being sprayed with citroboric acid (Fig. 5). F_{IV} was then applied to further testing with 72 h incubation period as the best incubation time (Fig. 7).

3.2. Effect of P. crocatum F_{IV} on collagen deposition of wHFs

To determine if F_{IV} has wound healing activity in producing the ECM, we assessed the collagen deposition induction of F_{IV} by using the Picro-Sirius Red method. The red staining represents collagen deposition. As a result, F_{IV} *P. crocatum* was found to possess wound healing activity *in vitro* trough ameliorating collagen deposition at the concentrations of (7.81, 15.62, 31.25, 62.5, and 125) μg/ml ($p < 0.0001$; ANOVA). As illustrated in Fig. 8, the lowest concentration of F_{IV} (7.81 μg/ml) exhibited the highest collagen deposition rate and showed significantly higher collagen deposition than control group ($p < 0.0001$; Dunnett's test). Moreover, collagen deposition arrangements were more compact and regular in the intervention groups (**Suppl.** Fig. 1).

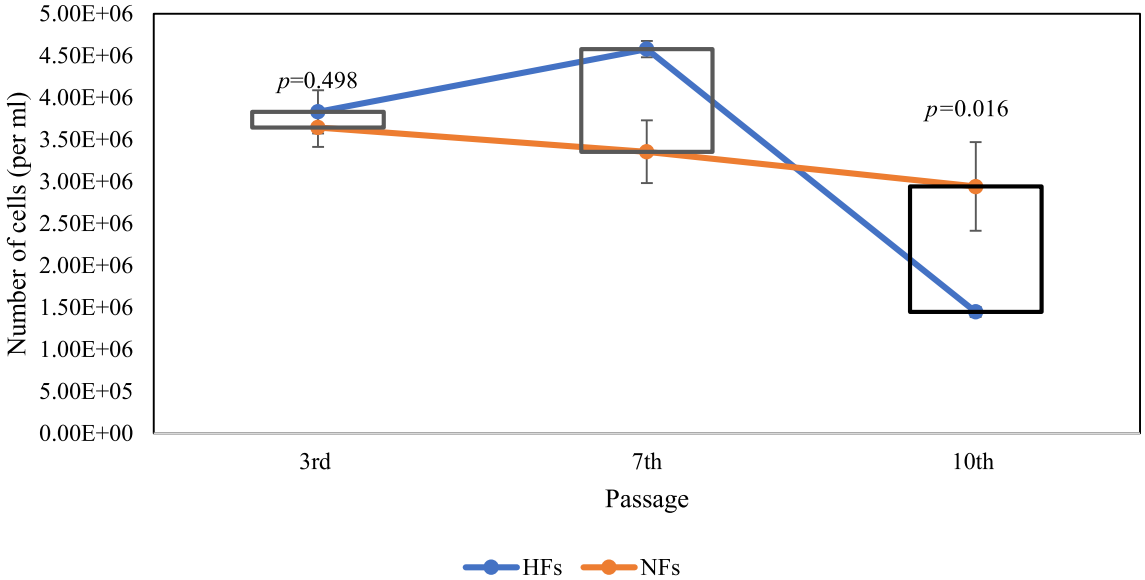


Fig. 1. The number of hyperglycemia fibroblasts (HFs) were significantly decreased at the 10th passage compared to normal fibroblasts (NFs) ($p = 0.016$; independent *t*-test).

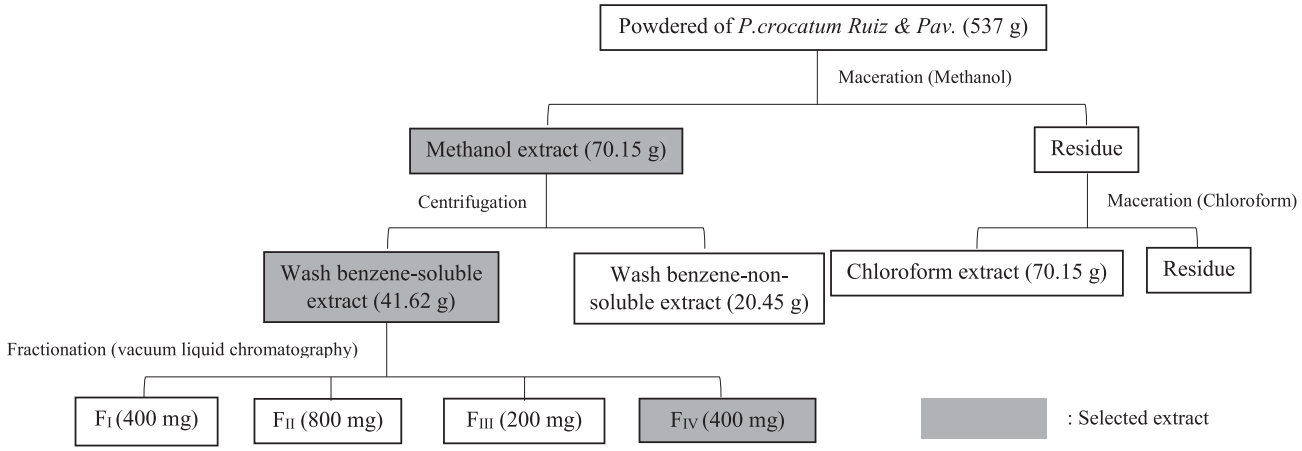


Fig. 2. The fractionation procedure.

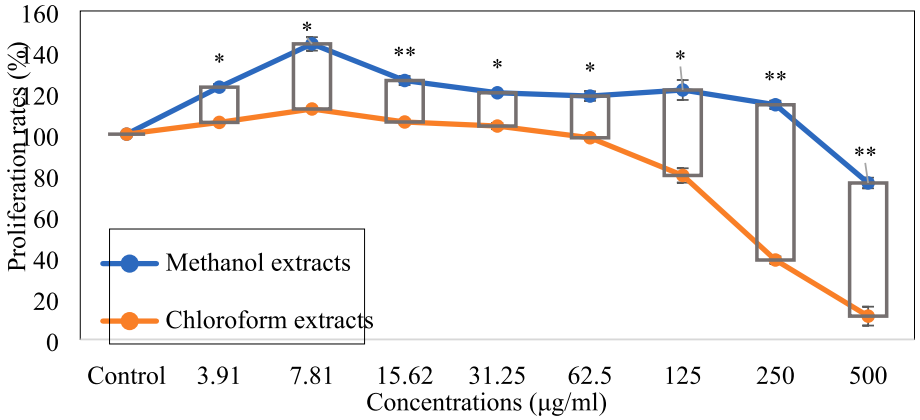


Fig. 3. F_{IV} of *P. crocatum* was identified as the most active fraction in promoting wHFs proliferation rates. Results shown in the graphs are mean ± SD from three independent experiments: The MeOH extracts were confirmed to promote significantly higher proliferation rates than $CHCl_3$ extracts (* $p < 0.05$, ** $p < 0.01$; Mann-Whitney U).

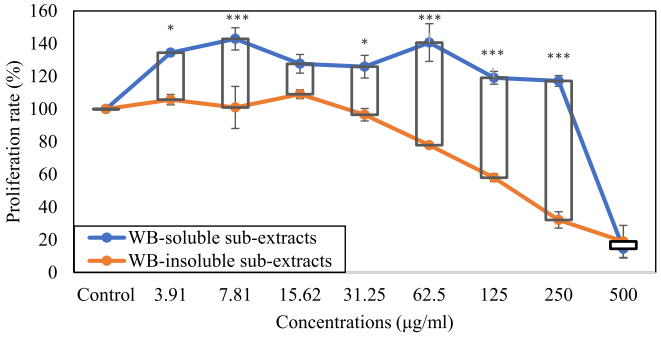


Fig. 4. WB-soluble sub-extracts were corroborated to induce significantly higher proliferation rate than WB-insoluble sub-extracts (* $p < 0.05$, ** $p < 0.001$; Tukey's test).

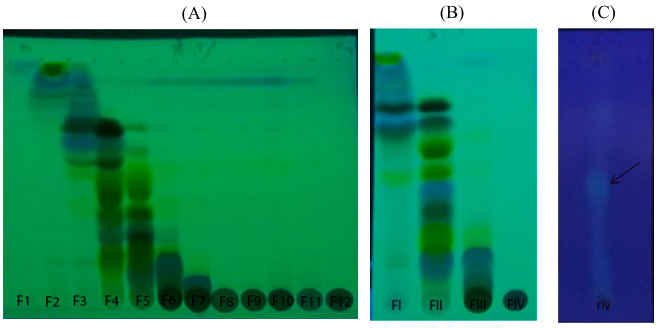


Fig. 5. The chromatogram of *P. crocatum* fractions: (A) The uncombined fractions ($F_{1\text{-}12}$) (silica gel 60 F_{254}; WB/ETOAc (3:1, v/v); λ 254 nm); (B) The combined fractions ($F_{I\text{-}IV}$) (silica gel 60 F_{254}; WB/ETOAc (3:1, v/v); λ 254 nm); (C) F_{IV} (silica gel 60 F_{254}; MeOH/CHCl3 (1:2, v/v) + two drops of glacial acetic acid; λ 366 nm).

3.3. Effect of P. crocatum F_{IV} on wound closure of wHFs

Scratch assay was performed to examine the wound closure of wHFs. Scratch assay is widely applied *in vitro* technique for understanding the wound healing properties of medicinally compound (Bolla et al., 2019). In the current study, wHFs were treated with five serial concentrations (7.81, 15.62, 31.25, 62.5, and 125 µg/ml) of F_{IV} *P. crocatum* for 72 h. The newly formed cell covering wound area was captured and calculated by Image J Software. The results indicated that F_{IV}, at all concentrations, closed the gap created by the scratch and the highest wound closure rate (98%) was achieved by F_{IV} at 7.81 µg/ml. Percentage of wound closure in intervention and control group at different concentrations have been represented in Fig. 9. **Suppl.** Fig. 2 shows the microscopic images of the intervention and control groups. The photographs show increased wound closure in the intervention groups.

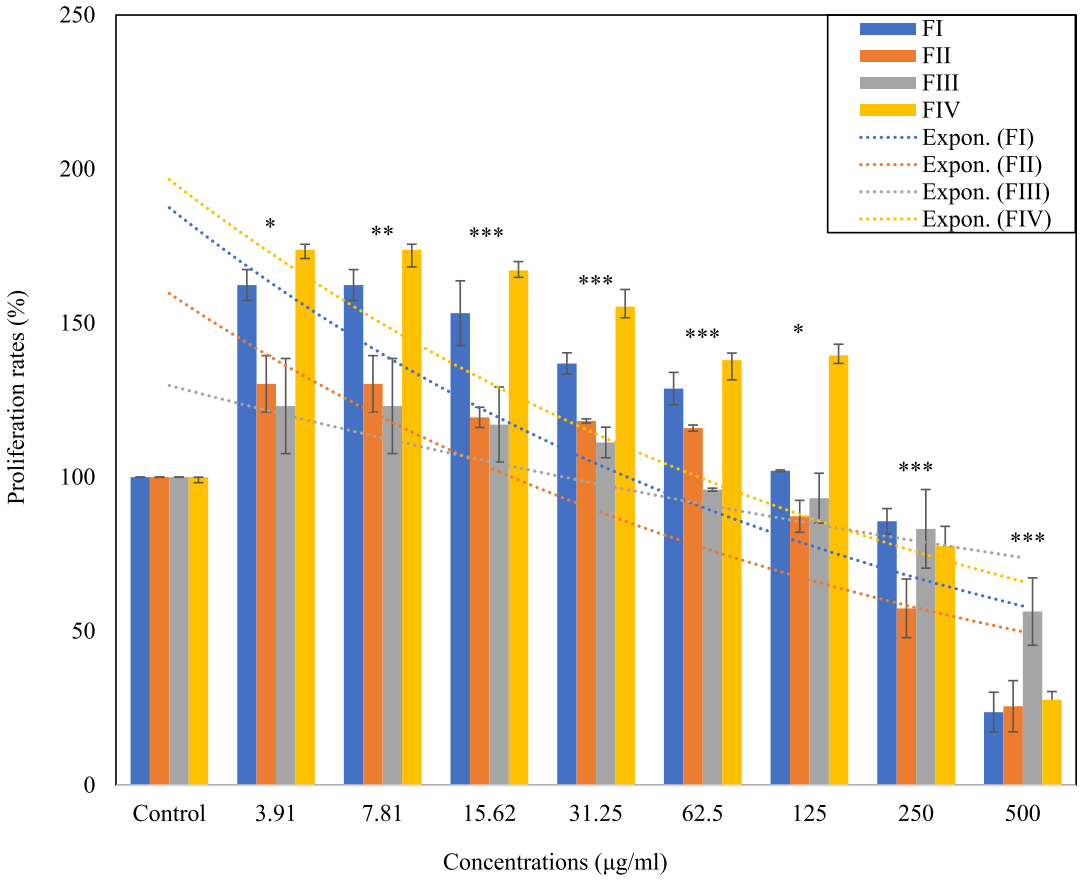


Fig. 6. The highest proliferation was stimulated by F_{IV} at the concentrations of 3.91, 7.81, 15.62, 31.25, 62.5, and 125 (*p < 0.05, *p < 0.01, ***p < 0.001; ANOVA).

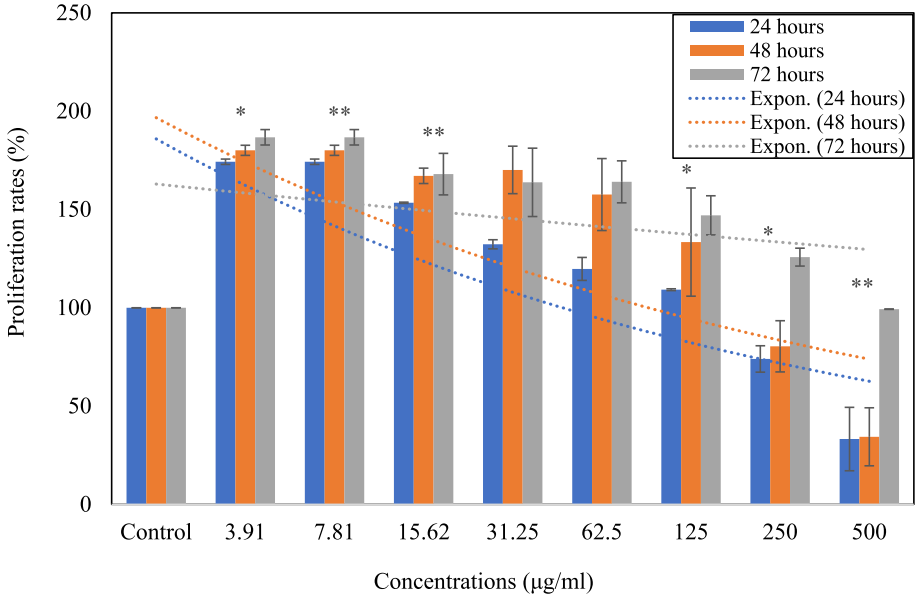


Fig. 7. F_{IV} applications within72 hours incubation were reported to stimulate the highest proliferation rate (*p < 0.05, **p < 0.001; ANOVA).

3.4. Down regulation of p53 and up regulation of αSMA, SOD1, and E-cadherin by F_{IV} P. crocatum leads to increased collagen deposition and wound closure of wHFs

We performed western blotting to observe the involvement of αSMA, p53, SOD1 and E-cadherin in collagen deposition and wound closure. wHFs were treated with three serial concentrations of F_{IV} (7.81, 15.62, and 31.25 µg/ml). After 72 h, proteins were extracted (refer to methods) and analyzed by western blot for levels of αSMA, p53, SOD1 and E-cadherin. Control cells, the untreated wHFs, and normal cells were analyzed in a similar manner.

The decreased expressions of p53 and the increased expressions of αSMA were generally considered as strong indicators of diabetic

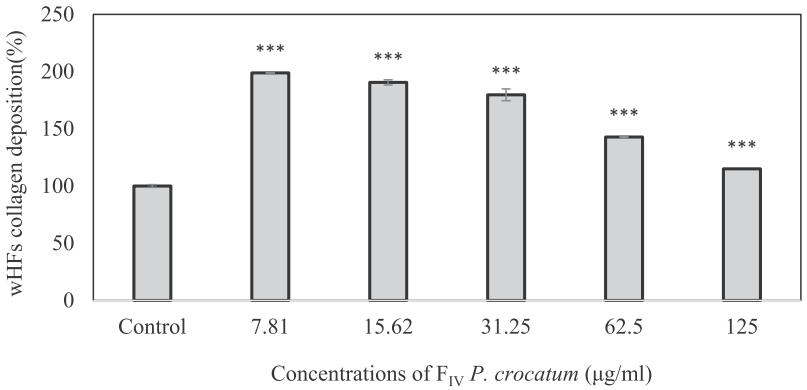


Fig. 8. F_{IV} of *P. crocatum* ameliorated collagen deposition of wHFs after 72 h exposure. Data are shown as mean ± SD: The intervention groups had a significantly higher collagen deposition than the control groups (*p < 0.001; Dunnett's test).

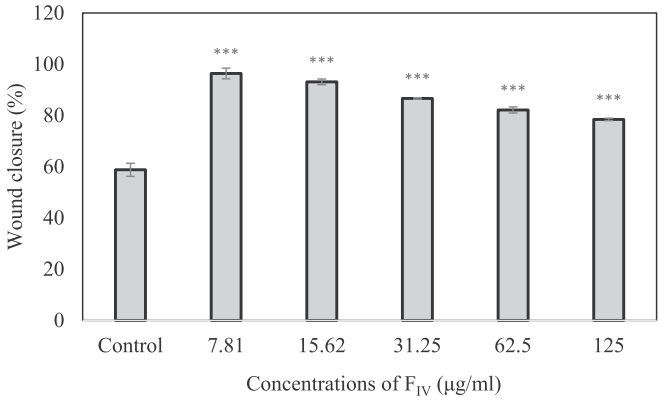


Fig. 9. F_{IV} of *P. crocatum* ameliorated wound closure of wHFs after 72 h exposure. Data are shown as mean ± SD: The intervention groups had significantly higher wound closure rate than the control groups (*p < 0.001; Dunnett's test).

wound healing progression with F_{IV} application at the concentrations of 7.81 and 15.62 µg/ml (Fig. 10). The increase in collagen deposition with F_{IV} treatment decisively correlated with a decrease in the expressions of p53 (p = 0.026, r = −0.864; Pearson correlation) and an increase in the expression of αSMA (p = 0.003, r = +0.957; Pearson correlation). In a similar way, the increase in wound closure with F_{IV} treatment was also firmly correlated with a decrease in the expressions of p53 (p = 0.032, r = -0.849; Pearson correlation) and correlated with an increase in the expression of αSMA (p = 0.018, r = 0.888; Pearson correlation).

According to our findings, the increased expressions of SOD1 and E-cadherin were also considered as a progress indicator of F_{IV} treatment (Fig. 11). The increase in SOD1 with F_{IV} treatment was also positively correlated with an increase of collagen deposition (p = 0.045; r = 0.685; Pearson correlation) at the concentrations of 7.81 and 15.62 µg/ml but did not correlate with an increase of wound closure (p = 0.257; Pearson correlation) as compared to the control cell. At the same time, the increased E-cadherin expressions were correlated with an increase of collagen deposition (p = 0.010, r = 0.919; Pearson correlation) and wound closure (p = 0.045, r = 0.803; Pearson correlation) as compared to the control cells.

For the purposes of the study, we concluded that the down regulation of p53 and the upregulation of αSMA, SOD1 and E-cadherin resulted in a significant increase of collagen deposition. Similarly, the increased wound closure rates were also triggered by the down regulation of p53 and upregulation of αSMA and E-cadherin but were not triggered by SOD1.

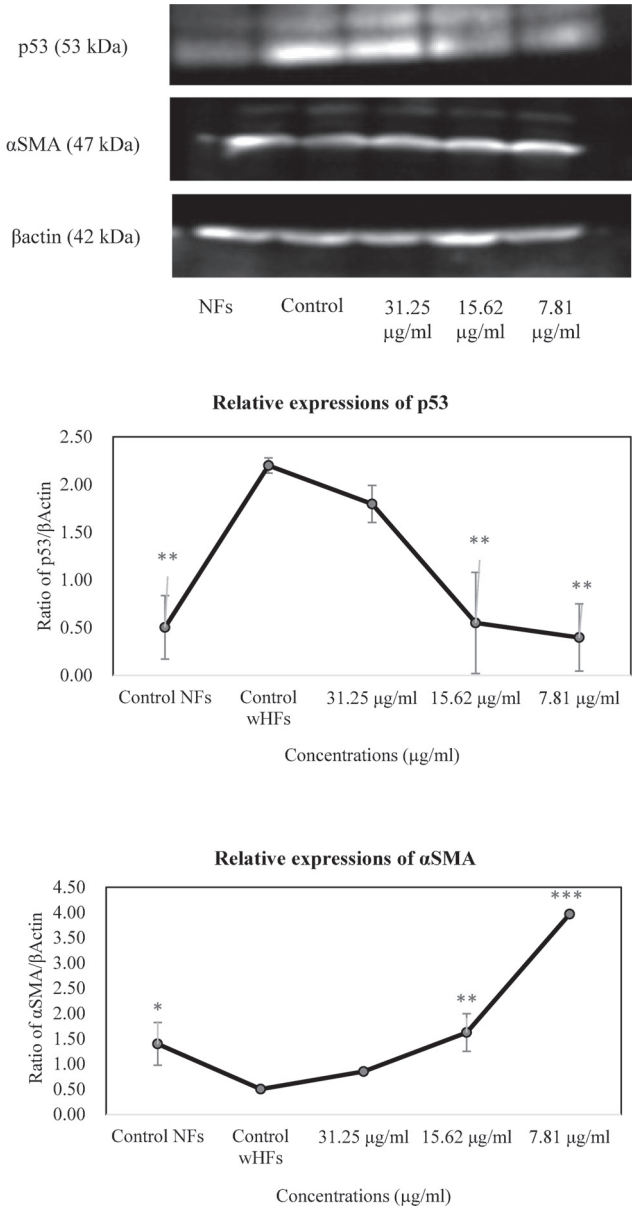
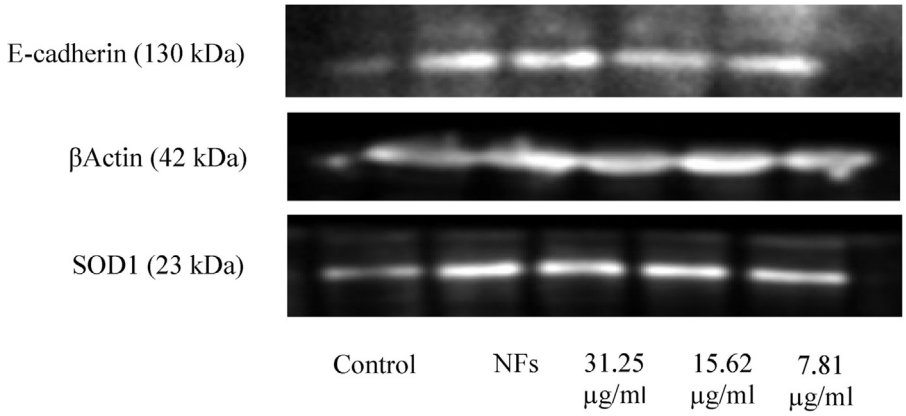
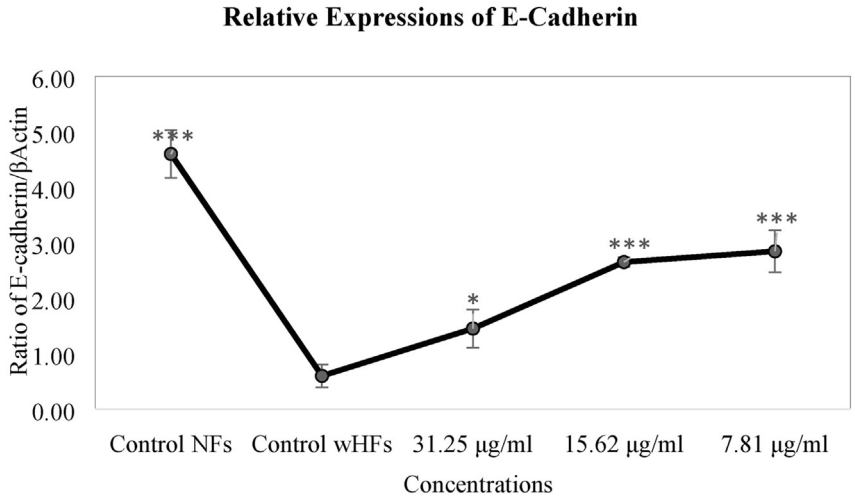


Fig. 10. The western blot analysis of p53 and αSMA protein bands were analyzed using Image J. Data are shown as mean ± SD (n = 3). The F_{IV} *P. crocatum* F_{IV} was confirmed to up regulate of αSMA and down regulate of p53 expressions (*p < 0.05, **p < 0.01, ***p < 0.001; Dunnett's test).





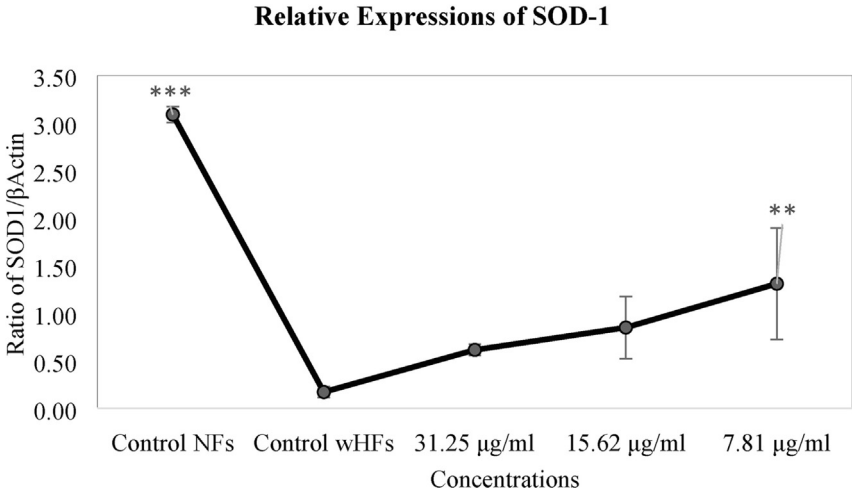


Fig. 11. The western blot analysis of SOD1 and E-cadherin protein bands were analyzed using Image J. Data are shown as mean ± SD (n = 3). The F_{IV} *P. crocatum* F_{IV} was confirmed to up regulate the SOD1 and E-cadherin (**$p < 0.01$, ***$p < 0.001$; Dunnett's test).

4. Discussion

Bioassay-guided fractionation was performed in our study to identify the active fractions through three steps (extraction, partition, and fractionation). This method was successful in separating the active compound groups in *Tithonia Diversifolia* and *Kappaphycus alvarezii* (Doty) Doty ex P.C.Silva algae (Syarif et al., 2018; Yulianti et al., 2021). One additional step from this method can be used to isolate the active compounds contained in natural materials, for example the Tagitinin C compound from *Tithonia diversifolia* (Wahyuningsih et al., 2015) and zerumbone from *Zingiber zerumbet* (Murini et al., 2018).

Our study confirmed that wHFs treated with MeOH extract had higher proliferation rate than wHFs treated with $CHCl_3$ extract. This finding agrees with a previous study which found that MeOH extract was not toxic (Chan et al., 2015). However, several studies have found a different result and reported that MeOH extract was toxic (Dhawan & Gupta, 2016; Truong et al., 2019). These differences in findings show that the MeOH extract contains many classes of compounds with opposing potential, so further separation needs to be done. Therefore, the MeOH extract was separated using wash-benzene to give simpler compounds.

wHFs treated with WB-soluble extracts had a higher proliferation rate than wHFs treated with WB-insolubleinsoluble-wash benzene. It has been published that the simpler the compound, the higher the level of activity (Jiang et al., 2017). Dissimilar with this report, we found that the WB-soluble extracts had lower proliferation rate compared to the MeOH extract. This circumstance could be due to the presence of various molecular weights. Centrifugation method used in this study ideally could be able to separate the secondary metabolites, but may still result in different molecular weights. The varying molecular weights will affect the level of activities (Ungureanu et al., 2013). However, our study revealed that four fractions (F_I, F_{II}, F_{III}, F_{IV}) obtained from WB-soluble extracts had a higher proliferation rate compared to the MeOH extract. This study confirmed that the most active fraction is F_{IV}, since it has the highest proliferation rate among all fractions treatments. Glycoside polyphenol contained in F_{IV} is a type of flavonoid that has a benzo-γ-pyrone structure and is responsible for the variety of pharmacology activities (Kumar and Pandey, 2013).

Our study revealed that treated wHFs had higher collagen deposition compared to untreated wHFs at all concentrations. In line with our study, flavonoids (morine, rutin and chrysin) from green tea also have an ability to increase collagen production by fibroblasts (Stipcevic et al., 2006). As observed in the previous study, the increase in collagen occurs in response to the free radical scavenging activity of hydroxyl chain in the flavonoid chemical structure (Buranasin et al., 2018; Prayitno et al., 2018). As one of the flavonoid types, polyphenol of F_{IV} *P. crocatum* was thought to play a role in the higher collagen deposition of treated group. One study that stated MeOH maceration has been known to attract more polyphenols is in accordance with the methods and findings of our study (Prayitno et al., 2018). We performed maceration three times to get extracts in large quantities by increasing diffusion and reducing density (Prayitno et al., 2018).

The study by Goulding (2015) affirmed that therapies that improve collagen production will have good impact for the next wound healing phase. We found that *P. crocatum* F_{IV} induces wound closure at all concentrations. The ability of cells to produce collagen affects the ability of cells to migrate in creating wound closure (Foster et al., 2018). Similar to the effect of *P. crocatum* on collagen deposition, we confirmed that *P. crocatum* accelerated wound closure at all concentrations (7.81, 15.62, 31.25, 62.5, and 125 μg/ml).

Additionally, we did not find the concentration-dependent of F_{IV} effects on collagen deposition and wound closure. However, the concentrations of F_{IV} used in this study were five concentrations that have been shown to have proliferative activity according to prior application of MeOH extracts and WB-soluble sub-extracts in wHFs. Showing anti-proliferative effect of methanol extracts and WB-soluble sub-extract at the concentration of 500 μg/ml, we suppose that F_{IV} ameliorated collagen deposition and wound healing in concentration-dependent way. In accordance with our view, a small dose of polyphenols from *Parapiptedenia rigida* was reported to increase fibroblast proliferation activity and suppressed proliferation at large doses (Muhammad et al., 2013). Another study reported that the high dose of polyphenols was shown to have anti-proliferative activity due to the inhibition of the STAT-3 signaling pathway (Huang et al., 2013). Accordingly, dosage must be carefully considered in investigating the effect of *P. crocatum* in the future studies.

Our study showed that the treated wHFs had a significantly higher expressions of αSMA, SOD1 and E-cadherin and had significantly lower expression of p53 compared to untreated wHFs. Since our study revealed significant relationship between expressions of p53 and wound closure rate, it is in agreement with the findings of Tombulturk et al (2019) which explained that p53 expression during the proliferation phase of diabetic wound healing needs to be decreased to accelerate wound closure. The increased activity of activated-protein 1 (AP-1) that is ubiquitously present in diabetic wounds causes over-expression of c-fos and c-jun. This condition leads to matrix metalloproteinase (MMPs) excess. The hydroxyl group in *P. crocatum* is supposed to have a high affinity of zinc contained in MMPs. This condition leads to MMPs declining and results in MMPs-p53 binding impairment. Consequently, the structure of p53 is impaired (Pang et al., 2016).

The decrease in p53 expression has an impact on deletion of apoptotic cells, resulting in enhancement of collagen production, myofibroblast differentiation and angiogenesis (Bhan et al., 2013). In line with this explanation, our study also confirmed that collagen deposition was significantly associated with p53 expression. As one of the limitations, our *in vitro* study was not able to identify the myofibroblast differentiation and angiogenesis. However, as a marker of fibroblast readiness to differentiate into myofibroblasts, αSMA expressions in treated wHFs were observed to be higher than untreated wHFs. Moreover, the increase of αSMA during wound healing is useful for maintaining collagen structure (Stojadinovic et al., 2012). In accordance with that study, we also observed the significant association between collagen deposition and αSMA expressions.

Collagen structure is also dependent on the quantity of E-cadherin as a cell adhesive molecule (CAM). E-cadherin which is expressed on the fibroblasts' surface has a vital function to strengthen the binding between collagen (Keshri et al., 2016; Wang and Shi, 2020). Hence, this description supports our finding of significant correlation between collagen deposition and E-cadherin expressions. The significant correlation between wound closure and E-cadherin expressions was also observed in our study ($p = 0.023$). In line with our findings, it has long been known that E-cadherin expression was observed in the cells, where migration was active (Kuwahara et al., 2001). However, E-cadherin production and distribution within cells require calcium intake (Hunter et al., 2015). Calcium-binding sites of glycoside polyphenol that have sensitive calcium signaling, result in the ability of F_{IV} *P. crocatum* to uptake more calcium from FBS containing media than their aglycons (Singh et al., 2021).

The tissues must remain stable during cell migration to get the complete wound closure. Therefore, the continuity of ECM production amid wound closure process needs to be maintained. One of the factors that play a role in ECM production is SOD1. SOD1 needs to be bound by Nrf2 to stimulate ECM production (Kelly, 2020; Li et al., 2020; Wade, 2012). In relevant, SOD-targeted therapy normalizes diabetic wound healing (Churgin et al., 2005). This finding supports the result of our study that observed significant correlation between SOD1 and collagen deposition. Differently, wound closure was not found to have a significant correlation with SOD1. However, our study corroborated that SOD expressions were inclined to be higher in treated wHFs than untreated wHFs. The mechanism was elucidated in several *in vivo* studies that found SOD1 maintains redox homeostasis in cells and tissues when injury occurs through interaction with bio-membranes to protect the cell from increased ROS activity and oxidizing them to become stable

molecules during the wound healing process (Kurahashi and Fujii, 2015; Shavandi et al., 2018; Wade, 2012).

Our results demonstrated that treated wHFs had significantly higher expressions of αSMA and lower expressions of p53 at 15.62 and 7.81 μg/ml, whereas SOD1 expressions were found to be significantly higher only on 7.81 μg/ml treated wHFs. E-cadherin expressions were significantly higher at all concentrations of treated wHFs than untreated wHFs. We confirmed that F_{IV} *P. crocatum* is a bio-selective compound that has a consistent effect in ameliorating the wound healing process in diabetic wounds mimicked in an *in vitro* model at concentration 7.81–31.25 μg/ml. Some polyphenols, in a dose dependent way, may regulate the wound healing process in accordance with its needs (Działo et al., 2016). By considering the results of our study and the advantages of developing these polyphenol-rich natural materials, such as low production costs, abundant supplies and few side effects (Bahramsoltani et al., 2014), *P. crocatum* F_{IV} has the potential to be developed into a novel wound healing technology. However, further *in vivo* research needs to be done to identify the effects of F_{IV} *P. crocatum* and future clinical study can identify its contribution in accelerating diabetic wound healing.

5. Conclusions

We concluded that F_{IV} *P. crocatum* increases collagen deposition and wound closure in wHFs through increasing αSMA, SOD1 and E-cadherin expressions as well as decreasing p53 expression at the best concentration of 7.18 μg/ml. However, *P. crocatum* F_{IV} has benefits on wHFs at concentrations of 7.18–31.25 μg/ml and is safe to use to treat wHFs until 125 μg/ml concentration.

Our study results provide biologically based evidence transferable to health practice and health education regarding the F_{IV} *P. crocatum* mechanisms in diabetic wound healing and safety concerns of concentration and manufacture of plant-based wound medicines. Moreover, further studies are required to identify the effects of F_{IV} *P. crocatum in vivo* and clinical manner in future study in contribution of accelerating diabetic wound healing

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sjbs.2021.08.039.

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