UK Journal of Pharmaceutical and Biosciences
Available at www.ukjpb.com
ISSN: 2347-9442

Bioassay-Guided Fractionation of Wound Healing Active Compounds From *Piper nigrum* L. Berries Extract in Malaysia

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Article Information
Received 20 April 2018
Received in revised form 07 Aug 2018
Accepted 08 August 2018

Keywords:
Piper nigrum L.,
Piperine,
Scratch assay,
Keratinocyte,
Wound healing activity

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Abstract

The study aimed to further evaluate the wound healing property of *P. nigrum* L. using bioassay-guided fractionation method. The ethanolic extract of *Piper nigrum* L. was fractionated in two stages using column chromatography and preparative reversed phase C18 HPLC, respectively. Significant wound healing properties screened using the scratch wound assay was observed through the cell migration assay in fraction number three (PNE3) out of the 14 fractions, exhibiting 36.7% and 43.8% closure of wound gap within 20 hours at concentration of 0.3 and 1.0 µg/mL, respectively. Sub-fractions which were further fractionated from PNE3 showed comparatively reduced wound healing activity using the same bioassay. The sub-fractions were also compared to Piperine, a major component of *P. nigrum* L. and the results were comparable. This experimental study revealed that Piperine works together with other compounds in *P. nigrum* L. to improve wound healing as claimed by those home remedy.

1 Introduction

Secondary metabolites are organic compounds produced in plant, which play an imperative role in defending the producing plant against attack by insect pests and other predators[1]. These secondary metabolites or phytochemicals possessed various therapeutic potential such as antioxidant, antimicrobial and antiviral activities[2,3]. Some of these compounds are beneficial to human beings as medicines and flavourings. The beneficial effects of plant based materials come from mixed activities of the secondary products found in the plant[4]. Alkaloids, phenols and phenolic compounds, terpenes, flavonoids and tannins are some of the many examples of phytochemicals[5]. The increasing demands for natural products over the years have led to the rising trend in natural product research.

Piper nigrum L. is the botanical name of black pepper, a perennial climbing vine in the family Piperaceae. Its origin is in Malabar, South India. Black pepper is grown primarily in India, Malaysia, Brazil, Indonesia, Sri Lanka and Vietnam, thus the main producing countries in the world. It is cultivated for its fruit, which is usually dried and used as spice and condiments. Black pepper has been traded internationally since ancient times due to their popular culinary role[6] and is known as “King of Spices”. Black pepper is valued for its pungent quality attributed to the naturally occurring essential oil and aromatic compounds as well as well-known alkaloid—piperine. These compounds are of significant values because of its antioxidant activity, carcinogenic activity, carminative property, antimicrobial activity, antibacterial activity and many more[7,8,9]. For centuries, black pepper is well-known as a traditional cure for minor cut and wound. This may be attributed to the many important activities of its natural compounds. Nowadays, in vitro assays are available to screen the activities of the plant biological properties, which include those that regulate the wound healing process. It was suggested that a natural remedy should have at

least two different scientifically proven processes to support its conventional use[2].

Wound healing process involves the coordination actions of several cell types, soluble cell mediators and extracellular matrix. This complex system calls for three phases that covers the process of inflammation, proliferation and reconstruction of the injured tissue[10]. Many plants are known to synthesize biologically active polyphenols that facilitate wound healing. Black pepper contains phenols and other compounds that contribute to its use in the folk medicine. These polyphenols in black pepper exhibit the effect of anti-inflammatory and antioxidant that lead to the healing activity of black pepper[11].

In the present study, the wound healing property of ethanolic crude extract of black pepper was further evaluated using bioassay-guided fractionation method. This recent study aimed to search for more accurate and inexpensive method for wound healing measures by using scratch assay approach. Optimization of a scratch assay was done, which generated the results in agreement with the previous study. The identification of the fraction containing the compounds responsible for wound healing activity was done. Piperine, an alkaloid, which was accountable for the wound healing activity were identified. Previous research concluded that the ethanolic crude extracts of black pepper exhibited promising wound healing properties[12]. Gel-dot method and plug method were adopted for the *in vitro* wound healing protocols in the previous study. Hence, the aim of this study is to determine and identify the chemical compound in the black pepper with wound healing activity.

2 Materials and Methods

2.1 Plant material

Processed black peppercorns were purchased from Saraspice®. The peppercorns were ground using a Waring Commercial laboratory blender. Extraction was performed using ethanol in a ratio of 1:10 solid to solvent ratio and left to stir in a vessel at room temperature overnight. The solvent extracts were then collected, filtered, centrifuged and evaporated to dryness. Every batch of samples was extracted twice. The crude extract was stored at 4°C.

2.2 Fraction preparation

2.2.1 First stage fractionation

The ethanolic crude extract that showed the potential for wound healing from the previous study[12] was subjected to chromatographic separation using column chromatography and thin layer chromatographic (TLC) techniques (Fig 1). Elution of the extract was done with solvent systems of gradually increasing polarity using hexane, chloroform, methanol and water. The fractions were collected in aliquots of 10-15mL in test tubes depending on the visible changes in the colorful bands eluted from the column. Each fraction thus obtained, was evaporated to dryness and subjected to wound healing assay.

2.2.2 Second stage fractionation

The active first stage fraction was further isolated and grouped based on the regular intervals of retention time. The fractionation was performed using a Gilson preparative high performance liquid chromatography (HPLC) equipped with GX-281 liquid handler and 156 UV detector. The column used was Waters Xterra RPC18 (19x 50mm, 5µm). Sample was prepared in ethanol at concentration of 200mg/mL. The sample was quickly filtered through a 0.45µm membrane filter. The mass injected was approximately 200mg. A UV detector was set at wavelength 210nm and 254nm. Again, each fraction obtained were evaporated to dryness and subjected to wound healing assay.

2.3 MTS assay

Cytotoxicity test was carried out prior to the scratch assay to determine the relative cell viability after being exposed to the extract. The CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay is a colorimetric method adopted in this study. This assay system is based on a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (MTS) being bioreduced by cells into a formazan product in the presence of phenazine methosulfate (PMS). The formazan product is soluble in cell culture medium and is quantified by the amount of absorbance at 490 nm. The conversion of MTS into aqueous, soluble formazan is accomplished by dehydrogenase enzymes found in metabolically active cells. Hence, its absorbance is directly proportional to the number of viable cells.

Viability % = [Absorbance sample / Absorbance control] x 100%

2.4 Wound healing assay

HaCaT cells were seeded into each well of 24 well-plates and incubated at 37°C with 5% CO_2 until confluent monolayer of cells was formed. The confluent monolayer in each well was scratched vertically with a sterile micropipette tip. The old media was discarded and the monolayer was rinsed with Phosphate Buffer Solution (PBS) to remove non adherent cells and debris. The media used was Dulbecco's Modified Eagle's Medium (DMEM) without Fetal Bovine Serum (FBS). Serum-free media was used to have a defined growth medium for the present study. Sample extracts of 200 µL at concentrations 0.3 µg/mL, 1.0 µg/mL, 3.3 µg/mL and 10.0 µg/mL were added accordingly. The plates were observed under microscope with magnification of 4X and images were taken at two points of the scratch for each well. Reference points were created and marked on the plates' cover lid with an ultrafine marker tip to obtain the same field during image acquisition at different time points. Cell migration was captured using Nikon D90 at three time-points of

0 h, 20 h and 44 h. Wound healing is measured as the distance the cell migrated to close the denuded area. The gap distance (cell migration) was then evaluated quantitatively using ImageJ, an automated image analysis software. Percentage of wound closure was calculated based on the unit pixel obtained from ImageJ in comparison to the control captured at time zero[13].

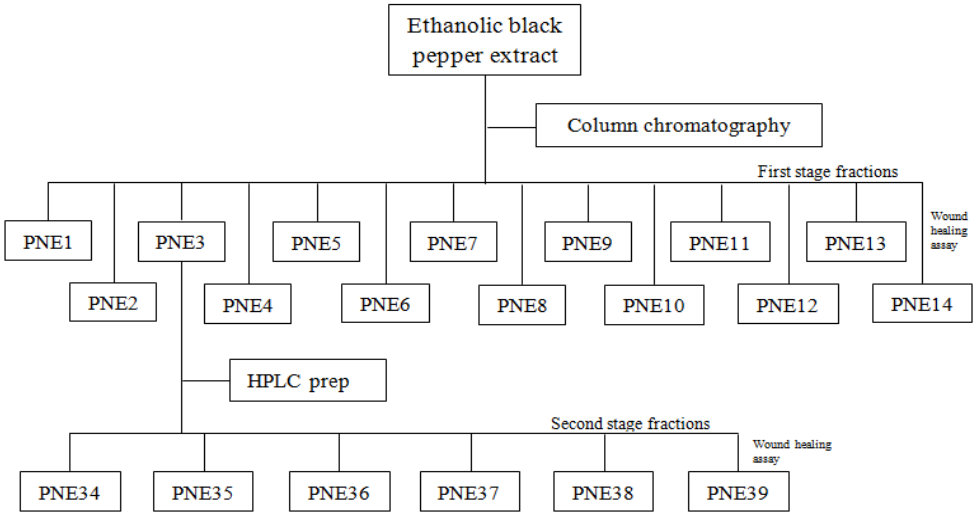

Fig 1: Schematic diagram of bioassay-guided isolation of potential wound healing compounds from *Piper nigrum* L.

2.5 LC-MS analysis

Stock solutions of 1mg/mL methanol for each sample were prepared. Serial dilutions were made to obtain a concentration of 32.125 ppm. The resulting solution was filtered through a 0.22 µm membrane filter prior to analysis.

LC-UV analysis was performed using the Accela™ UHPLC System (Thermo Scientific, San Jose, USA) equipped with quaternary pump, a build degasser, a PDA detector and an autosampler. A hypersil GOLD RP C18 column (3 µm, 2.2 mm I.D. x 150 mm). The step gradient and isocratic solvent composition of acetonitrile : water at a flow rate of 200 µL/min over 30 minutes was adopted. Sample injection volume was 10 µL. After acquisition of the UV spectra, the eluent was redirected to electrospray interface of a mass spectrometer.

LC-MS analysis was done using LTQ Orbitrap mass spectrometer (Thermo Scientific, San Jose, USA). The spectral m/z from 50-1000 was recorded. The electrospray ionization conditions were source accelerating voltage, 4.0kV; capillary temperature, 280ºC; sheath gas flow, 40arb; auxiliary gas, 20arb. Analysis was performed in the positive mode.

2.6 DPPH free radical scavenging assay

According to Mensor et al[16], 1 mL from 0.3 mM ethanol solution of 2, 2 -diphenyl-1-pycrylhydrazyl (DPPH) was added into 2.5 mL sample or standard reference. The solution was mixed and left to stand at room temperature for 30 minutes in the dark. The mixture was measured spectrophotometrically at 518 nm. The antioxidant activity (AA) was calculated as below:

AA% = 100 – [(Abs. sample – Abs. empty sample) / Abs. control] x 100

whereby Abs. is absorbance.

IC_{50}, the amount of sample extracted into 1 mL solution necessary to decrease by 50% the initial DPPH concentration was derived from the % disappearance versus concentration plot which at this point, concentration means mg of sample extracted into 1.0 mL solution. The results were also expressed as ascorbic acid equivalent antioxidant capacity (AEAC)[14] using the following equation:

AEAC (mg Ascorbic acid/100g) = [(IC_{50} Ascorbic acid/IC_{50} Sample) x 10^5]

2.7 Statistical analysis

All values are represented as mean ± SE. Analysis of variance (One-way ANOVA) was used to test any difference in DPPH assay with $p<0.05$. Duncan test was used to determine the significant differences. For wound healing assay, all images were analyzed quantitatively using automated image analysis software called ImageJ. Statistical significance of differences among samples were accessed using student's t-test with $p<0.05$.

3 Results and Discussions

3.1 Cytotoxicity

The eluted fractions yielded by column chromatography in the first stage of isolation were pooled into 14 fractions based on their TLC profile similarity. Sample concentrations that were found to exert low or no cytotoxicity were selected for further activity. The maximum acceptable concentrations were determined from the pre-screening for cell viability and the test concentration range selected was 0.3 µg/mL – 10.0 µg/mL for all the fractions. In the second stage of isolation, cytotoxicity test was again carried out prior to the wound healing assay to determine the relative cell viability after being exposed to the

extract. The maximum acceptable concentrations were determined from the pre-screening for cell viability, and the test concentration range selected was 0.3 µg/mL – 10.0 µg/mL for all the fractions, which was similar to the range used in the first stage bioassay.

3.2 Wound healing assay

3.2.1 First stage fractions

The distance travelled by migrating cell in the wound healing assay was used to measure the healing of a wound. In the first fractionation, out of the 14 fractions, PNE3 has the most bioactive component of cell migration by exhibiting 43.8% and 40.1% closing of 'wound' gap within 20 hours at concentrations of 1.0 µg/mL and 3.3 µg/mL respectively. This was followed by PNE4 whereby it closed the gap by 37.2% and 41.0% within the same time frame and concentrations as PNE3 (Fig 2 and 3).

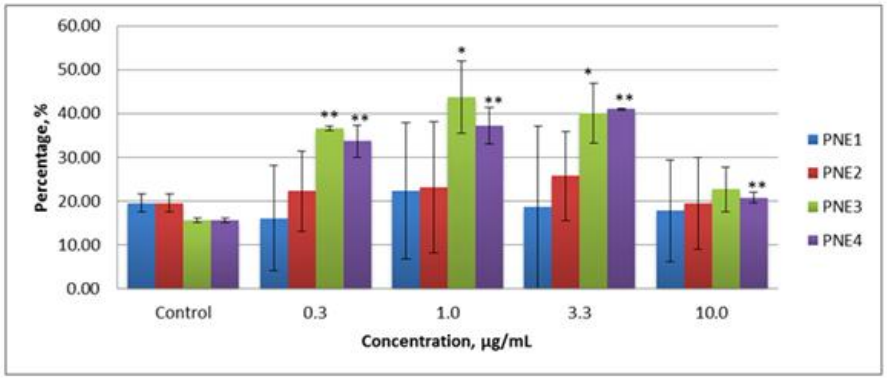

Fig 2: Percentage of scratch 'wound' gap closure after 20 hours of treatment with different concentrations of fractions (* denotes significant difference of p<0.10 and ** denotes significant difference of p<0.05)

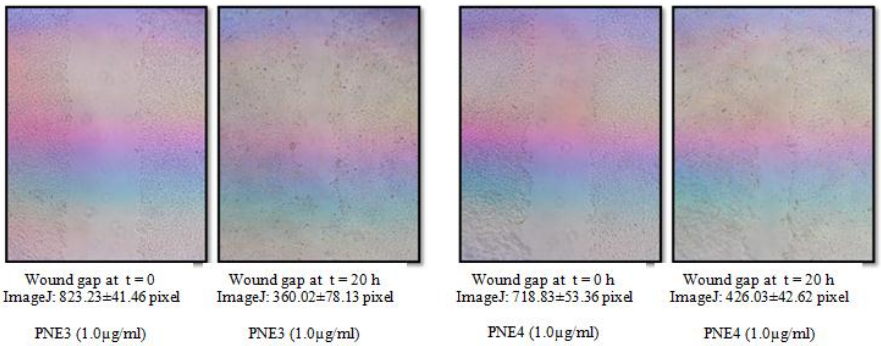

Fig 3: Cell migration at 0 hour and at 20 hours after the treatment of PNE3 and PNE4 at 1.0 µg/ml [Magnification 4X]

At lower concentrations, PNE5 and PNE6 also showed some wound healing activity though the evidence is not as strong as those shown by PNE3 and PNE4. These results confirmed the pre-screening assay done whereby the bioactivity started to decrease when the extract concentration was increased. On the other hand, two fractions, i.e. PNE9 and PNE10 tested in the wound healing assay suggested active inhibition of cell migration at higher test concentrations despite being tested at concentrations which do not induce cytotoxicity. This is shown at both time points after 20 hours and 44 hours (Fig 4 and 5). The remaining eight fractions showed negligible activity.

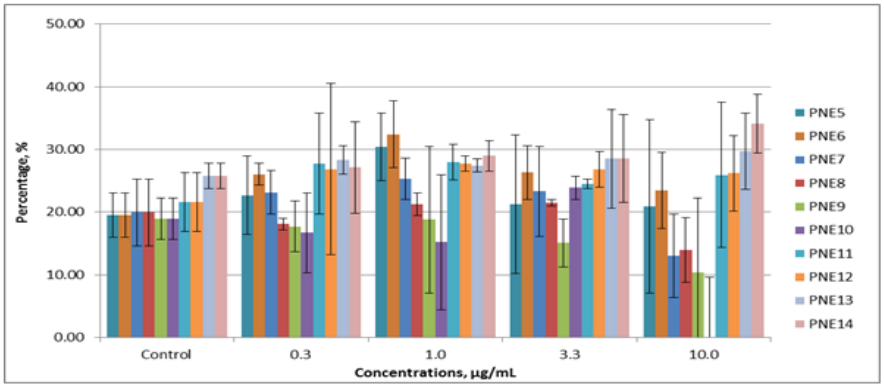

Fig 4: Percentage of scratch 'wound' gap closure at various concentrations for PNE5 – PNE14 after 20 hours

3.2.2 Second stage fractions

From the first stage of isolation and bioassay performed, the two most active fractions detected were PNE3 and PNE4. PNE3 with higher bioactivity was further purified to isolate the compounds which have the wound healing property. The fraction PNE3 was fractionated into nine fractions. However, only six out of nine fractions were further studied in the second stage bioassay as no significant amount of samples were collected for the first three fractions during fractionation (Fig 1).

Almost all of the fractions showed a similar percentage of cell migration by exhibiting 6% - 10% and 22% - 27% closure of wound gap at 1.0 µg/mL after 20 hours and 44 hours respectively, with allowance given for matching vehicle control (Fig 6 and 7). Wound healing activity for all the fractions decreased as the concentrations increased. Fraction PNE37 showed an exceptionally high wound gap closure of 50% at concentration of 10.0 µg/mL after 44 hours which was about two-fold of its matching control while fraction PNE38 showed inhibition to closure of the scratch wound gap (Fig 7).

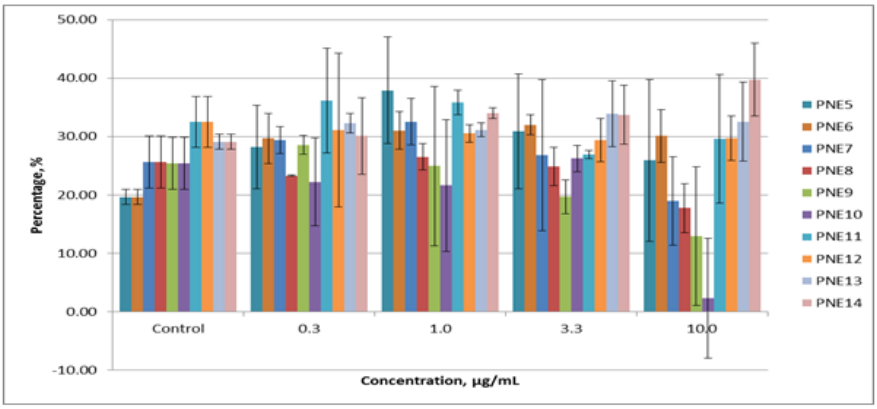

Fig 5: Percentage of scratch 'wound' gap closure for various concentrations of PNE5 to PNE14 after 44 hours

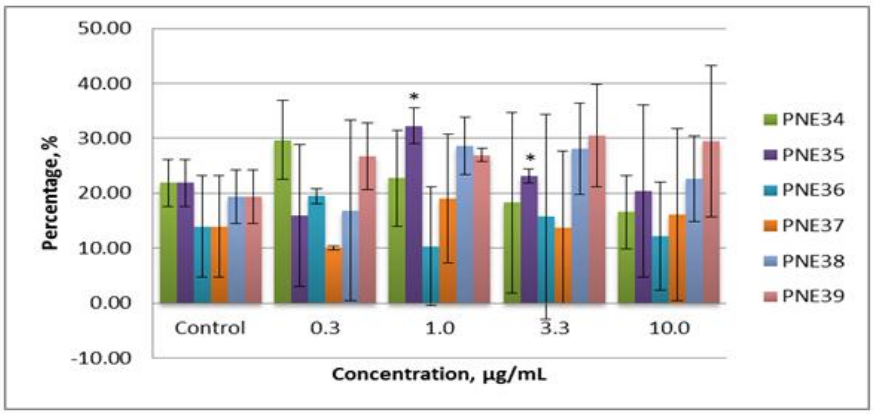

Fig 6: Percentage of scratch wound gap closure of various concentrations for PNE34 – PNE39 after 20 hours. * denotes significant difference of p<0.10

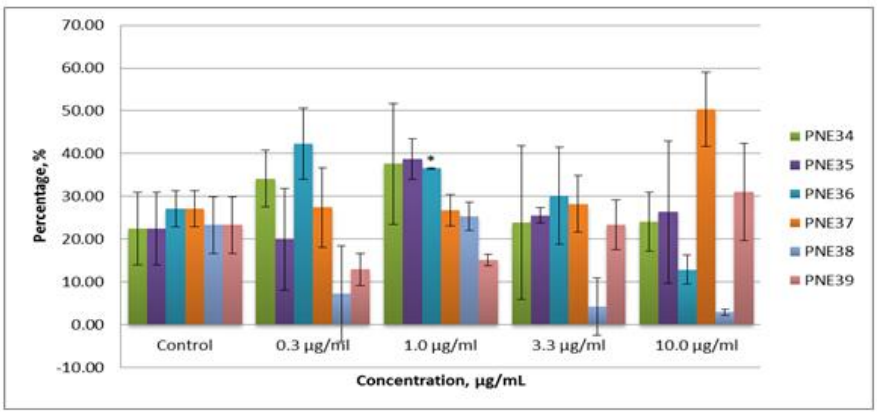

Fig 7: Percentage of scratch wound gap closure of various concentrations for PNE34 – PNE39 after 44 hours. * denotes significant difference of p<0.10

Piperine, a major component found in black pepper (*Piper nigrum* L.) was also tested in the bioassay at this stage. Two sources of piperine were used, one commercially available and the other extracted from peppercorn in the laboratory. The piperine was dissolved in dimethyl sulfoxide (DMSO) whilst all the samples were dissolved in ethanol. Surprisingly, both piperine showed more than 35% closing of the wound gap after 20 hours and increased to more than 42% after 44 hour as compared to its control (DMSO) which closes the wound at about 31% and 38% respectively (Fig 8 and 9).

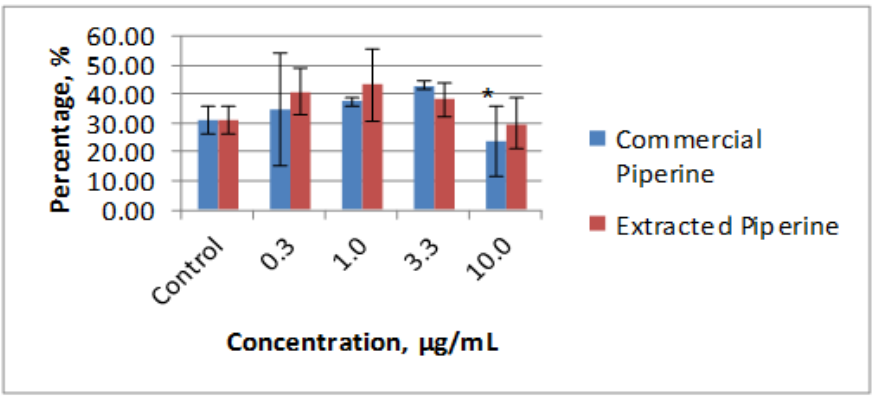

Fig 8: Percentage of scratch wound gap closure at various concentrations for commercial and extracted piperine after 20 hours. * denotes significant difference of p<0.10

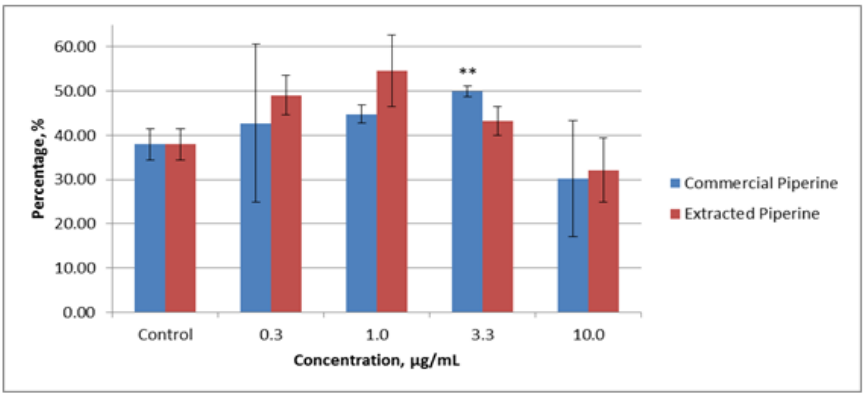

Fig 9: Percentage of scratch wound gap closure at various concentrations for commercial and extracted piperine after 44 hours. ** denotes significant difference of p<0.05

3.3 LC-MS and TLC analysis

The efficacy of wound gap closure exhibited by PNE37 was comparable to those shown by piperine extract. This may be due to the presence of piperine in PNE37. The presence of piperine in black pepper extract and sub-fraction PNE37 were shown by LC-MS (Fig 10;[15]) and TLC profile (Fig 11). TLC chromatogram showed the absence of piperine in PNE38 (Fig 11). From the chromatogram obtained from preparative HPLC (Fig 11), PNE37 was shown as a broad band with several peaks. It shows that this fraction still contain mixtures of compounds that might boost cell migration in the wound healing process.

3.4 DPPH free radical scavenging assay

DPPH is a popular assay used in natural product antioxidant studies. It is used to evaluate the ability of compounds, which in this case was black pepper compounds to act as free radical scavengers or hydrogen donors and thereafter, to measure the antioxidant activity of the pepper extract. Antioxidant activity was determined by the mixture of DPPH solution with black pepper extract that can donate a hydrogen atom (the 2,2-diphenyl-1-picrylhydrazyl (DPPH•)). This gave rise to the reduced form with the loss of the violet colour to a residual pale yellow at absorbance 518nm.

Antioxidant activity can be determined by monitoring the decrease of absorbance[16]. Results were recorded as the inhibition concentration (IC_{50}), which is the amount of antioxidant necessary to decrease by 50% the initial DPPH concentration[17]. The lowest IC_{50} indicates the strongest ability of the extracts to act as DPPH scavengers. In this study, piperine, BHT and quercetin were used as standard references. S2 extract showed the comparable antioxidant activity with BHT at the concentration of 12.50 µg/ml with 64,000 µg/ml AEAC and 66,700 µg/ml AEAC respectively (Table 1). These indicated that the S2 extract was as good DPPH scavenger as BHT at the concentration of 12.5 µg/ml. The result revealed that S2 extracts exhibited higher antioxidant activity than piperine in the

DPPH assay model, at all of six concentration levels tested (3.13 to 100 µg/ml). Piperine was tested together with the sample as it is one of the major components found in black pepper.

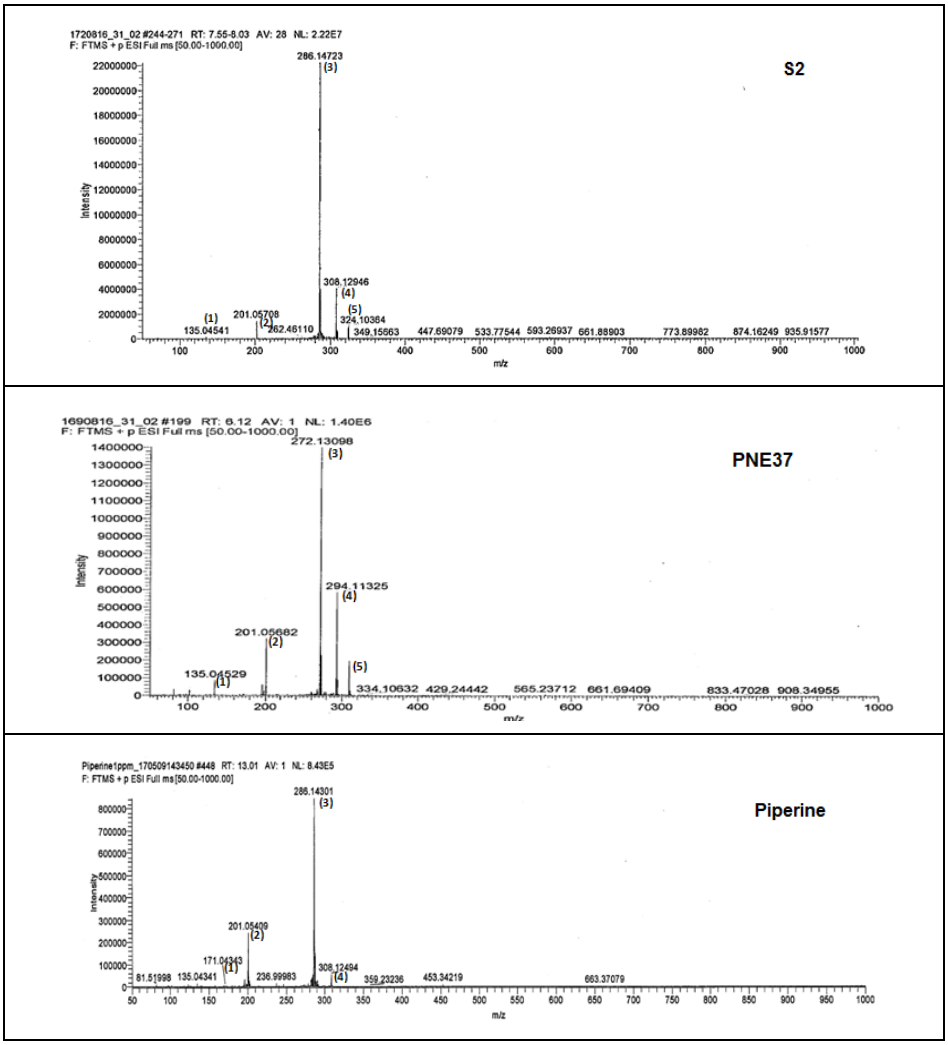

Fig 10: Mass spectrum of crude ethanolic extract of black pepper (S2), sub-fraction (PNE37) and piperine standard

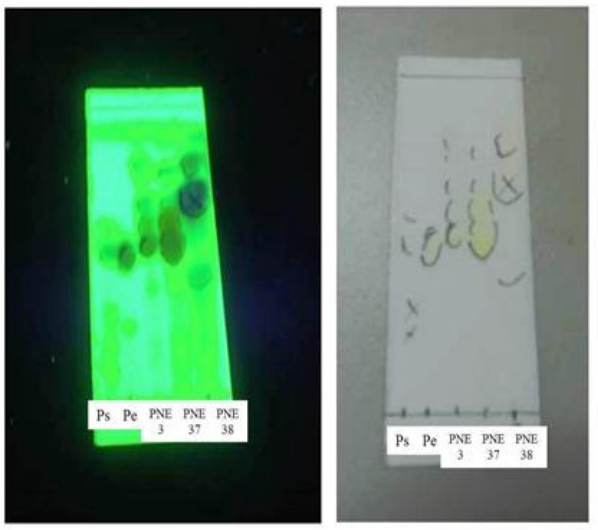

Fig 11: TLC analysis showed the presence of piperine in fraction PNE3 and sub-fraction PNE37 as compared to the standard Piperine (Ps) and extracted piperine (Pe). Piperine was absent in sub-fraction PNE38

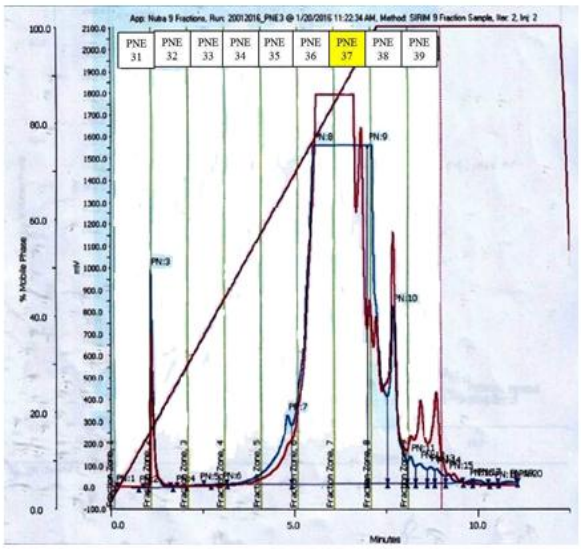

Fig 12: Preparative HPLC chromatogram shows sub-fraction PNE37 as broad band with mixture of compounds that work together to boost wound healing activity

3.5 Wound healing activity

In this recent study, the potential of black pepper (*P. nigrum* L.) in wound healing was demonstrated using the in vitro cell based assay. The scaled down cell based assay systems are constructed to mimic in vivo behaviour and are cost efficient and most of all the increase of the accuracies of predicting in the process of drug discovery. Simple, rapid and specific in vitro assays is viewed as a favourable approach in the breakthrough of new drug as it provides an evaluation stage for cell based and whole animal study. Moreover, in vivo test concern the use of experimental animals that are subject to variation and code of ethics. Thus, in vitro or cell based assay is desirable compared to the in vivo approach[18].

Table 1: DPPH radical scavenging activity of the black pepper

Sample	% Scavenging activity against DPPH free radical at various concentrations (µg/ml)						AEAC (µg/ml)
	3.13	6.25	12.5	25.00	50.00	100.00	
Piper nigrum (S2)	40.46 ± 0.79^{abcd}	50.32 ± 0.19^{fgh}	54.45 ± 0.71^{ghi}	54.83 ± 1.08^{ghi}	57.69 ± 0.50^{hij}	62.02 ± 0.30^{ijk}	6.40×10^4
Piperine	36.72 ± 3.45^{ab}	33.96 ± 2.90^{a}	36.38 ± 0.50^{ab}	36.95 ± 0.60^{abc}	34.30 ± 0.37^{a}	36.18 ± 0.66^{ab}	ND
BHT	42.71 ± 1.13^{bcdef}	48.18 ± 1.35^{defg}	53.87 ± 0.53^{gh}	62.96 ± 0.87^{jk}	77.96 ± 1.09^{l}	86.11 ± 2.54^{m}	6.67×10^4
Quercetin	89.49 ± 1.04^{mn}	91.03 ± 1.06^{mno}	92.83 ± 1.28^{mnop}	93.09 ± 0.08^{mnop}	94.65 ± 1.01^{nop}	95.28 ± 1.03^{nop}	ND

Values represents mean ± S.E (n=3). Means with different letters were significantly different at the level of $p<0.05$.; ND – Not detected at the concentration tested

The wound healing assay is an easy to perform, cheap and well-developed method to measure cell migration in vitro. It reflects cell migration during in vivo wound healing. The technique involves creating a 'wound' by scratching in a cell monolayer, capturing the images at time zero and then at regular intervals during which the cells migrate and grow towards the center of the gap to close the scratch 'wound', and quantifying the migration rate of the cells by comparing the images. The in vitro scratch assay. In the present study, the wound healing assay was performed by observing the migration of keratinocyte HaCaT cells. HaCaT cells are widely used in wound healing assay due to its high capacity to differentiate and proliferate in vitro.

The presence of flavonoids, sesquiterpenes and diarylheptanoids were reported by past researchers as anti-inflammatory, antioxidant agents and promote cell proliferation and migration of fibroblasts. Moreover, flavonoids and sterol could enhance collagen production[19].

Flavonoids were reported to boost the build-up and chemical bonding of collagen by stimulating the alteration to insoluble collagen from soluble one and also, by decelerate the breakdown of soluble collagen[20]. Ethanolic black pepper extract was reported with alkaloids, flavonoids and triterpenes phytochemicals compounds[12]. With that, it was further fractionated at two stages. The most recent study showed significant wound healing activities on the sub-fraction PNE37. PNE37 which has undergone LC-MS analysis showed the presence of piperine as major compound. Piperine was believed to have accelerated the wound healing with its anti-inflammatory and anti-microbial properties[7, 8]. Research on the anti-inflammatory and anti-microbial properties of piperine and the positive fractions are in progress.

4 Conclusion

Sub-fraction PNE37 isolated from fraction PNE3, which is from the crude ethanolic extract of *Piper nigrum* L. berries was found to contain the most profound bioactive wound healing property. It exhibited a remarkably high bioactivity which is comparable to that of piperine, a major component in black pepper. In future, mechanism study, especially, gene expression approach will be pursued to reveal the signalling pathway that responsible for healing process.

5 Acknowledgement

Appreciation goes to Ms. Eri Nagatsuka, attachment student at Swinburne University of Technology (Sarawak) for the partial optimization of the scratch assay and Mr. Macmillan Fidelis, research assistant of MPB for measurement of cell migration using ImageJ.

6 Funding Source

This research was funded by Malaysian Pepper Board.

7 Conflict of interest

The authors declare that there is no conflict of interest.

8 Author's contributions

All authors designed the work study, analysed and interpreted the work data. WCM and LJJ did the experimental work, drafted and revised the work and manuscript. PMN and HSS reviewed the drafted work and manuscript.

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