Ethanol extract of *Imperata Cylindrica* leaves inhibits proliferation and migration of HSC-3 cell lines

Moehamad Orliando Roeslan¹ and Gabriella Tasha²
¹Department of Oral Biology, Faculty of Dentistry, Trisakti University, Jakarta, Indonesia
²Undergraduate Student, Faculty of Dentistry, Trisakti University, Jakarta, Indonesia

**ABSTRACT**

**Background:** Squamous cell carcinoma (SCC) is classified as the most common type of oral cancer up to 90% of all malignant neoplasm in the oral cavity. Currently, the only treatments for SCC are surgery and/or radiation or chemotherapy, which can cause various side effects. Cogon grass leaves (*Imperata cylindrica*) have been considered an alternative cancer treatment that may reduce side effects. *Imperata cylindrica* (*I. cylindrica*) leaf extract can inhibit cancer cell proliferation and migration by withholding the cell cycle in the gap 1/synthesis (G1/S) and gap 2/mitosis (G2/M) phases. Therefore, the levels of matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) is decreased and cancer is not progressing.

**Purpose:** The study aims to determine the effect of *I. cylindrica* leaf extract on the proliferation and migration of human oral squamous carcinoma-3 (HSC-3) cell lines. **Methods:** This in vitro experimental study was conducted with nine study groups. The treatment group was divided into seven concentrations—640 ppm, 320 ppm, 160 ppm, 80 ppm, 40 ppm, 20 ppm and 10 ppm. 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium-bromide (MTT) assay and scratch assay were carried out to assess the effect of *I. cylindrica* leaf extract on HSC-3 cell proliferation and migration. **Results:** Ethanol extract of *I. cylindrica* has a significant effect compared to the negative control towards the proliferation and migration of HSC-3 cells. **Conclusion:** This study shows that *I. cylindrica* ethanol leaf extract can inhibit proliferation and migration of HSC-3 cells.

**Keywords:** HSC-3 cell; *Imperata cylindrica*; MTT assay; oral cancer; scratch assay

Correspondence: Moehamad Orliando Roeslan, Department of Oral Biology, Faculty of Dentistry, Trisakti University. Jl. Kyai Tapa No. 1, Jakarta Barat 11440, Jakarta, Indonesia. Email: orliando.roeslan@trisakti.ac.id

**INTRODUCTION**

Oral cavity SCC is a type of cancer in humans that has a high prevalence in comparison to all malignant oral cancers.¹ The incidence of SCC mainly occurs in people over 40 years of age. Its prevalence in men is greater than in women. The aetiology of oral SCC is multi-factorial—a combination of genetic and predisposing factors.² The lateral side of the tongue, gingival mucosa, tongue ventral and the floor of the mouth are the predilections of SCC.³ Referring to World Health Organization (WHO) data in 2018, the death rate for oral cancer was ranked at 15 from those of 33 types of cancer, namely 177,384 deaths.⁴ Currently, the treatment for SCC is surgery alone, or it can be combined with radiation or chemotherapy.⁵ In the world of modern medicine, traditional medicine is starting to be developed again because of the high incidence of oral cancer.⁶,⁷ One of the natural ingredients that has anti-cancer activity is cogon grass leaves (*I. cylindrica*).⁸ Previous research showed that the leaf extract of *I. cylindrica* has an effect on the SCC-9 cell line by inhibiting cell proliferation.⁹ Cell migration can be reduced by the presence of flavonoids and alkaloids in the leaves of *I. cylindrica*. They achieve this by decreasing the expression of MMP-2 and MMP-9, which are proenzyme members of zinc-endopeptidases and play a role in cancer development.¹⁰ However, there was no similar study that used leaf extract of *I. cylindrica* on the HSC-3 cell line. Cancer cell line HSC-3 cells are a type of the human oral SCC cell culture that is suitable for use as a study model. This cell line has the highest metastatic ability among cells other cultures, such as HSC-2, HSC-4 and HSC-7.¹¹ The aim of this study is to determine effect of *I. cylindrica* leaf extract on the proliferation and migration of HSC-3 cell lines.
MATERIALS AND METHODS

The leaves of *I. cylindrica* were grown and taken from Bogor, West Java, Indonesia. The leaves, stem and root of the *I. cylindrica* were sent to the Indonesian Institute of Science for authentication. After the leaves dried under indirect sunlight, they were ground to powder. Five hundred millilitres of ethanol was used to extract 50 g of leaf powder into a Soxhlet extractor at 79°C for 70 hours. After that, the liquid extract was evaporated in a rotary evaporator at 50°C under vacuum. The leaf extract of *I. cylindrica* was stored at 4°C in the refrigerator until it was used.\(^1\)\(^2\)

Qualitative phytochemical screening was done to assess the constituents in ethanol extract of the *I. cylindrica* leaves. Five percent of the sodium hydroxide (NaOH) solution was mixed to 1 mL of crude extract, which generated a yellow colour. The presence of flavonoid was confirmed after hydrogen chloride (HCl) was added and the yellow colour disappeared.\(^3\)

One milligram of extract was dissolved with 10 mL of chloroform, then an equal volume of sulphuric acid (H\(_2\)SO\(_4\)) was added. The presence of steroid was confirmed when the H\(_2\)SO\(_4\) layer turned yellow with green fluorescence and the layer above it turned red.\(^4\)

The crude extracts were mixed with 1 mL of chloroform and H\(_2\)SO\(_4\). The presence of terpenoid was confirmed after it generated a red-brown colour.\(^5\)

Fifty grams of crude extract was mixed with 5% ferric chloride (FeCl\(_3\)). The presence of tannin was confirmed after the development of a bluish black colour.\(^6\)

Dragendorff’s solution was added to 0.1 g of extract. The development of an orange-red precipitate confirmed the presence of alkaloid.\(^7\)

The HSC-3 cell line was cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, USA) with an addition of 10% fetal bovine serum (FBS; Gibco, USA) and 1% penicillin-streptomycin (Invitrogen, USA). The cells were incubated under humidified conditions at 37°C and in an atmosphere of 5% CO\(_2\). Then dimethyl sulfoxide (Sigma Aldrich, USA) was added and a microplate reader (Tecan, Salzburg, Austria) was used to measure the absorbance at 570 nm. The formula to calculate cell viability (%) is:

\[
\text{Percentage gap closure} = \frac{[\text{A}_\text{test} - \text{A}_\text{control}] \times 100}{\text{A}_\text{test}}
\]

Where \([A]_\text{test}\) is the absorbance of the test sample and \([A]\) control is the absorbance of the control.

The in vitro scratch assay was used to test the effects of the *I. cylindrica* leaf extract on the migration of HSC-3.\(^8\)

The cells (1 x 10\(^4\) cells/well) were cultured in 6-well plates containing DMEM with an addition of 10% FBS and 1% penicillin-streptomycin. After it reached 90% confluence, the scratches were created vertically and horizontally in the centre of the well and monolayers of each well. Phosphate-buffered saline was used to wash cellular debris from the scratch. The cells in the well were then treated with various concentrations (10, 20, 40, 80, 160 and 640 ppm) of *I. cylindrica* leaf extract. The scratches were observed under an inverted microscope periodically (0, 6, 12 and 24 hours). The negative control was DMEM without extracts. Three \(\mu\)M of doxorubicin was used as the positive control. Image J software (National Institutes of Health, Bethesda, MD) was used to calculate the gap area in the monolayers after pictures were taken. The formula to calculate gap closure is:

\[
\text{Percentage gap closure} = \frac{[\text{A}_\text{test} - \text{A}_\text{control}] \times 100}{\text{A}_\text{test}}
\]

Where, \(A\) test is the area of the wound measured immediately after scratching and \(A\) control is the area of the wound measured 24 hours after scratching.

The Shapiro-Wilk test was used to assess normality. The differences between experimental groups were analysed using one-way and two-way analysis of variance (ANOVA) with the Post-Hoc test. A p-value (p<0.05) was considered statistically significant.

RESULTS

Qualitative phytochemical assay showed that *I. cylindrica* ethanol leaf extract contains flavonoid, steroid, terpenoid, tannin and alkaloid (Table 1). The results of the cell proliferation assay indicated that the *I. cylindrica* ethanol leaf extract at all concentrations was significantly different compared to the negative control (Figure 1). While compared to the positive control, concentrations 640, 320, 160, 80 and 40 ppm had no significant difference.

The results of the scratch assay showed that a small percentage means that the gap is closing. The negative control group showed that at six hours, the gap started to close by 61.82% and at 12 hours the whole gap was closed.

| Assay          | Results |
|---------------|---------|
| Flavonoid     | +       |
| Steroid       | +       |
| Terpenoid     | +       |
| Tannin        | +       |
| Alkaloid      | +       |

Table 1. Qualitative phytochemical assay of ethanol extracts from *I. cylindrica*
already closed by the cells. On the contrary, the positive control and concentration 640 ppm groups showed that at six hours, cells had already undergone apoptosis. While at concentration 320 ppm, from six to 24 hours, the gap was more open (114% to 145%). This means that after a longer time, cells started to undergo apoptosis. At concentration 10 to 160 ppm, it showed that after a longer time, the gap was closing. It means that concentration 10 to 160 ppm could not inhibit HSC-3 migration (Table 2).

TABLE 2. Scratch assay of HSC-3 under various concentrations. The data is expressed as mean ± SD (n = 3)

| Concentration | Hour | Viable Cells (%) |
|---------------|------|-----------------|
| Control (-)   | 0    | 100             |
|               | 6    | 61.82 ± 49.25   |
|               | 12   | Cell already closed the gap |
|               | 24   | Cell already closed the gap |
| Control (+)   | 0    | 100             |
|               | 6    | Apoptosis       |
|               | 12   | Apoptosis       |
|               | 24   | Apoptosis       |
| 640 ppm       | 0    | 100             |
|               | 6    | 114.64 ± 19.64  |
|               | 12   | 125.53 ± 19.64  |
|               | 24   | 145.55 ± 19.64  |
| 320 ppm       | 0    | 100             |
|               | 6    | 93.69 ± 6.26    |
|               | 12   | 89.5 ± 6.26     |
|               | 24   | 85.31 ± 6.26    |
| 160 ppm       | 0    | 100             |
|               | 6    | 87.62 ± 9.48    |
|               | 12   | 87.19 ± 9.48    |
|               | 24   | 76.81 ± 9.48    |
| 80 ppm        | 0    | 100             |
|               | 6    | 75.099 ± 29.3   |
|               | 12   | 69.13 ± 29.3    |
|               | 24   | 29.2 ± 29.3     |
| 40 ppm        | 0    | 100             |
|               | 6    | 73.05 ± 43.99   |
|               | 12   | 33.13 ± 43.99   |
|               | 24   | Cell already closed the gap |
| 20 ppm        | 0    | 100             |
|               | 6    | 77.01 ± 43.22   |
|               | 12   | 46.48 ± 43.22   |
|               | 24   | Cell already closed the gap |
| 10 ppm        | 0    | 100             |
|               | 6    | 75.099 ± 29.3   |
|               | 12   | 69.13 ± 29.3    |
|               | 24   | 29.2 ± 29.3     |

Figure 1. Cell proliferation after treated with ethanol extracts of *I. cylindrica* leaves with various concentration. Negative control was without treatment. Positive control was with 3 µM of doxorubicin. The data was expressed as mean ± SD (n = 3). * Indicates a significant difference versus the negative control group (p<0.05).

DISCUSSION

In previous studies, *I. cylindrica* leaf extract which contained flavonoid and alkaloid showed anti-cancer activity by inhibiting proliferation, inflammation, angiogenesis, invasion and metastasis.18,19 Flavonoid and alkaloid work by inducing the inhibition of the cell cycle in the gap 1/synthesis (G1/S) and gap 2/mitosis (G2/M) phases and cell apoptosis. This ultimately leads to inhibition of cancer cell proliferation.20 The results of the cytotoxicity assay showed that concentrations 40 to 640 ppm had no significant difference compared to the positive control. This showed that as low as at concentration 40 ppm, *I. cylindrica* leaf extract could inhibit proliferation of HSC-3. This result is in line with previous research which stated that *I. cylindrica* leaf extract could inhibit proliferation and induce apoptosis of SCC-9.21 However, in that research the highest inhibition was approximately 50%, while in this research the highest inhibition was 74.5%. This could be because of cell difference and solvent difference for extracting from the plant. Previous research also showed that *I. cylindrica* leaf extract could decrease proliferation (inhibition up to 50%) of the colorectal cancer cell line (HT-29).22

The benefit of this scratch assay is that it can study the regulation of cell migration through cell–extracellular matrix (ECM) interactions and cell–cell interactions.23 In this study, the concentration of 640 ppm at 6, 12 and 24 hours could induce HSC-3 to undergo apoptosis, the same as the positive control. At concentration 320 ppm, some of the cells already started to undergo apoptosis at...
6 hours. At 12 and 24 hours more cells had undergone apoptosis. Meanwhile, at concentration 10 to 160 ppm, *I. cylindrica* leaf extract could not induce HSC-3 to undergo apoptosis.

Vimentin, N-cadherin and β-catenin are proteins that play a role in HSC-3 becoming less invasive. These proteins affect cancer invasion and tumour metastasis. The alteration of cell state in HSC-3 cells was also caused by epithelial-mesenchymal transition (EMT) programs that are characterized by a mesenchymal marker. A previous study showed that inhibition of EMT promoters, the epithelial cell adhesion molecule (EpCAM) and survivin-1 also inhibit cancer cell migration.

Based on the qualitative phytochemical assay, the *I. cylindrica* leaf extract that is used in this research contains flavonoid, steroid, terpenoid, tannin and alkaloid. In carcinogenesis, flavonoid are secondary plant metabolites that have the ability to decrease proliferation, metastasis and angiogenesis and to increase apoptosis by interfering in multiple signal transducing pathways. Flavonoid that are distributed mainly in leaves are flavones. Apigenin, one of the flavones, induced the SCC25 cell to apoptosis by leading to halting the cell at the G2/M phase via an increase level of reactive oxygen species (ROS). In a previous study, apigenin induced ACHN (kidney-cancer-derived cell line), 786-0 cell lines to undergo apoptosis via cell arrest at the G2/M phase, DNA damage and p53 upregulation. Luteolin, another flavones, induced HeLa cells to undergo apoptosis by the escalation of caspase-3 and -8, receptor (Fas/FasL, DR5/TRAIL, and FADD) upregulation and inhibition of B-cell lymphoma 2 (Bcl-2) and B-cell lymphoma-extra large (Bcl-XL) expression.

Terpenoids consist of 4 large groups—steroids, terpenes, saponins and cardiac glycosides. Every year new terpenoid are being discovered. There are 40,000 compounds that are included in terpenoids. A previous study showed *Annona cherimola*, with a high content of terpenoid, induced apoptosis of acute myeloid leukemia cell lines through a Bax/Bcl-2 dependent mechanism. The shifting of Bax in cytosol to mitochondria through the Bax pores caused apoptosis via DNA damage. A previous study showed that steroid induced apoptosis via enhancement of caspase-3.

Alkaloid isolated from *Zanthoxylum nitidum* possesses the ability to induce apoptosis on HSC-3 and HSC-4 cell lines via blockage expression of signal transducer and activator of transcription 3 (STAT3). The STAT3 signalling pathway showed a contribution to the progression of a tumor. Another previous study on alkaloid from a medicinal plant, showed apoptosis activity on Jurkat T cells by upregulating caspase-8 and -9 and effector caspases-3 and -6. It also enhanced expression of Bax and p53.

Flavonoid, steroid, terpenoid, and alkaloid from medicinal plants have many mechanisms to induce apoptosis on various cancer cell lines. These compounds are found in *I. cylindrica* leaf extract and promising as anti-cancer agents. In the future, we need to investigate the predominant compound in *I. cylindrica* that possesses activity to induce apoptosis in cancer cells. In conclusion, *I. cylindrica* leaf ethanol extract could inhibit HSC-3 cell proliferation at concentration 10 ppm and higher. This extract also could inhibit HSC-3 migration at concentration 320 ppm at 6 hours and higher.

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