**In Vitro Study of Wound Healing Potential in Black Pepper (Piper nigrum L.)**

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**Abstract**

*Piper nigrum* L. is a perennial climbing vine and its dried; ground berries make one of the most common spices in worldwide cuisine. Besides its extensive culinary uses, black pepper is commonly mixed in home remedies to heal wound and cut. Hence, the current research study was aimed at discovering potential wound healing properties of black pepper berries. A preliminary study was also carried out to determine some major phytochemicals in black pepper berries extracted with different solvents. *In vitro* cell-based assays were adopted for the observation of wound healing activity. The present study demonstrated that the extracts of the black berries of *Piper nigrum* L. (0.32-1.0 µg/ml) encouraged cell migration activity, notwithstanding existence of contrasting activity as the concentration increased. The observed wound healing activity was most probably due to the presence of phytochemicals, viz.; flavonoids and triterpenes.

**Keywords:** *Piper nigrum* L., Phytochemicals, Wound healing activity

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1 Introduction

Since ancient times, plants are widely used as folk’s medicine for wound healing¹. Plant products are the potent healer because they are natural, broadly available and effective as crude preparations². The occurrence of bioactive compounds in plant parts have been revealed and proven through studies on the phytochemicals of the plant crude extracts. These secondary metabolites possess protective or disease preventive characteristics. Although the plant produces the non-nutritive chemical compounds to shield itself, investigations showed that these compounds are capable of promoting health³. For example, Manach et al. reported that generally, the secondary metabolites of plants defend against ultraviolet radiation or aggression by pathogens⁴.

In the recent years, much research interest is fixated on the promising characteristic of phytochemicals in the prevention and treatment of many diseases⁵. Many molecules originated from natural product especially plant have demonstrated encouraging medicinal effects⁶. The medicinal values of the medicinal plants are attributed to the bioactive phytochemical compounds that yield normal physiological action on the human body. Phytochemicals have been linked to the prevention and handling of diabetes, cardiovascular diseases and hypertension⁷. Phenolics, for example, have been reported to exert antioxidant, anti-inflammatory, antmutagenic and anticarcinogenic activities owing to their valuable biological and pharmalogical properties. Besides phenolics, flavonoids are another group of phytochemicals that have been recognized for its antimicrobial activity, anti-inflammatory activity, anti-allergic activity and antitumor activity⁸. Alkaloids, essential oils, tannins, terpenoids, saponins and phenolic compounds are some other examples of the most useful and important bioactive phytochemical constituents⁹. These phytochemicals are believed to exert a significant characteristic in wound healing.

Black pepper (*Piper nigrum* L.) also known as the King of Spice is one of nature’s goodies that served important culinary purposes. It is widely cultivated in its native land of Southern India. For decades, black pepper has been introduced and extensively cultivated as one of the major commodity crops in tropical countries such as Malaysia, Vietnam as well as Indonesia. Peppercorn, the dried seed of black or white pepper is used as a condiment to flavor food¹⁰,¹¹. It also has vast medical potentials that are often being overlooked. For centuries, black pepper remains as a traditional cure for minor cut and wound¹²,¹³. It is known to disinfect the wound and stimulates blood coagulation, and scab formation. Black pepper not only disinfects the injury and seals the wound, but it also helps the wound to heal faster and lessens the chances of scars as well. However,
there is scarce scientific literature on the application of peppercorn in wound healing\textsuperscript{14}.

The commencement of this work was to assess the presence of phytochemicals in \textit{Piper nigrum} L. berries extracts. The wound healing activity of the extracts was evaluated using \textit{in vitro} methods.

\textbf{2 Materials and Methods}

\textbf{2.1 Plant Material}

Dried and processed black berries of \textit{Piper nigrum} L. was procured from SaraSpice\textsuperscript{\textregistered}. The berries were ground by using a laboratory blender and stored in airtight container until used.

\textbf{2.2 Preparation of Extracts}

The black berries that were ground were subjected to Soxhlet extraction process. 40 g of the ground berries was submitted to successive solvent extraction separately with 200 mL each of hexane, ethanol and methanol at room temperature for eight hours. The solvent extract was collected and then evaporated to dryness using a rotary evaporator at 60 °C and stored at 4 °C until further analysis. The percentage of yield was calculated from the extract that was obtained after evaporation\textsuperscript{15}.

\textbf{2.3 Phytochemical Screening}

Preliminary phytochemical screening was carried out on the extracts for detection of some vital constituents such as alkaloids, flavonoids, tannins and triterpenes. The qualitative screenings of the extracts for phytochemicals were carried out using the standard chemical tests\textsuperscript{16, 17}.

\textbf{2.4 Wound Healing Activity}

Wound healing activity was evaluated by using the Oris™ Pro Cell Migration Assay Tissue Culture Treated Plate (Platypus Technologies). The manufacturer's protocol was adopted. Human Epidermal Keratinocytes was seeded in culture dishes under standard growth conditions at 37 °C /5% CO\textsubscript{2}. A single layer of cell was cultured in a specific culture dish with a central barrier that mimic the condition of wound surface that were devoid of cells. Then, in two separate experiments, the central “wound areas” were formed by different means, with wound healing cell migration measured in different ways.

\textbf{2.4.1 Sample Pre-Screening}

Prior to the experiment, the samples were tested for non-specific interference. Pre-screening for sample cell damage (cytotoxicity) was done to determine maximum acceptable test concentration.

\textbf{3 Results and Discussion}

\textbf{3.1 Percentage of Yield}

The average percentage yield of various extracts of \textit{Piper nigrum} L. calculated is as shown in Table 1. From the percentage yield, it was observed that the hexane and ethanol extracts gave the most yields whereas the methanol extracts yielded the least.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
Sample & Extracts & Nature of extracts & Color & Yield (%w/w) \\
\hline
MPB/S1 & Hexane & Liquid & Reddish brown & 2.88 \\
MPB/S2 & Ethanol & Semisolid & Dark olive green & 2.86 \\
MPB/S3 & Methanol & Semisolid & Light olive green & 0.86 \\
MPB/S4 & Hexane & Solid & Yellowish & 0.65 \\
\hline
\end{tabular}
\caption{Percentage yield of various extracts of \textit{Piper nigrum} L.}
\end{table}

\textbf{3.2 Phytochemical Screening}

Four samples were evaluated qualitatively for the presence of several phytochemical compounds as reported. Overall, the preliminary screening observed the presence of alkaloids, flavonoids, triterpenes and steroids in berries of \textit{Piper nigrum} L. (Table 2).
Flavonoids and triterpenes were detected in all the extracts whereas saponins and tannins were found absent.

### 3.3 Pre-Screening Study

#### 3.3.1 Assay Compatibility

The assay compatibility test was done in a cell-free assay system and had identified all the samples interference with assay readout within an acceptable range of 1.0–0.01 mg/ml (Table 3). The samples then undergone preliminary phytochemical screenings in order to observe the chemical nature of its active constituents prior to the wound healing assay.

#### 3.3.2 Cytotoxicity

An assay involving the release of cytosolic dehydrogenase enzyme from the cells into the culture medium was done to determine levels of cell damage. Sample concentrations that were found to exert low or no cytotoxicity were selected for further activity (Table 4).

### Table 2: Phytochemicals screening of the black berries extracts of *Piper nigrum* L

<table>
<thead>
<tr>
<th>Sample</th>
<th>Alkaloids</th>
<th>Saponins</th>
<th>Flavonoids</th>
<th>Tannins</th>
<th>Triterpenes</th>
<th>Steroids</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPB/S1</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MPB/S2</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>MPB/S3</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>MPB/S4</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Note: (+) = present; (-) = absent

### Table 3: Sample assay compatibility concentrations

<table>
<thead>
<tr>
<th>Sample</th>
<th>Assay readout concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPB/S1</td>
<td>1.0–0.01 mg/ml</td>
</tr>
<tr>
<td>MPB/S2</td>
<td>1.0–0.01 mg/ml</td>
</tr>
<tr>
<td>MPB/S3</td>
<td>1.0–0.01 mg/ml</td>
</tr>
<tr>
<td>MPB/S4</td>
<td>1.0–0.01 mg/ml</td>
</tr>
</tbody>
</table>

### Table 4: Sample concentrations with low or no cytotoxicity

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sample concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPB/S1</td>
<td>31.6 µg/ml</td>
</tr>
<tr>
<td>MPB/S2</td>
<td>10.0 µg/ml</td>
</tr>
<tr>
<td>MPB/S3</td>
<td>10.0 µg/ml</td>
</tr>
<tr>
<td>MPB/S4</td>
<td>31.6 µg/ml</td>
</tr>
</tbody>
</table>

### Table 5: Sample concentration ranges for wound healing activity

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sample concentration ranges</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPB/S1</td>
<td>31.6–0.316 µg/ml</td>
</tr>
<tr>
<td>MPB/S2</td>
<td>10–0.1 µg/ml</td>
</tr>
<tr>
<td>MPB/S3</td>
<td>10–0.1 µg/ml</td>
</tr>
<tr>
<td>MPB/S4</td>
<td>31.6–0.316 µg/ml</td>
</tr>
</tbody>
</table>

#### 3.3.3 Sample Test Range

Based on the pre-testing, the following sample concentration ranges (Table 5) were selected for the wound healing activity.

### 3.4 Wound Healing Activity

For this study, the potential of black pepper (*Piper nigrum* L.) in wound healing was demonstrated by using the *in vitro* cell-based assay. The application of cell-based assays in the drug discovery process is regarded as promising screening approaches, allowing a platform of evaluations between gene- or protein-based studies and whole animal models. It is also believed that the scaled-down cell-based assay systems are constructed specifically to mimic *in vivo* behavior and are able to reduce costs whilst add efficiencies and most of all increase the accuracies of predicting in the process of drug discovery. In addition, *in vivo* tests using small laboratory animals often have variables and are highly debated by ethical considerations of animal welfare. However, the cell-based assay method should be as simple, rapid and specific as possible.
The cell migration in relative fluorescence unit (RFU) for the four extracts with each tested in five different concentration ranges and depicted in figure 1. The analyses were done in comparison to a positive (serum) and a negative control. The present investigation revealed that even at low concentrations, the liquid extract of hexane (S1) showed negligible activity. On the other hand, the ethanolic (S2), methanolic (S3) and solid extract of hexane (S4) showed some evidence of wound healing ability at one or more, lower concentration(s) by using the plug method of the wound healing assay. These are represented by the positive bars in figure 1.

Figure 2 (the gel-dot method) showed the sample effect compared to control cultures containing sample solvent vehicle only. This method was performed to compare the reproducibility of the assay measurements. Positive values indicate stimulation of migration while negative values indicate adverse cell effect. At low concentrations of 0.32-1.0 µg/ml, all the samples tend to promote cell migration. And in every case, the positive effect declines with increasing concentration. Samples S2 and S3 appeared to be stimulatory at the lowest concentration tested at 100 ng/ml.

The phytochemical result and cell migration activity suggested that the wound healing potential in black pepper could be attributed to the presence of alkaloids, flavonoids and triterpenes. Flavonoids, which contain one carbonyl group in their structure includes flavones and flavonols. Studies with other plants have shown that these phytochemicals demonstrate wound healing properties due to their antibacterial and antioxidant properties\(^\text{21}\). Triterpenes also have been recognized as the agent that promotes wound healing. They seem to be responsible for wound contractions and increasing the rate of epithelialization because of their astringent and antimicrobial properties\(^\text{22}\).

Despite being tested at concentrations which do not induce cytotoxicity, there is evidence of active inhibition of cell migration at higher test concentrations (shown as high negative values in fig. 1 and 2). The tendency to increase cell migration at low concentration and the declination of the positive effect with the increase of concentration suggests that each extract contains a cell-inhibiting activity which progressively counteracts the positive effect of a relatively potent stimulatory component. Together, the results indicated that the extracts may have adverse cell effect at high concentrations, which may mask a stimulation of migration, i.e. suggesting wound healing potential as seen in some samples at low concentrations.

4 Conclusions

The extracts of black berries of \textit{Piper nigrum} L. encouraged cell migration activity under conditions in which no direct cytotoxicity was observed. This is due to the phytochemical constituents found in the extracts that are believed to play a major role in promoting the wound healing activity. However, further isolation and identification of the phytochemicals are necessary to figure out the active compound(s) accountable for the pharmacological activity. All extracts also seem to contain other activities which actively counteract stimulation of cell migration. Selection and chemical fractionation of samples that showed stimulation of cell migration may allow dissociation of the stimulatory and inhibitory activities, hence allowing the biological, i.e. wound healing potential of the active agent(s) to be more accurately assessed. Based on the results of this study, the ethanolic extracts (MPB/S2) and methanolic extract (MPB/S3) are recommended for further investigation.

5 Acknowledgments

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6 References


Wong et al. Wound Healing Potential in Black Pepper


