Journal of Current Science and Technology, September - December 2023 Vol. 13 No. 3, pp. 672-682

ISSN 2630-0656 (Online)

Cite this article: Thongphasuk, P. & Limsitthichaikoon, S. (2023). Feasibility study of *Neptunia javanica* Miq. extract as an alternative medicine for wound healing. *Journal of Current Science and Technology*, *13*(3), 672-682. https://doi.org/10.59796/jcst.V13N3.2023.691

Journal of Current Science and Technology

Journal homepage: https://jcst.rsu.ac.th

Feasibility Study of *Neptunia javanica* Miq. Extract as an Alternative Medicine for Wound Healing

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Received 1 March, 2023; Revised 21 June, 2023; Accepted 30 June, 2023;
Published online 30 August, 2023

Abstract

Neptunia javanica Miq. (NJ) locally known as Kra-Chood, is an unwanted weed that belongs to the family Mimosaceae. This study aims to investigate the feasibility of NJ extract being used as an alternative medicine for wound healing. Preliminary phytochemical analysis was carried out using standard phytochemical screening methods and the NJ ethanolic extract demonstrated alkaloid, flavonoid, tannin, and phenolic compounds. The total phenolic content of the NJ extract was 117.05 ± 0.03 mg GAE/g crude extract, and the total flavonoid contents were 0.63 ± 0.0231 mg RE/g crude extract. The antioxidant activities (IC_{50}) of NJ were 9.29 ± 0.04 µg/mL which is lower than the IC_{50} of quercetin (38.49 ± 5.38 µg/mL) which represented the potent antioxidation activity of the NJ extract. The metal chelating activity (IC_{50}) of NJ was 1.8584 ± 0.2708 mg/mL. The extract was also found to have α-glucosidase inhibitory and the IC_{50} was 1.89± 0.19 mg/mL which has a potency equal to 0.13 of the standard acarbose (0.25± 0.09 mg/mL). Moreover, the extract disturbed the human dermal fibroblast viability in a dose-dependent manner. Low concentration of the NJ extract (0.001-0.01 mg/mL) showed no cytotoxicity compared to the negative control ($p < 0.05$) while high concentration of the extract (0.5 and 1 mg/mL) significantly reduced the cell viability compared to the control ($p < 0.05$) and the low concentration ($p < 0.05$). Moreover, the fibroblast exposed to the NJ extract concentration of 0.001-0.01 mg/mL was found to significantly enhance cellular movement and wound gap closure compared to the negative control and gallic acid ($p < 0.05$). Thus, all these results could develop complementary and integrative benefits, and more investigation in wound healing assay and a subject for should be conducted further studies in clinical trials.

Keywords**:** *Kra-Chood; Neptunia javanica Miq.; Scratch wound assay; Wound healing*

1. Introduction

Neptunia javanica Miq. (NJ) known as Kra-Chood in Thai, belongs to the Mimosaceae family which is the same genus as *Neptunia oleracea* Lour. (*N. oleracea)* or Water Mimosa or Kra-Ched in Thai. *N. oleracea* is a plant cultivated as a famous vegetable in Southeast Asia, especially in Thailand. A considerable number of studies have also revealed that *N. oleracea* demonstrated several biological activities including properties as antioxidants (Lee et al., 2019), antiulcer (Bhoomannavar et al., 2011a), antiviral (Nakamura et al., 1996), hepatoprotective (Bhoomannavar et al., 2011b), α-glucosidase inhibitory (Lee et al., 2019), 5α-reductase inhibitory (Kumar & Chaiyasut, 2017), anticancer (Bhumireddy et al., 2022), analgesic and anti-inflammatory activities (Paul et al., 2012). Moreover, numerous studies have reported the phytochemical compositions (Ouedraogo et al., 2019) and biological activities of *N. oleracea.* On the other hand, NJ is regarded as an unwanted weed as it is abundantly found, andit has

a high rate of regeneration, adaptability, and tolerance to adverse climates. NJ is an edible vegetable, but it is tough and unpopular for cooking. Thus, there have been several studies and information on the bioactive compounds, phytochemical components, and biological activities of NJ. The variety of biological characteristics of medicinal plants is known to be attributed to phytochemical constituents which exist in those plants. Based on the same genus of *N. oleracea*, Kra-Chood might contain the same bioactive substances as Kra-Ched. Utilizing these problematic weeds which are sustainable and renewable will provide economical and environmentally viable bioproducts and help increase the value of this unwanted plant.

2. Objective

The main objective of the study was to investigate the feasibility of NJ extract being used as an alternative medicine for wound healing. Thus, the NJ extract was subject to rigurous investigation to validate its healing properties including anlysis and testing of the phytochemical constituents, solubility, total phenolic content, DPPH radical scavenging activity, α-glucosidase inhibitory assay, *in vitro* cytotoxicity, and scratch wound healing assay.

3. Materials and methods

3.1 Extract preparation

The aerial part of NJ was identified by Piyanut Thongphasuk and collected from Pathum Thani province. The voucher specimen (RxRSU190519) was deposited at the Department of Pharmacognosy, College of Pharmacy, Rangsit University. The plant was washed, oven-dried at 50 °C for 12 hours, then, crushed to be fined powder using an electric blender. The fined powder of NJ was kept in a closed container until used. The NJ powder was extracted by using 95% ethanol in a ratio of 1:10 (w/v) and sonicated in an ultrasonic bath (Crest Ultrasonics CP230T) at 35°C for 15 min. The extract solution was filtered using a 0.45 µm filter paper, air-dried, and evaporated at room temperature. The NJ extract powder was kept in a closed container until used.

3.2 Phytochemical analysis

The preliminary phytochemical analysis was conducted according to standard phytochemical screening methods (Manzo et al., 2017; Erdenechimeg et al., 2017). Alkaloid test with Dragendorff's reagents; anthraquinone test with modified Borntrager's test; deoxy sugar test with Keller-Kiliani test; iridoid test with Trim Hill reagent; phenolic test with ferric chloride test; saponin test with froth test; tannin test with lead acetate test.

3.3 Solubility test

The solubilty of the NJ extract was investigated . The NJ powder was weighted (0.10 g) and mixed with 10 mL of various solvents such as deionized (DI) water, propylene glycol (PG), polyethylene glycol (PEG 400), glycerin, 95% ethanol, 10% dimethyl sulphoxide (DMSO), and 5% tween80 at room temperature (25±5 °C). The mixture solution was vortexed and observed the clarity of the mixture extracted. If a precipitate of the mixture solvent was found, 10 mL of these solvents was added, vortexed, and observed clarity. The classification of solubility was explained following USP 43 – NF38 solubility chart guideline (United States Pharmacopoeia, 2020).

3.4 Determination of total phenolic content

The total phenolic content was detected by using the Folin–Ciocalteu method (Velioglu et al., 1998; Ji-u, & Apisittiwong, 2022). A total of 20 µL of various concentrations of samples and 100 µL of the Folin-Ciocalteu reagent were mixed in the 96-well plate and incubated for 5 min. Then, 80 µL of 7.5% w/w sodium carbonate solution was combined. After 2 hours, the UV absorbance of the NJ extract mixture was observed at 765 nm. All testings were performed in triplicate (n = 3). A standard curve of gallic acid was achieved to calculate the TPC and the results were shown in mg GAE/g crude extract.

3.5 Determination of total flavonoid content

The total flavonoid content of the NJ extract was determined by using the aluminum trichloride method (Brighente, Dias, Verdi, & Pizzolatti, 2007). The NJ ethanolic extract was mixed with aluminum trichloride solution (2% $AlCl_3$) and the mixture was incubated at room temperature for 1 h. The absorbance of mixtures was measured at 420 nm by using the microplate reader. The results were shown in equivalents of rutin (mg RE/g crude extract).

Figure 1 *Neptunia javanica* Miq. (NJ) known as Kra-Chood and its aerial part which was selected, extracted, and investigated

3.6 Free radical- scavenging assay by DPPH

DPPH radical scavenging activity was determined by a modification of the protocol suggested by Cavin et al., (1998) and Kumar et al., (2023). Briefly, 100 µL of the sample solution in different concentrations was mixed with 100 µL of methanolic containing DPPH radicals (0.1 mM). The assay was performed on a 96-well plate. The plate was then incubated in the dark for 30 min and examined for absorbance at 517 nm using a microplate reader. The analysis was achieved in triplicate (n = 3). The antioxidant activity was presented as IC_{50} of DPPH radical scavenging activity by calculating the 50% inhibitory concentration using the calibration curve. The IC_{50} value was statistically compared by t-test analysis ($p < 0.05$).

3.7 Determination of metal chelating activity

The metal chelating activity was measured by mixing the NJ extract with 0.1 mM $FeSO_4$ in an equal ratio and incubating for 1 min at room temperature. Then, 0.25 mM ferrozine was added to the mixture and incubated for 1 min at room temperature. The mixture was recorded at 562 nm by using the microplate reader. EDTA was used as the standard equivalent (Chew et al., 2009; Dinis et al., 1994). The metal chelating activity was calculated using the equation below.

$$\text{Metal chelating activity} = \frac{A_{blank} - A_{extract}}{A_{blank}} \times 100$$

Where A_{blank} is the absorbance of blank control (without adding the NJ extract) and $A_{extract}$ is the absorbance of the NJ extract.

3.8 Analysis of α-glucosidase inhibitory assay

The α-glucosidase inhibitory activity was performed using the modified method (Dewi et al., 2007; Elya et al., 2012). The NJ extract sample was dissolved in phosphate buffer and varied concentrations as 0.001, 0.01, 0.1, 1, and 10 mg/mL, respectively. While standard acarbose dissolved in phosphate buffer varied the concentration to 0.0005, 0.005, 0.05, 0.5, and 5 mg/mL. The α-glucosidase inhibitory assay was performed by using *ρ*–nitro phenyl–alpha–D–glucopyranose (Sigma-Aldrich, U.S.A.) as a substrate. The α-glucosidase activity was determined by using a microplate reader (Shimadzu 265, Jepang) at 415 nm and calculating the concentration of the NJ extract required to inhibit 50% of α-glucosidase activity as the IC_{50} value.

3.9 *In vitro* studies of human dermal fibroblast

A confluent monolayer of human dermal fibroblasts (HDF, Merck, U.S.A.) was cultured in a completed medium containing high glucose Dulbecco's Modified Eagle's (DMEM) medium (Invitrogen, CA, U.S.A.) with 2 mM of l-glutamine, 100 U/mL of penicillin, 0.1 mg/mL of streptomycin, 0.025 mg/mL of amphotericin B

(Invitrogen, CA, U.S.A.) and 10% fetal bovine serum (FBS, Gibco, CA, U.S.A.) and then the cell culture was incubated in the incubator (Shel Lab, Oregon, U.S.A.) under 37°C, 5% CO_2 and 95% RH. All experiments were performed after the HDF cells reached a confluent monolayer of ~10,000 cells per well in a 96-well plate for cytotoxicity test or ~40,000 cells per well in a 24-well plate for scratch wound assay (Damrongrungruang et al., 2021).

3.9.1 Determination of cell viability using MTT assay

The HDF cells were detached and cultivated in a 96-well plate at ~10,000 cells/well overnight. The former medium was replaced by the complete medium (DMEM with 10% of FBS) as a negative control, hydrogen peroxide (0.1 mg/mL, H_2O_2) was used as the positive control, and with or without samples in a concentration of 0.001-1.00 mg/mL for 24 h (Damrongrungruang et al., 2021). The assay medium was discarded and MTT (400 µg/mL) was added, and the cells were incubated for 4 h. The MTT solution was discarded and replaced by 100 µL per well of DMSO. The intensity of formazan color was determined by spectrophotometry at 570 nm (Bio-Rad Laboratories). Cell viability was obtained, using the equation below.

$$\text{Cell viability (\%)} = \frac{A_{extract}}{A_{blank}} \times 100$$

Where A_{blank} is the absorbance of blank control (without adding the NJ extract)

$A_{extract}$ is the absorbance of the NJ extract.

3.9.2 Determination of In vitro scratch wound assay

The *In vitro* scratch wound assay was performed by a modified method (Dewi et al., 2007). In brief, the HDF cells were detached and cultivated in a 24-well plate using 40,000 cells/well overnight. The attached cells were scratched using a yellow (20-200 µL) pipette tip. The old medium was replaced by the new complete medium (DMEM with 10% FBS) with or without samples (Damrongrungruang et al., 2021). The pictures of cell gap areas (wound gaps) were magnified using bright field microscopy (10x) at 0, 16, 20, and 24 h, and the wound gaps were measured using the Image J program.

3.10 Statistical analysis

All categorical variable numbers were evaluated as percentages (%). The continuous variable information (n = 3-6) was described as averages with standard deviations (SD) and the normality was tested. Student t-tests and analysis of variance (ANOVA) were completed to test differences between or among experimental groups using the SPSS software (version 13, SPSS Inc, Chicago, IL, U.S.A.). Statistical significance is described as $p < 0.05$.

4. Results and discussion

The phytochemical screening of NJ extract exhibited the presence of alkaloid, flavonoid, deoxy sugar, phenolic, and tannin and the absence of anthraquinone, saponin, and iridoid as shown in Table 1. Based on a lot of phytochemical substances found in the NJ extract, further studies on solubility and antioxidant activity were carried out.

The solubility of NJ extract, shown in Table 2, demonstrated that the extract was insoluble in DI water, PG, DMSO, and glycerin. Surfactant and co-solvent were introduced to help solubility as the extract was very soluble in ethanol, PEG400, and tween80 which might be by using ethanol in the extraction process. Then, surfactant and co-solvent enhanced the extract solubility. Moreover, the use of a co-solvent system may provide a benefit in the process of pre-formulation development.

Table 3 displays the total phenolic content of the ethanolic extract of the NJ. The results were shown as mg equivalents of gallic acid per gram of crude extract. The results revealed that the total phenolic content of the ethanolic extract was 117.05 ± 0.03 mg GAE per gram of the NJ crude extract and the total flavonoid contents were 0.63 ± 0.0231 mg RE per gram of the NJ crude extract. Moreover, the free radical scavenging activities of the ethanolic extracts of NJ by DPPH showed that the IC_{50} value of ethanolic extracts was 9.29 ± 0.04 µg/mL, whereas the IC_{50} value of quercetin was 38.49 ± 5.38 µg/mL. The results revealed that the aerial part of NJ contained phenolic and flavonoids which were responsible for several biological functions and health benefits. The ethanolic extracts of NJ have significant ($p < 0.05$) antioxidant activity as compared with quercetin. As a result, the NJ may serve as a possible source of antioxidants.

Table 1 Phytochemical constituents of *Neptunia javanica* Miq. (NJ).

Tests	***Neptunia javanica* Miq. (NJ) extracted**
Alkaloid	presence
Anthraquinone	absence
Deoxy sugar	presence
Flavonoid	presence
Iridoids	absence
Phenolic	presence
Saponin	absence
Tannin	presence

Table 2 Solubility assay of *Neptunia javanica* Miq. (NJ) extract.

Solvents	**Solubility**
DI water	-
95% ethanol	++
PG	+
10% DMSO	-
PEG400	++
glycerin	-
5% tween80	++

Note: The solubility testing followed USP 43 – NF38 solubility chart guidelines. The results were shown as ++ (very soluble), + (slightly soluble), and - (insoluble).

Table 3 Percentage of yield, total phenolic content, DPPH radical scavenging activity, and an α-glucosidase inhibitory assay of the ethanolic extract of *Neptunia javanica* Miq. (NJ).

Tests	**NJ extract**	**Quercetin**	**EDTA**	**Acarbose**
Yield (%)	1.58 ± 0.08	-	-	-
Total phenolic content (mg GAE /g extract)	117.05 ± 0.03	-	-	-
Total flavonoid content (mg RE / g extract)	0.63 ± 0.02	-	-	-
IC_{50} (DPPH assay) (μg/mL)	9.29 ± 0.04	38.49 ± 5.38	-	-
IC_{50} of metal chelating activity (mg/mL)	1.86 ± 0.27	-	0.004±0.0001	-
IC_{50} α-glucosidase inhibitory assay (mg/mL)	1.89 ± 0.19	-	-	0.25 ± 0.09

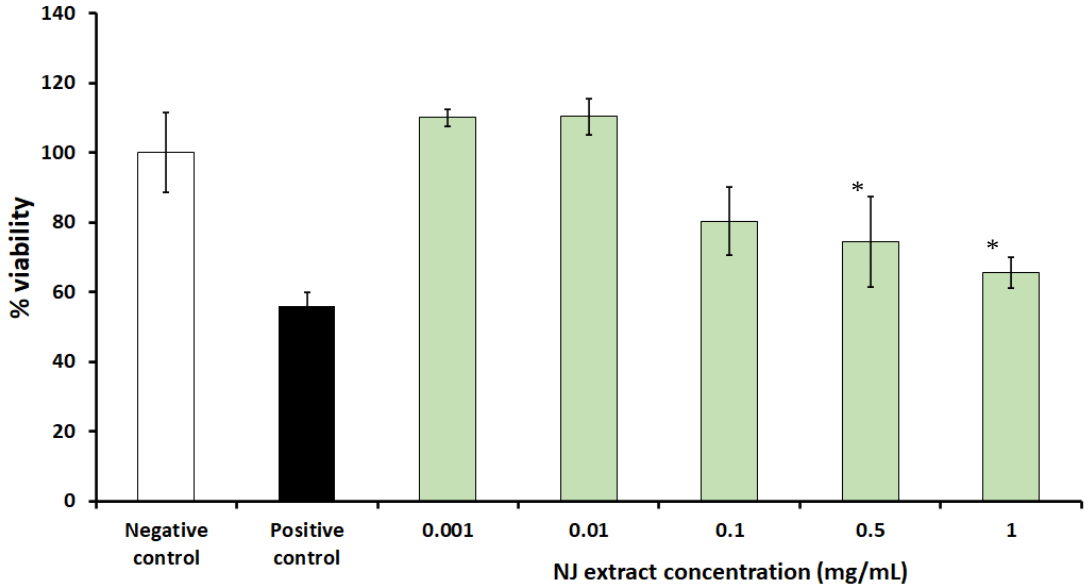

Figure 2 Percentage of human dermal fibroblast viability after 24 hours exposed to the *Neptunia javanica* Miq. (NJ) extract at the concentration of 0.001 – 1.0 mg/mL compared to the negative control treated with completed medium (DMEM) and positive control treated with hydrogen peroxide (H_2O_2). Error bars represented standard deviations, * indicates a statistically significant difference at $p < 0.05$ compared to the negative controls using the student t-tests and analysis of variance (ANOVA).

The metal chelating activity (IC_{50}) of NJ was 1.8584 ± 0.2708 mg/mL. Taken together with antioxidant activity, the NJ extract might have wound healing activity, which correlated with the chelating capacity towards ferrous (Fe^{+2}) ions (Korkina et al., 2007). Moreover, the NJ extract was found to have an α-glucosidase inhibitory activity which the IC_{50} value was 1.89 ± 0.19 mg/mL which has a potency equal to 0.13 of the standard acarbose (IC_{50} values was 0.25 ± 0.09 mg/mL) indicating that the NJ ethanolic extract might be a candidate substance for diabetes wound treatment (Han et al., 2017).

Fibroblasts are the important cells involved in wound repair and healing (Politis et al., 2016). Fibroblast proliferation and migration is a necessary step in healing management and is the beginning of the *in vitro* scratch assay to evaluate wound healing (Liang et al., 2007). The scratch wound assay is reproducible and can be used to evaluate the qualitative potential therapeutic properties of extracts (Stewart et al., 1979). The NJ extract showed no toxicity to human dermal fibroblast at the concentration of 0.001 and 0.01 mg/mL (Figure 2) and affected cytotoxicity at specific concentrations in a dose-dependent manner. The high concentration of NJ (0.5 and 1 mg/mL) disturbed the environment of cell proliferation which can cause a decrease in the cell viability compared to the negative control and the NJ treatment at a lower concentration.

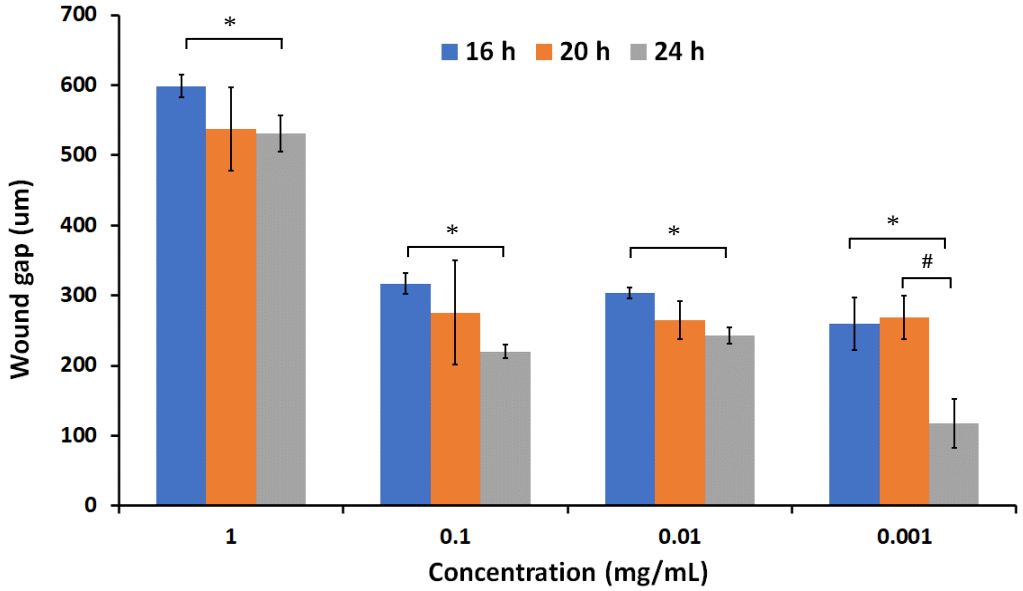

Figure 3 Wound gap measurement of human dermal fibroblast exposed to the *Neptunia javanica* Miq. (NJ) extract in a concentration of 0.001-1.00 mg/mL at 16, 20, and 24 hours (h). Error bars represented standard deviations, * indicates a statistically significant difference at $p < 0.05$ compared to the treatment measured at 16 h), and # indicates $p < 0.05$ compared to the treatment measured at 20 h using the student t-tests and analysis of variance (ANOVA).

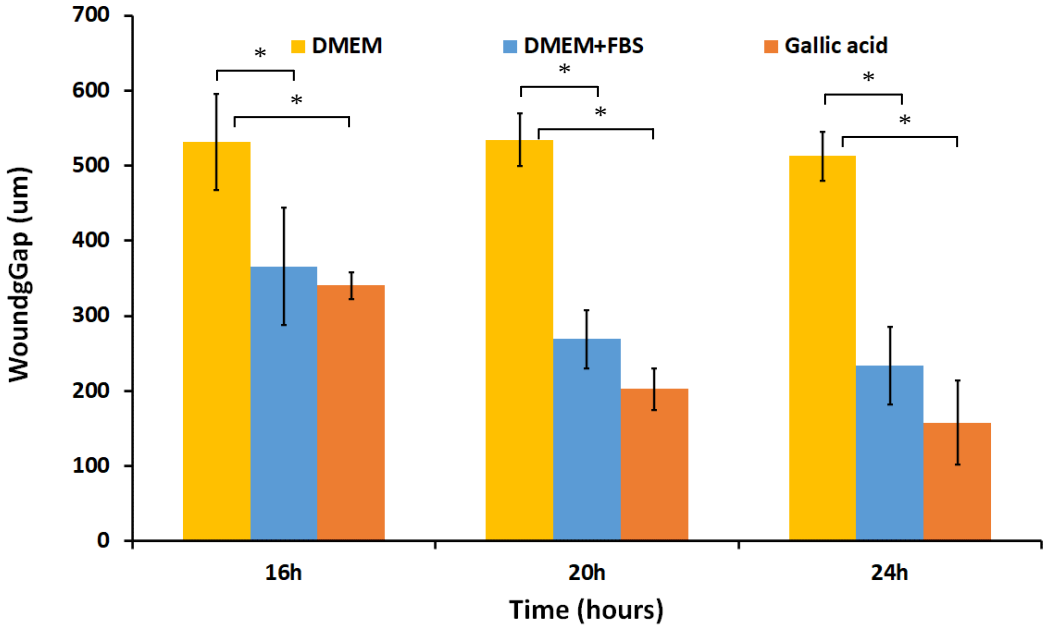

Figure 4 Wound gap measurement (µm) of human dermal fibroblast (HDF) exposed to the cell culture medium (DMEM), complete medium (DMEM+FBS), and gallic acid at 16, 20, and 24 hours (h). Error bars represented standard deviations. The * indicates a statistically significant difference at $p < 0.05$ compared to the negative controls (DMEM) using the student t-tests and analysis of variance (ANOVA).

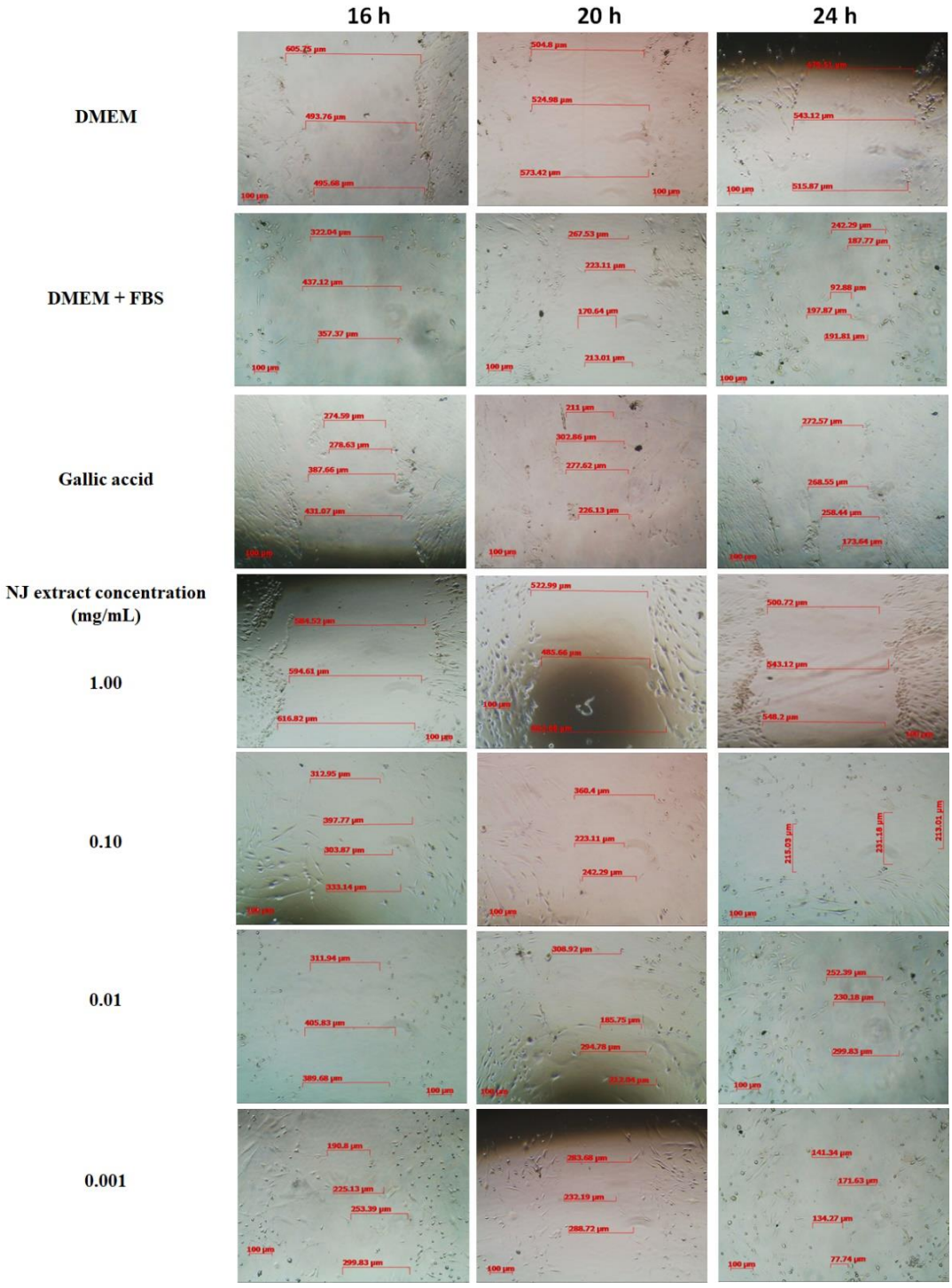

Figure 5 Cellular movement and wound gap closure of human dermal fibroblast exposed to the *Neptunia javanica* Miq. (NJ) extract compared to the cell culture medium (DMEM), complete medium (DMEM+FBS), and gallic acid at 16, 20, and 24 hours (h).

The NJ extract concentration of 0.001-1.00 mg/mL (shown in Figure 3) was subjected to investigation of cellular movement and wound gap closure. It was found that the NJ extract promoted cell migration in 24 hours and the gap closure was started at 16 hours. In comparison to the cell culture medium (DMEM), complete medium (DMEM+ FBS), and gallic acid, at 16 hours of the treatment, the NJ extract concentration of 0.001-0.01 mg//mL helped increase cellular movement insignificantly different from the cells treated with DMEM+FBS and gallic acid ($p > 0.05$) and significantly higher than the cells treated with the DMEM ($p < 0.05$) as shown in Figure 3 and Figure 4. However, the fibroblast exposed to the NJ extract at a concentration of 0.001 mg/mL for 24 hours was shown to have significantly affected decreasing the wound gap and enhanced cellular movement better than those treated with the DMEM, DMEM+FBS, and gallic acid ($p < 0.05$) (Figure 5) (Damrongrungruang et al., 2021). The range of concentration used to treat cytotoxicity and cell migration was the same as 0.001-1.00 mg/mL. Figure 2 showed that the cytotoxicity of HDF depended on the NJ extract concentration in a dose-dependent manner and a concentration between 0.001-0.01 mg/mL did not alter the cell viability while a concentration range of 0.1-1.0 mg/mL significantly decreased the cell viability ($p < 0.05$). Therefore, the high concentration of the NJ extract disturbed the viability of the fibroblast and altered the cells migration which caused the wide wound gap of the cells as shown in Figure 3 and Figure 4. Low concentration (0.001-0.01 mg/mL), in contrast, did not trigger cytotoxicity but initiated the cellular movement of the fibroblast resulting in decreasing wound gap within 24 h and showed a non-significant difference compared to gallic acid ($p > 0.05$).

All of these results in total phenolic contents, antioxidant activity, α-glucosidase inhibitory assay, *in vitro* cytotoxicity, and scratch wound healing assay of the NJ extract might be significant from phenolic compounds as flavonoid and tannin are the major bioactive components. However, further investigation on the bioactivity of bioactive compounds in other wound healing assays and also for their safe use should be investigated in future studies.

5. Conclusion

In this present research, the phytochemical screening test showed that the NJ extract contained a lot of compounds such as alkaloids, flavonoids, deoxy sugar, phenolic compounds, and tannin. The ethanolic extracts of NJ exhibited stronger antioxidant activities than quercetin, and α-glucosidase inhibitory was significantly higher than standard acarbose. Moreover, the extract was found to have no cytotoxicity and increased cellular movement and wound closure. Thus, all these results could develop complementary and integrative benefits, and more investigation in wound healing assay and a subject for should be conducted further studies in clinical trials.

6. Acknowledgements

The authors are grateful for the financial support from the Research Institute of Rangsit University (Grant No. 85/61). Miss Natnicha Muangsan, Miss Suttahathai Thamwijitkul, Miss Teraporn Changkaew, Mr. Phoonyawat Santipongpanit and Mr. Teekatass Thaworn are thanked for technical assistance.

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