

# High Antitumor Activity of Ethanolic Extracts of Papua's Ant Nest Plant (*Myrmecodia tuberosa*) on an Oral Carcinoma (KB) Cell Line

Sartari Entin Yuletnawati<sup>1</sup>, Edi Meiyanto<sup>2</sup>, Dewi Agustina<sup>3</sup>

<sup>1</sup>Post-graduate student, Faculty of Dentistry, Universitas Gadjah Mada, Indonesia

<sup>2</sup>Cancer Chemoprevention Research Center, Faculty of Pharmacy, Universitas Gadjah Mada, Indonesia

<sup>3</sup>Department of Oral Medicine, Faculty of Dentistry, Universitas Gadjah Mada, Indonesia

**Abstract:** Papua's ant nest (*Myrmecodia tuberosa*) is a traditional plant that is known to have the strong anticancer compound. Efficacy of ant nest was shown by the content of active substances include flavonoids, polyphenols, tannins and glycosides. Ant nest plant has been used to treat various diseases traditionally and safely without any side effects. Aims of the study were to examine the high antitumor activity of ethanolic extracts of Papua's ant nest plant on an oral carcinoma cells through the growth inhibition and apoptosis induction of KB cells. In the present study, KB cells were treated with various concentration of ethanol extract of Papua's ant nest plant. MTT assay was conducted to determine the growth inhibition of KB cells. The double-staining analysis was carried out to evaluate the induction of cells apoptosis. The results revealed the growth of KB cells treated with ant nest extract was remarkably suppressed. The percentage of cell inhibition was known at 84.2%. Therefore, apoptosis of KB cells was markedly induced characterized by an increase in death cells. Percentage of apoptosis was detected at 88%. In addition, the fifty-inhibitory concentration (IC<sub>50</sub>) of Papua's ant nest was reported at 215 µg/ml in KB cell line. These results indicated that Papua's ant nest plant exhibited a high potential antitumor activity in oral carcinoma KB cells.

**Keywords:** Papua's ant nest plant, ethanolic extract, growth inhibition, apoptosis, KB cells.

## 1. Introduction

Cell growth is controlled through an intricate network of extracellular and intracellular signaling pathways which process both negative and positive growth signals. The ultimate recipients of many of these signals are cyclin-dependent kinases (CDKs), a family of enzymes which catalyze events required for individual cell-cycle transitions. CDKs require association with cyclins for activation, and the timing of CDK activation is dependent largely upon the timing of cyclin expression.<sup>1</sup> The activity of cyclins-CDKs complexes is affected by two groups of CDK inhibitors (CKIs) which have an inhibitory effect on cell cycle progression. Both families have a conserved amino-terminal domain that is sufficient for both binding to cyclin/CDK complexes and inhibiting CDK catalytic activity.<sup>2</sup>

The KB cell line was established from a removal of human mouth epidermal carcinoma cells. These cells were derived from human carcinoma of the nasopharynx. KB cells exhibit an epithelial cell morphology and are known to produce keratin. It was reported that HeLa cell line marker chromosomes have identified in the KB cell line, and through DNA fingerprinting, KB cells are now thought to have been produced due to HeLa cell contamination.<sup>3</sup>

*Myrmecodia tuberosa* (*M. tuberosa*) are plants that are attached to other plants (epiphyte) and thrived in the highlands of Wamena, Papua, Indonesia. Originally, *M. tuberosa* was introduced in the inland of Papua, and commonly used as a medicine by local residents. *M. tuberosa* was used by the people of Papua since the 1950s for a mixture porridge and boiled drink to enhance the body

immunity<sup>4</sup>. Interestingly, *M. tuberosa* has been used to treat various diseases traditionally and safely without any side effects by some tribes in Wamena inland for a long time. It was reported that *M. tuberosa* can inhibit various types of human cancers including brain, breast, lung, colon, liver, uterus, prostate, skins, cervical and blood cancers<sup>5,6</sup>. In addition, the fifty-inhibitory concentration (IC<sub>50</sub>) of extract of *M. tuberosa* was reported at 33.28 µg/ml in cervix cancer cell line (HeLa cell), and 353.18 µg/ml in breast cancer cell line (MCF-7 cell).<sup>5</sup> Recently reported that *M. tuberosa* could treat a variety of systemic diseases such as leukemia, heart diseases, tuberculosis, kidney and prostate, various allergies, migraine, rheumatism, hemorrhoid, and infection diseases<sup>7</sup>. Although *M. tuberosa* can inhibit various types of human cancers, its antitumor effect on KB cell is still unclear. In the present study, the high antitumor activity of *M. tuberosa* extract through growth inhibition and apoptosis induction on KB cell line was examined.

## 2. Materials and Methods

### Cell and cell culture

KB cells (ATCC CCL-17) were delivered from LPPT-UGM, Yogyakarta, Indonesia, and maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO, USA) supplemented with 10% fetal calf serum (FCS; Moregate BioTech, Bulimba, Australia), 100 µg/ml streptomycin, and 100 U/ml penicillin (Invitrogen Corp., Carlsbad, CA, USA). Cells were incubated at 37°C with 5% CO<sub>2</sub>.

**Preparation of crude ethanol extract**

Fresh Papua's ant nest plants were collected from Wamena inland, Papua, Indonesia, and identified at the Department of Biology Pharmaceutical, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta, Indonesia. Plants were cut into small pieces and dried inside the incubator at 55°C. Five hundred gram of pieces were soaked in 500 ml of 80% ethanol and put on the shaker at 150 rpm for 3 days at ambient temperature. The mixtures were then filtered. The filtrate were evaporated using vacuum rotary evaporator (BÜCHI Rotavapor R-200/205, Model R205V800). Stock solutions of crude ethanol extracts were prepared by diluting the extracts with suspending agent (200 µl of dimethyl sulfoxide) followed with aquabidest solution to obtain a final concentration of 100 µg/ml.

**Suppression of cell growth (MTT assay)**

KB cells were seeded on 96-well plates (Falcon, NJ, USA) at  $2 \times 10^4$  cells per well in DMEM containing 10% FBS. After 24 h, the culture medium was replaced with new medium containing various concentrations (0, 62.5, 125, 250, 500, and 1000 µg/ml) of ethanol extract of ant nest plants and 12.5 µg/ml of docetaxel hydrate (Aventis Pharma, Japan) as a positive control. After 24 h, 20 µl fresh 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide [MTT, 5 mg/ml phosphate-buffered saline (PBS)] (Sigma-Aldrich, USA) was added to each well. After 4 h of incubation, the culture media were discarded, 100 µl of dimethyl sulfoxide (DMSO: Nacalai tesque, Inc., Kyoto, Japan) was added to each well and vibrated to dissolve the MTT formazan. The optical density was measured at 540 nm with a BioRad microplate reader (BioRad Laboratories, Hercules, CA, U.S.A.). Each determination was performed in triplicate.

**Induction of apoptosis using double-staining analysis**

Acridine orange (AO) and ethidium bromide (EB) double staining were carried out in this study. DNA-binding dyes AO and EB (Sigma, USA) were used for the morphological detection of apoptotic cells. AO is taken up by both viable and non-viable cells and emits green fluorescence if

intercalated into double stranded nucleic acid (DNA). EB is taken up only by non-viable cells and emits red fluorescence by intercalation into DNA. After treatment with different concentrations of ant nest extract for 24 h, the cells were detached, washed by cold PBS and then stained with a mixture of AO (100 µg/ml) and EB (100 µg/ml) at room temperature for 10 min. The stained cells were observed by a fluorescence microscope (Zeiss, Germany) at 100 × magnifications. The cells were divided into three categories as follows: living cells (normal green nucleus), early apoptotic (bright green nucleus with condensed or fragmented chromatin), late apoptotic (orange-stained nuclei with chromatin condensation or fragmentation). In each experiment, more than 100 cells/sample were counted.<sup>8</sup>

**Statistical Analysis**

Statistical differences between the means for the different groups were evaluated with Stat View 4.5 (version 5.0J, SAS Institute Inc, Cary, NC, USA) using one-way ANOVA and a post hoc *t-test*. The significance level was set at 5% for each analysis.

**3. Results**

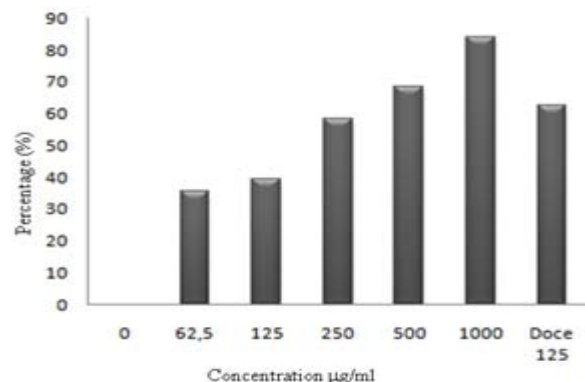
**Cell growth *in vitro***

KB cells treated with various concentrations of Papua's ant nest extract were examined by the MTT [(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Relative cell number was evaluated by comparing the absorbance in each cell. It was detected that the more increasing concentration of the extract, the more decreasing the number of viable KB cell based on the colorimetric absorbance values. Data indicated the negative control has the highest absorbance at  $1.416 \pm 0.05$ , whereas the positive control (Docetaxel  $IC_{50} = 12.5 \mu\text{g/ml}$ ) has an absorbance at  $0.514 \pm 0.20$ . Concentration of 500 to 1000 µg/ml of extract has the lower absorbance at  $0.587 \pm 0.04$  and  $0.365 \pm 0.03$ , respectively (Table 1).

**Table 1:** Mean and standard deviation of KB cell growth after treatment with the various concentration of ant nest plant extract for 24 hours.

Negative control (Aquadest)	62.5 µg/ml	125 µg/ml	250 µg/ml	500 µg/ml	1000 µg/ml	Positive control (Docetaxel) $IC_{50} = 12.5 \mu\text{g/ml}$
1.416±0.05	0.909±0.03	0.863±0.01	0.732±0.02	0.587±0.04	0.365±0.03	0.514±0.20

The percentage increase in KB cell growth inhibition by extracts concentration of 62.5, 125, 250, 500 and 1000 µg/ml was known at 35.5%, 39.5%, 58.3%, 68.5% and 84.2% compared to the negative control, whereas 12.5 µg/ml of docetaxel was detected at 62.4% (Figure1). In addition,  $IC_{50}$  of ant nest extract was found at concentration 215 µg/ml. These data showed that extract of Papua's ant nest was effective and has the strong potential to inhibit the growth of KB cells.

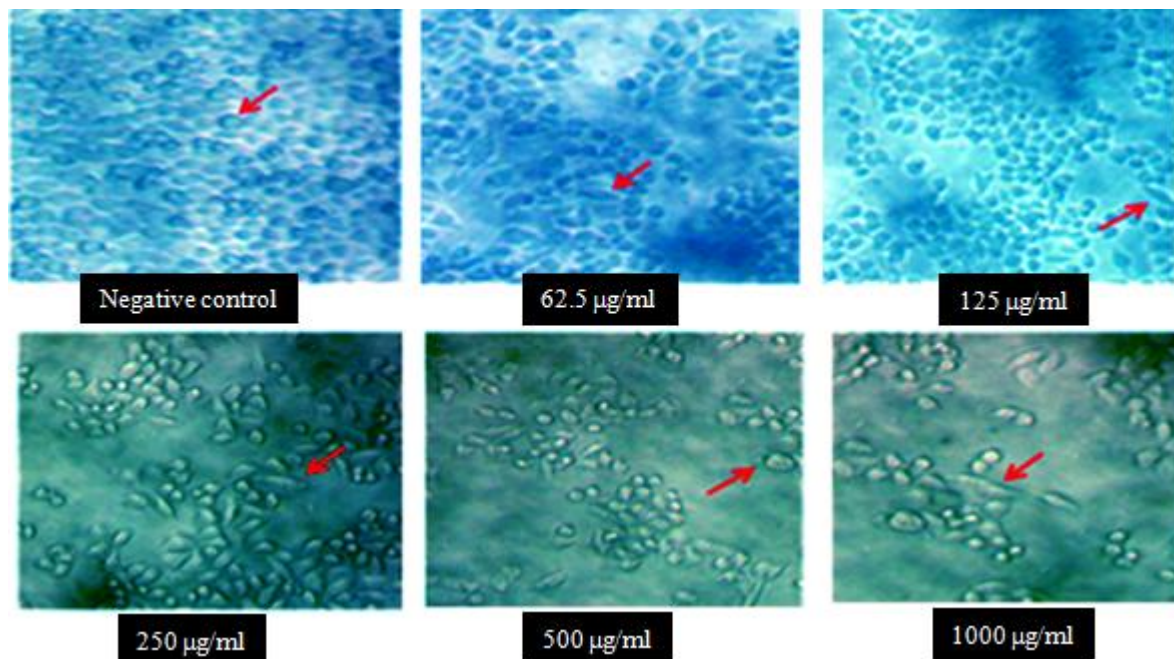


**Figure 1:** The percentage increase in growth inhibition of

KB cells after treated with various concentrations of extract for 24 hours

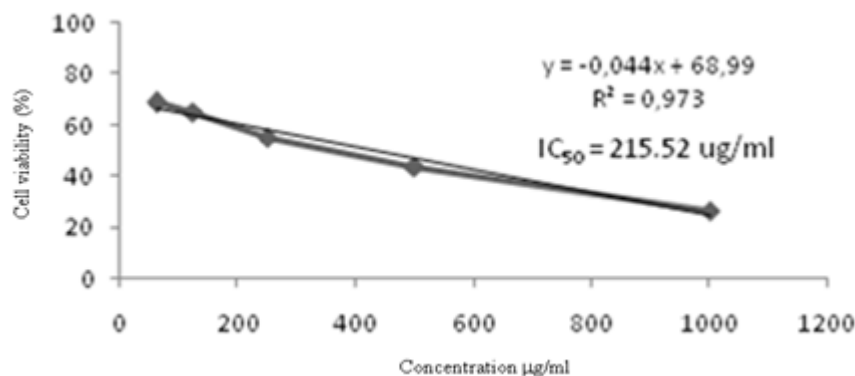
In the culture cell, the growth of KB cell was inhibited with the various concentration of ant nest plant extract. The

greater concentration of the extract was appeared the greater the KB cell growth inhibition. In addition, the morphology of KB cells that turn into long, flat, oval and irregular form (Figure 2).



**Figure 2:** Differences in the cell number and changes in KB cell morphology incubated with different concentrations of the extract for 24 hours.

Linear regression between the extract concentrations of ant nest plant with the percentage of KB cell viability were shown in Figure 3.



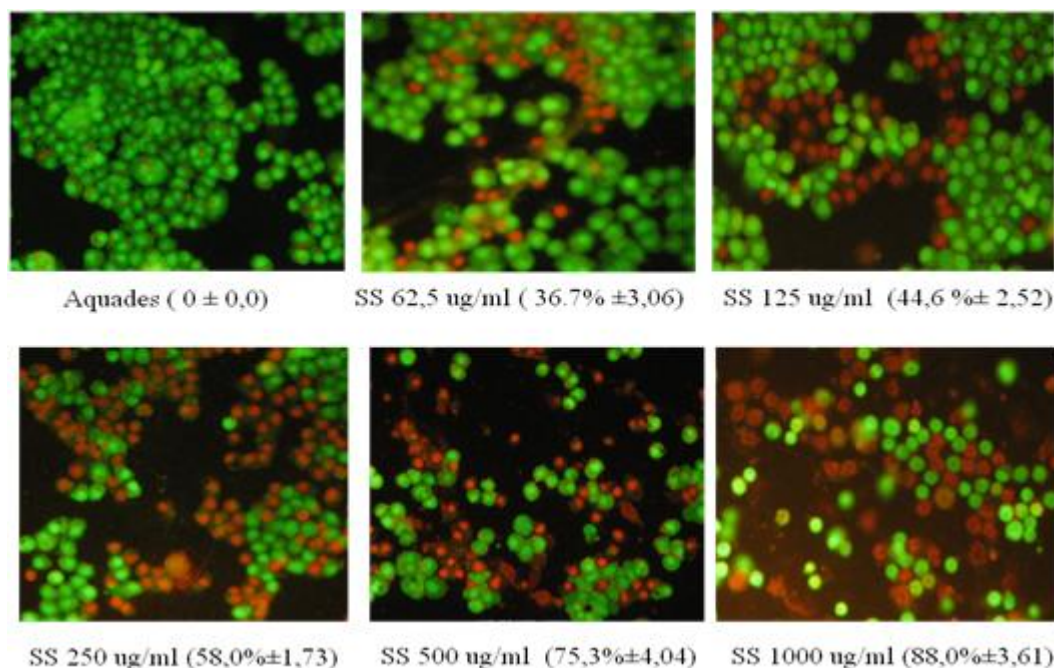
**Figure 3:** Linear regression between extract concentrations of ant nest plant with KB cell viability

Results of linear regression analysis of the extract concentrations of ant nest plant with the percentage of KB cell viability has a regression coefficient  $r = 0.973$ . These results indicated a very strong relationship between the concentrations of the extract with KB cell viability. Given the linear regression equation  $y = 0,044x + 68.99$ .

**Induction of apoptosis**

In this study, the acridine orange-ethyidium bromide (AO-EB) double-staining test was used to evaluate the apoptotic potential of Papua’s ant nest extract on an oral carcinoma

KB cells. These dyes emit different shades of fluorescence and possess a different ability to penetrate cells. AO penetrates into living cells, emitting green fluorescence after intercalation into DNA. The second dye, EB emits red fluorescence in the cells with an altered cell membrane. Morphological features of apoptosis such as chromatin condensation, nuclear fragmentation, alterations in the size and the shape of cells, as revealed by fluorescence microscopic analysis, were observed predominantly after ant nest extract treatment in 24 h.



**Figure 4:** KB cell apoptosis after incubation with ethanol extract of ant nest plant for 24 hours using a double-staining method magnification 40x. Normal cells (green) and cell apoptosis (orange-red).

As shown in Figures 4, ant nest extract treatment in the different concentrations (62.5, 125, 250, 500 and 1000  $\mu\text{g/ml}$ ) has an effect on apoptosis, while the maximum percentage of apoptotic cells was observed in 1000  $\mu\text{g/ml}$  concentration of extract ( $88.00 \pm 3.61\%$ ). However, the minimum percentage of apoptotic cells was detected in 62.5  $\mu\text{g/ml}$  extract concentration ( $36.67 \pm 3.06\%$ ) [ $F = 356.607$ ;  $P < 0.000$ ].

#### 4. Discussion

The normal operation of cell cycle requires a delicate balance between positive and negative regulatory factors. Any alteration in this balance can result in abnormal cell proliferation, which may lead to malignant transformation. Loss of normal cell cycle control plays a crucial role in the genesis of most types of cancer<sup>1</sup>. The cell cycle is modulated by the interaction of multiple cell cycle molecules, including cyclins, cyclin-activating kinase (CAK), cyclin-dependent kinases (CDK) and cyclin-dependent kinases inhibitors (CDIs)<sup>9</sup>. Disruption of the cell cycle can cause cell growth abnormalities. In the present study, concentration-dependent of Papua's ant nest extract has strong capability to suppress the growth of KB cells. These result may originate from secondary metabolite of ant nest plant include flavonoid, polyphenol, tocopherol and tannin compound contained in extract. Flavonoid is a class of natural compound which exhibits activity as reductor to hydroxyl, superoxide and peroxy radicals<sup>10</sup>. Flavonoids and polyphenols have been proved to inhibit proliferation and angiogenesis of human tumor cells *in vitro*<sup>11</sup>, as well as in experiment animals<sup>12</sup>. Engidaa *et al.*<sup>13</sup> reported that Papua's ant nest plant has five flavonoid compounds through HPLC examination, namely kaempferol, luteolin, rutin, quercetin and apigenin. Interestingly, kaempferol was found to have the highest concentration (13.77 mg/ g). It was reported that kaempferol has strong antitumor activity proved by inhibition of various types of human cancer cells

through suppressed the angiogenesis, signal transduction and decreased the expression of a wide variety of protein growth factors, including ovarian cancer<sup>14</sup>, kidney<sup>15</sup>, breast<sup>16</sup>, colon<sup>17</sup> and lung<sup>18</sup>. In the present study, ant nest plant extract inhibited the KB cells growth by 68.5% to 84.2%. In addition,  $IC_{50}$  of ant nest extract in this study was found exactly at 215  $\mu\text{g/ml}$ . These suggest that ant nest plant has sufficient cytotoxic properties by category active<sup>19</sup>. Recent study reported that flavonoids would increase p53 protein characterized by apoptosis induction<sup>20</sup>, and tannins would augment p27 protein that caused the cell cycle arrest<sup>21</sup>.

Since components of apoptotic programs represent promising targets for anticancer therapy, increasing cell cycle arrest by ant nest plant extract could be a useful apoptosis-modulating strategy for treatment of oral cancers. High levels of apoptosis in cancer cells are strongly associated with chemotherapeutic sensitivity<sup>22</sup>. Therefore, the main purpose of double staining AO-EB is to detect apoptosis. Multiple methods, such as PI, *in situ* nick translation, terminal deoxynucleotidyl, acidic denaturation, and thermal denaturation assays, have been developed to detect apoptosis by monitoring changes in cell morphology and surface markers<sup>23</sup>. These methods identify cell death; however, they also have drawbacks. These methods involve multi-step procedures, including diversion, washing, and transfer of samples, using both time and materials<sup>24</sup>. In our study, increased apoptosis KB cells marked with orange-red color, appropriate with the increased in the extract concentration. Ant nest plant extract at 1000  $\mu\text{g/ml}$  of concentration has induced apoptosis by 88%. Liu *et al.*<sup>25</sup> reported AO penetrated normal and early apoptotic cells with intact membranes, fluorescing green when bound to DNA. EB only entered cells with damaged membranes, such as late apoptotic and dead cells, emitting orange-red fluorescence when bound to concentrated DNA fragments or apoptotic bodies<sup>24</sup>. Fluorescent staining using AO alone has been used in the past; however, detection of cell apoptosis

using AO-EB is a relatively new approach, and few papers have reported its use<sup>23</sup>. In comparison to AO staining, the AO/EB method improves the detection of apoptosis and can distinguish between late apoptotic and dead cells<sup>25</sup>.

In conclusion, extract of Papua's ant nest plant exhibited a high potential antitumor activity in oral carcinoma KB cells. In addition, IC<sub>50</sub> of ant nest extract was detected at 215 µg/ml.

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