

# Differential Scanning Calorimeter Profiling for the Determination of Nanoparticle Ethanol Extract of Clove (*Syzygium aromaticum* L.) Toward Michigan Cancer Foundation-7 Human Breast Cancer Cell Lines

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## Abstract

**Aim:** This research was conducted to evaluate *in vitro* assay of nanoparticles ethanol extract of cloves toward Michigan Cancer Foundation-7 human breast cancer cell lines. **Materials and Methods:** In this research, we have synthesized bud clove extract nanoparticles and analyzed with differential scanning calorimetry. The cytotoxic activity of extract, nanoparticles, and its bioactive component was investigated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and analyzed by ELISA reader. **Results:** The *in vitro* test showed that the IC<sub>50</sub> values of the extract and the nanoparticles were 20.13 µg/mL and 7.6 µg/mL, respectively. **Conclusion:** The ethanol extracts of clove (*Syzygium aromaticum* L.) could be formulated in nanoparticles form. From cytotoxicity assay, nanoparticle of clove extract showed better cytotoxic activity than the ethanol extract.

**Key words:** 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, clove (*Syzygium aromaticum* L.), direct scanning calorimetry, Michigan Cancer Foundation-7, nanoparticle

## INTRODUCTION

Breast cancer is the most common type of cancer in women around the world. Incidence of breast cancer in 2012 was as many as 1.671.149 and was recorded as the second most cancer in the world (25% of the total incidence of cancer).<sup>[1,2]</sup> Breast cancer was recorded to 521.907 deaths in the world in 2012.<sup>[3]</sup> Statistical data in America on 2017 show that breast cancer is a type of cancer with the second highest mortality rate in women, which is as many as 40.610 deaths, equivalent to 14% of all deaths due to cancer.

The high incidence and mortality of breast cancer in the world show that a better therapy is needed for breast cancer patients. Breast

cancer therapy currently consists of surgery, radiation therapy, chemotherapy, hormonal therapy, and target therapy depending on the type, stage, and other special situations of the disease.<sup>[4]</sup> However, various therapies are still not good enough to cure advanced patients. Therapy in patients with advanced breast cancer can generally inhibit cancer growth, but overtime, there is a tendency for therapy to slowly stop

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responding.<sup>[5]</sup> Chemotherapy in breast cancer patients also provides quite a number of unwanted side effects.<sup>[6]</sup>

The ineffectiveness of chemotherapy treatment in cancer patients has led to the development of treatment from plant materials that are quite convincing as a preventive and curative agent for cancer. *Syzygium aromaticum* L. flower buds or clove, a Myrtaceae family, is a traditional Chinese and Indian medicine that has begun to investigate its anticancer effects. Cloves contain several bioactive compounds such as eugenol,  $\beta$ -caryophyllene, humulene, chavicol, methyl salicylate,  $\alpha$ -ylangene, and eugenone. Clove extracts show potent cytotoxic activity against several cancer cell lines. Ethyl acetate extract of clove retains the G0/G1 cell cycle and induces apoptosis at certain doses.<sup>[7]</sup> Cloves also exhibit cytotoxic activity against Michigan Cancer Foundation (MCF)-7 cells and are an important resource in the development of anticancer agents.<sup>[8]</sup>

Bioactivity of compounds is directly related to the glass dissolution rate; it is obvious that it will also be dependent on its morphology. The higher the specific surface area, i.e., the contact surface between the material and the physiological fluid, the greater the glass bioactivity. Bioactive glass nanoparticles (20–500 nm) are thus of tremendous interest not only because they present a larger specific surface area but also a higher surface energy compared to micrometric-sized particles.<sup>[9,10]</sup> In addition, their nanometric size allows material shaping versatility. The bioactive nanoparticles can be dispersed in a polymer scaffold. Finally, it is worth noting that their size also permits them to be internalized by different types of cells (macrophages, bone cells, cancer cells, etc.). Analysis effectiveness of nanoparticle used direct scanning calorimetry (DSC).<sup>[11]</sup> DSC has been applied as an analysis tool for biological samples such as blood plasma and other biological fluids.<sup>[12]</sup> Therefore, DSC can also be used to determine the thermal profile of breast cancer cells in various treatments.

Based on these problems, we are encouraged to conduct a research that can support the use of cloves extract and nanoparticle of extract as an anticancer agent. This research was carried out by conducting clove cytotoxic tests on MCF-7 breast cancer cells *in vitro*. Next, we determined the thermal profile of breast cancer cells that had been given using DSC.

## MATERIALS AND METHODS

### Preparation sample for ethanol extract of *S. aromaticum* L.

The clove (*S. aromaticum* L.) was collected and determined by Balai Penelitian Tanaman Rempah dan Obat (BALITRO), Bogor - Indonesia, in 2017. Cloves were cut and dried in oven at 60°C before ground into powder. The sample powder

(100 g) was macerated in 1 L of 95% ethanol (Merck) for 48 h at room temperature. The solution was filtered and the sample was macerated again 95% with ethanol (1 L). The procedures were repeated for 3 times. A total volume of 3 L solution was collected and dried freeze-drying under reduced pressure. The yield of the ethanolic extract was 3.18 g.

### Nanoparticle of ethanol extract (NEC) clove (*S. aromaticum* L.)

The sodium alginate (Sigma-Aldrich) and calcium chloride (Sigma-Aldrich) solutions were prepared by dissolving the chemicals in distilled water. The pH of the sodium alginate solution was adjusted to 5.1 using hydrochloric acid (Mallinckrodt). Briefly, a known amount of chitosan was dissolved in 1% acetic acid solution and pH was modified to 5.4 using NaOH (Merck).

The method used to prepare the nanoparticles was in two steps. Adapted from Rajaonarivony's method of preparing alginate nanoparticles.<sup>[13]</sup> Aqueous calcium chloride (2 ml of 3.35 mg/ml) was added dropwise to 10 ml aqueous sodium alginate (3.0 mg/ml) while stirring for 30 min (two-way homoisothermy magnetic stirrer, 1200 rpm), and then, 4 mL chitosan solution (0.8 mg/mL) was added into the resultant calcium alginate pre-gel and stirred for an additional 1 h. The resultant opalescent suspension was equilibrated overnight to allow nanoparticles to form uniform particle size.

### Preparation of clove *S. aromaticum* L. loaded chitosan-alginate nanoparticles

A constant volume (300  $\mu$ l) of clove *S. aromaticum* L. solution in a dehydrated alcohol/water mixture (1:1, 1.105 mg/ml) was incorporated into the calcium chloride solution; then, the other processes were the same as the preparation of blank chitosan-alginate nanoparticles.

### Morphology and structure characterization

The morphology and particles size measurements of the nanoparticles were performed by transmission electron microscope (TEM) JEOL Type 1010, nanoparticles separated from suspension were dried by oven and their thermogram of nanoparticle was taken with DSC.

### Cell line culture and MTT Assay Procedure

MCF-7 breast cancer cells were obtained from the Pathological Anatomic Medical Faculty of Universitas Indonesia. Cells were cultured in Roswell Park Memorial Institute 1640 media supplemented with 10% fetal bovine serum (FBS), glutamine (2 mm), and 1% penicillin-streptomycin in static 75 cm<sup>2</sup> TFlask (GIBCO, USA). The cells were incubated in a humidified atmosphere with 5% CO<sub>2</sub> at 37°C.

Cells were plated in a 96-well plate with  $1 \times 10^5$  cells/well of concentration. The cells were left to adhere for 48 h before exposed to the plant extracts (0–1000  $\mu\text{g/ml}$ ) administered in media containing 1% of FBS and returned to the incubator for 48 h. Subsequently, MTT (Merck) reagent (0.5 mg/mL in sterile phosphate-buffered saline) was added directly to the wells. Cells were returned to the incubator for 4 h. The formation of insoluble purple formazan from yellowish MTT by enzymatic reduction was dissolved in dimethylsulfoxide after removal of supernatant. The optical density of solution was measured at 590 nm using a microplate reader.<sup>[14]</sup>

### Cell Viability Assays

After treatment with the plant extracts, the cells were pooled together and the remaining attached cells were detached from the culture plates by exposure to trypsin-EDTA (GIBCO, USA). The resultant cells were then stained with trypan blue at the concentration of 0.2%. Then, the trypan blue-excluded viable cells were counted using a microplate reader under microscope.

Finally, the absorbance was monitored by a microplate reader at a wavelength of 570 nm. The percentage of viable cells was plotted versus the concentration of the test compound. The  $\text{IC}_{50}$  value was determined using linear regression analysis.

## RESULTS AND DISCUSSION

### Preparation and phytochemical analysis from ethanol extract of clove

Phytochemical analysis results of clove *S. aromaticum* L. taken at the Nuts and Tuber Crops Research Institute (BALITKABI) of Indonesia by analysis the presence of phytochemical compounds which include alkaloids, triterpenoids, steroids, flavonoids, and phenolics. The phytochemical analysis results are shown in Table 1.

The ethanol extract of the buds of *S. aromaticum* L. gave a brown oil with a characteristic clove odor. The phytochemical compounds of the ethanol extract were determined by screening of phytochemistry analyses. Table 1 shows that phytochemical compounds of the ethanol extract of *S. aromaticum* contain terpenes, steroid, flavonoids, and phenolic compounds. Gas chromatography–mass

spectrometry analyses also established the percentage composition of the 16 volatiles detected in the extract of the buds; eugenol (71.56%) and eugenol acetate (8.99%) were the major components.<sup>[15]</sup>

### Structure morphology of nanoparticles (NEC)

Nanoparticle of herbal extract is prepared from plant extracts or their therapeutically active constituents. Nano drug delivery systems help in better bioavailability, decrease side effects and toxicity. Some of the marketed nano herbal medicines are described as below. Herbal extract in the nanocarriers will increase its potential for the treatment of various chronic diseases and health benefits. Nanoparticles composed of biodegradable polymers with poly(lactic-co-glycolic acid) have been used to encapsulate the drug within the nanoparticles, using emulsifiers such as tween 20.

### The morphology structure of Nanoparticles using TEM

TEM analysis was confirmed the presence of nanoparticle and provided morphological information of the typical loaded of extract with alginate-chitosan. Analysis with TEM, the particle was seen to be spherical, distinct, and regular. The nanoparticle coating plays a predominant role in protecting the active substance incorporated and the release profile. That TEM analysis, a particle is defined as a small object that behaves as a whole unit with respect to its transport and properties. This nanoparticle conducted by glassy ionic with polymers colloidal particles 10–1000. From the results of the synthesis of nanoparticles with this method, it was successfully carried out with a 200 nm size as shown in Figure 1.

Thermal analysis can also be used to analyze the incorporation of herbal into nanoparticles through examining enthalpy change. Organic polymer has been used to penetrate skin for drug delivery and localized drug delivery. DSC is one of the primary tools used for the characterization of the matrix state. Nanoparticles tend to have a decreased melting temperature compared to bulk material that is not in the nanometer size. Results of thermogram profile analysis of extract nanoparticles are shown in Figure 2.

Nanoparticles composed of biodegradable polymers have been used to encapsulate the herbal within the nanoparticles using emulsifiers. DSC allowed for comparison of the

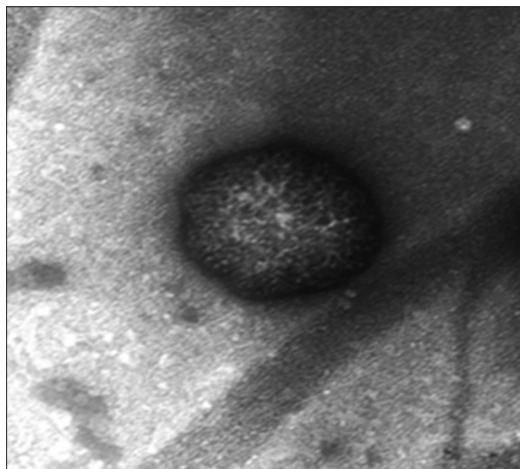
**Table 1:** Analysis of the phytochemical compounds of clove

Screening of phytochemistry	Result of test (Ref.)	Result of experimental test	Conclusion
Alkaloid	Orange precipitate (Dragendorff)	Yellow	Negative
Terpene	Ring brown color (Liebermann–Burchard)	Ring brown color	Positive
Steroid	Blue or green color	Green color	Positive
Flavonoids	Magenta color	Magenta color	Positive
Phenolic	Fluorescence at 366 nm (Taubeck)	Fluorescence at 366 nm	Positive

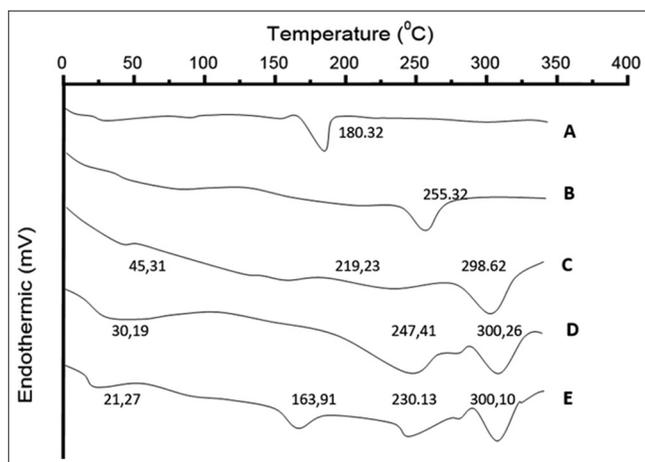
**Table 2: MTT assay clove *S. aromaticum* L. against MCF-7 cell line**

Cytotoxicity	Ethanol extract	Nanoparticle of extract	Doxorubicin
IC <sub>50</sub>	20.13 µg/mL	7.6 µg/mL	0.023

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, *S. aromaticum*: *Syzygium aromaticum*, MCF: Michigan Cancer Foundation



**Figure 1:** Transmission electron microscope morphology of nanoparticles of ethanol extract of clove with  $\times 30.000$  and  $d = 200$  nm



**Figure 2:** Thermogram profiling with direct scanning calorimetry analysis. (A) Clove extract, (B) alginate, (C) chitosan, (D) alginate-chitosan (E) loaded of extract with chitosan-alginate nanoparticles of the ethanol extract of clove

thermodynamic properties, as the  $T_m$  of herbals of NEC and the nanoparticle carriers were analyzed, to screen for undesirable changes to the herbals. DSC was also used to record the transition of compounds from a crystalline to an amorphous state on encapsulation, accompanied by a decrease in endothermic transition overtime and progressive scanning.

Solid lipid nanoparticles are emerging as a potential application in drug delivery, due to their low toxicity and their ability to maximize drug incorporation for secondary and tertiary drug targeting. Thermograms of crystalline compounds and the drug-loaded particle showed that the

melting peak for the drug was not observed in the loaded. The polarity and charge of the head group, the pH values, and ionic strength become major factors governing the  $T_m$  of the loaded particle main transition. Low ionic strengths are characterized by a large gel-fluid transition approximately from 18 to 35°C, which produces an optically transparent solution due to rearrangements in lipid packing.

### Cytotoxic activity

Cell growth profile in MTT assay, MTT assay is a rapid and high accuracy colorimetric approach that widely used to determine cell growth and cell cytotoxicity, particularly in the development of new drug. It measures cell membrane integrity by determining mitochondrial activity through enzymatic reaction on the reduction of MTT to formazan.

After completion of extraction, cytotoxic activity of bark extract was determined by MTT cell proliferation assay against HCT-116 cell line, cytotoxic activity represented by IC<sub>50</sub>. The smaller IC<sub>50</sub> value, the higher cytotoxic activity. Anticancer activity is represented by an IC<sub>50</sub> value (µg/mL). IC<sub>50</sub> value <100 is considered as an active compound with anticancer activity. IC<sub>50</sub> value in ranging from 100 to 300 is considered as weak anticancer activity, whereas the IC<sub>50</sub> value over than 300 is considered as inactive compounds. Cytotoxicity assay of bark extract is summarized in Table 2 as follows.

The cytotoxic activity of the ethanol extracts of clove *S. aromaticum* L. on MCF-7 cells from human breast cancer was investigated *in vitro* by MTT. The results showed decreased cell viability and cell growth inhibition in a dose-dependent manner. The IC<sub>50</sub> value of standard cisplatin, gossypol, ethanol extract, and nanoparticle of ethanol result of the *in vitro* test showed that the IC<sub>50</sub> values of the extract and the nanoparticles were 20.13 µg/mL and 7.6 µg/mL, respectively. Nanoparticles of *S. aromaticum* L. extract were very potential as anti-breast cancer. Ethanol extracts and nanoparticle extract of clove *S. aromaticum* L. demonstrated antiproliferative activities. Many studies in the past have assumed that the free phenolic groups of the other polyphenols are necessary for biological effects.<sup>[16]</sup> Flavonoid have the structure that required for antiproliferative activity on breast cancer cells.<sup>[17]</sup>

### CONCLUSION

The ethanol extracts of clove (*S. aromaticum* L.) could be formulated in nanoparticles form. From cytotoxicity assay,

nanoparticle of clove extract showed better cytotoxic activity than the ethanol extract. Based on the findings, further studies need to be carried out to investigate *in vivo* assays and toxicological studies of NEC.

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