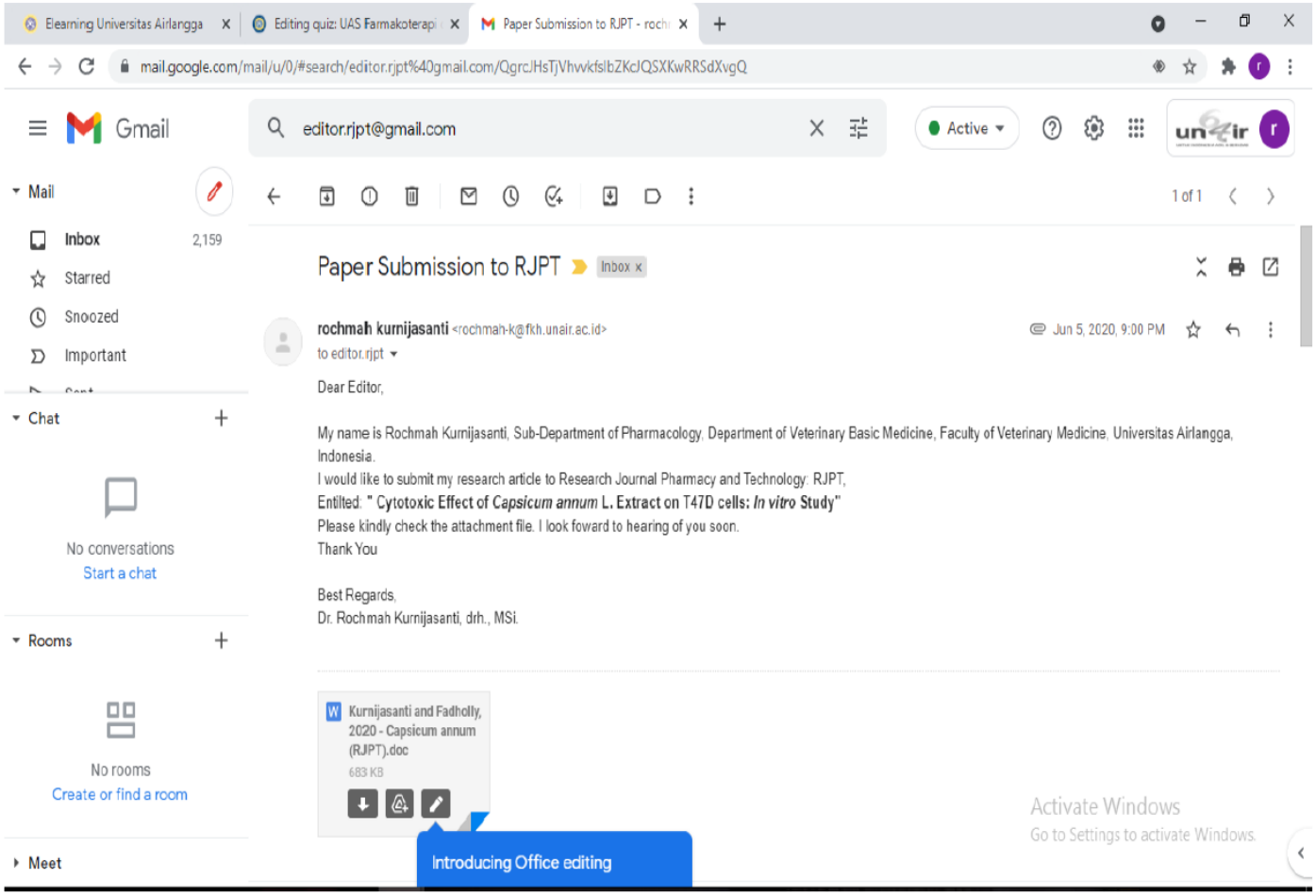
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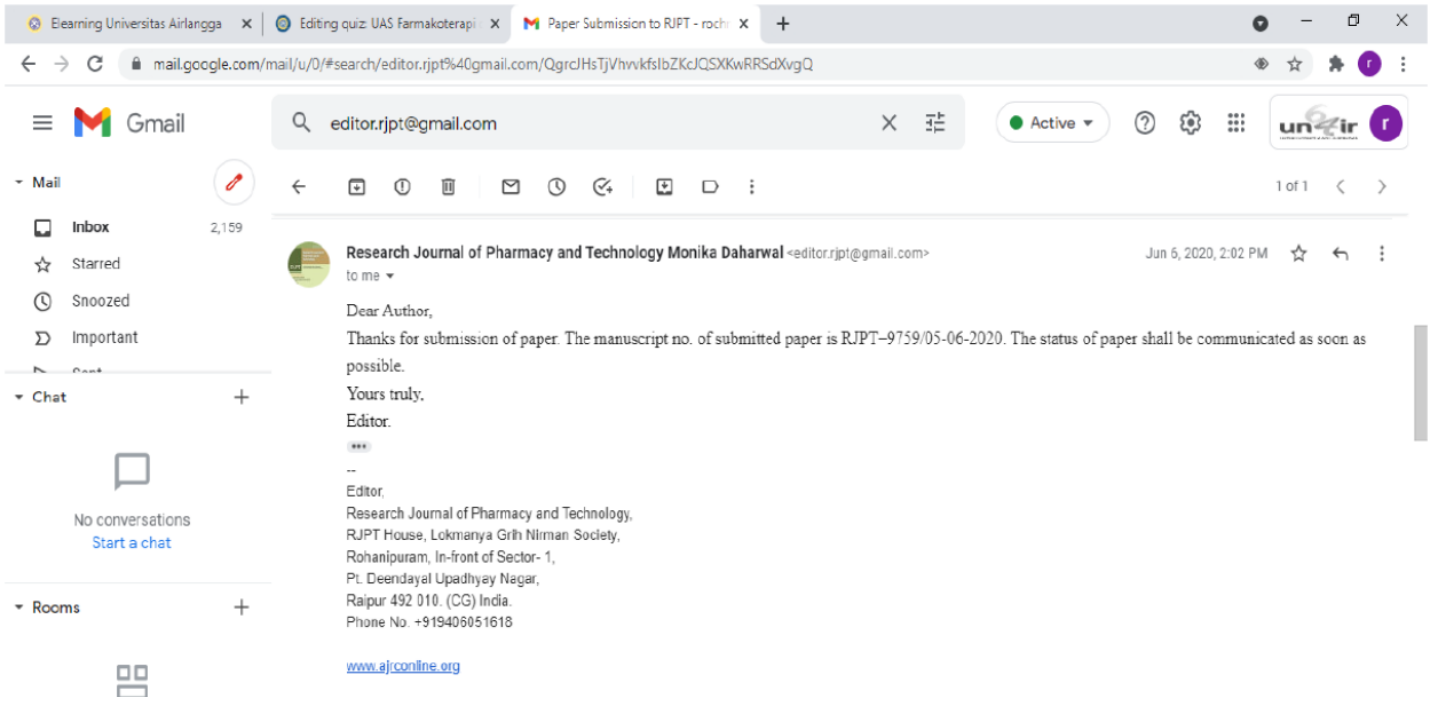
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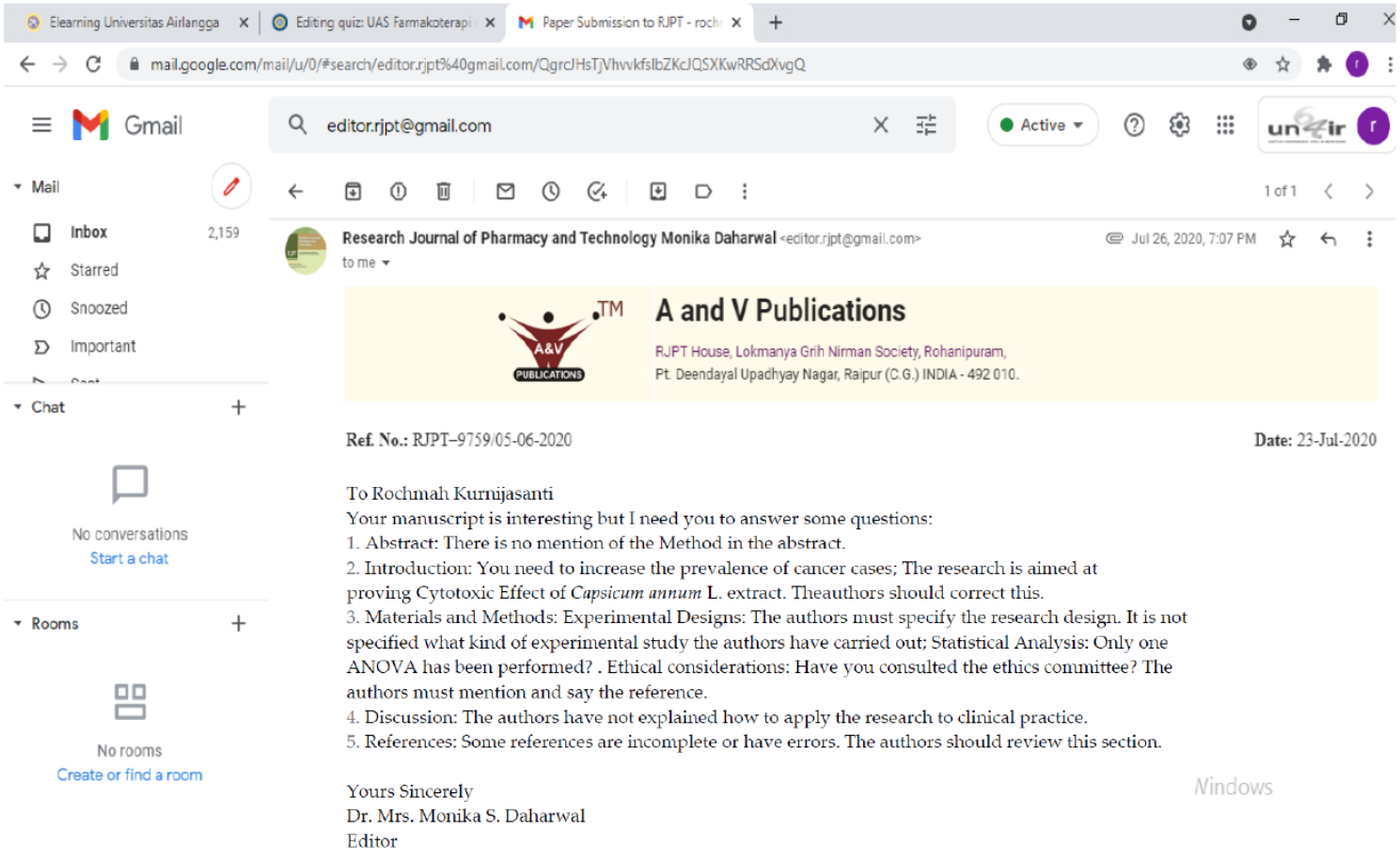

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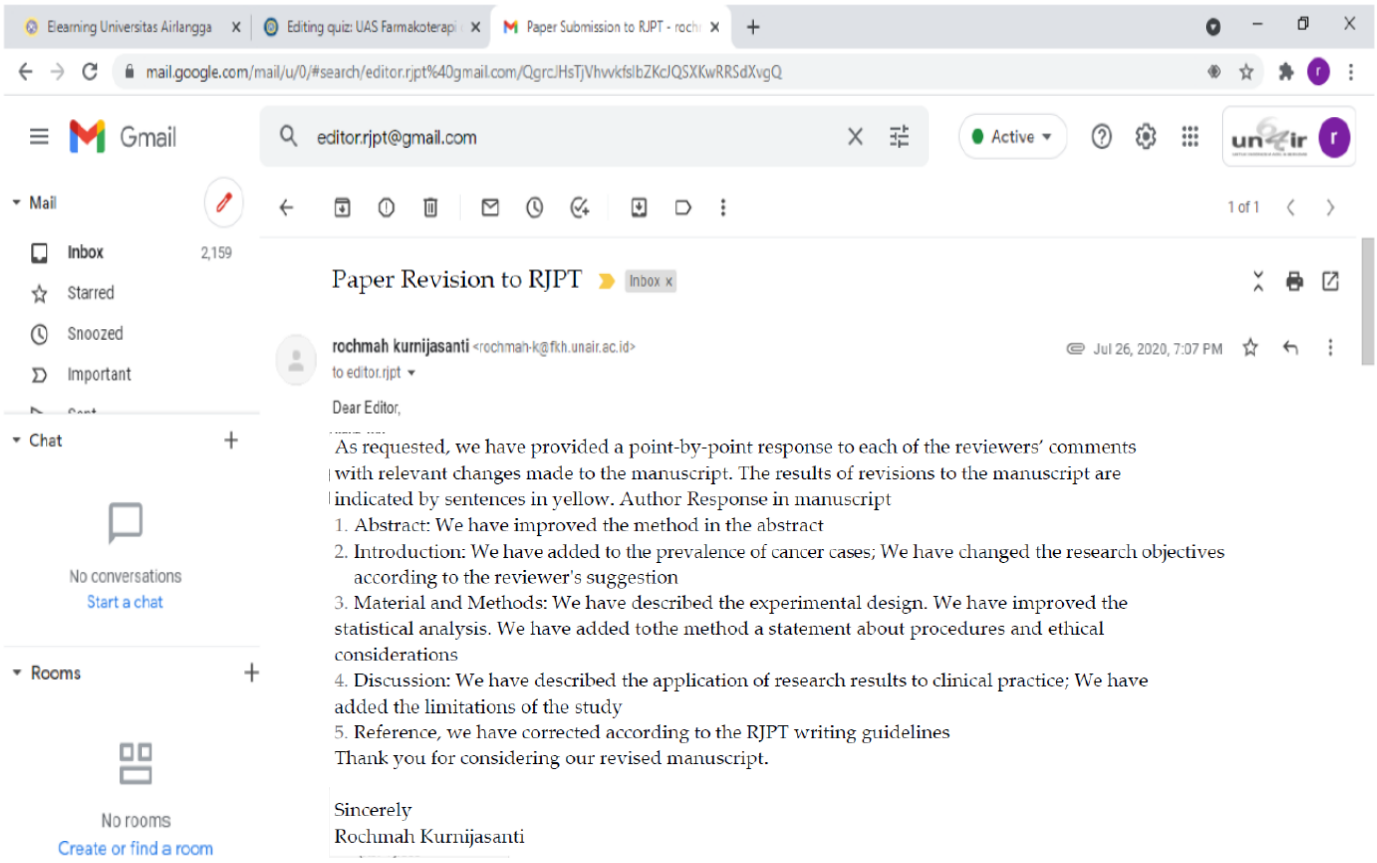

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Dear Editor,

As requested, we have provided a point-by-point response to each of the reviewers' comments with relevant changes made to the manuscript. The results of revisions to the manuscript are indicated by sentences in yellow. Author Response in manuscript
1. Abstract: We have improved the method in the abstract
2. Introduction: We have added to the prevalence of cancer cases; We have changed the research objectives according to the reviewer's suggestion
3. Material and Methods: We have described the experimental design. We have improved the statistical analysis. We have added tothe method a statement about procedures and ethical considerations
4. Discussion: We have described the application of research results to clinical practice; We have added the limitations of the study
5. Reference, we have corrected according to the RJPT writing guidelines
Thank you for considering our revised manuscript.

Sincerely
Rochmah Kurnijasanti

RESEARCH ARTICLE

Cytotoxic Effect of *Capsicum annum* L. extract on T47D Cells: *In vitro* Study

ABSTRACT:

Capsicum annum L. is a potential natural plant that have a lot of various pharmacological effects, including as anticancer agent. This study aim to analyze *Capsicum annum* extract (CAE) on T47D cells. CAE (10,20,40,60,80 µg/mL) tretaed on T47D cells to determined IC_{50} value by MTT assay. Apoptosis induction is also investigated through caspase-3 expressions (IC_{50}, $2IC_{50}$). The present study showed that CAE surpress T47Dcells proliferation with IC_{50} value of 75.81 µg/mL. The caspase-3 expression on $2IC_{50}$ is higher (67.16%) than IC_{50} (52.16%). This results indicate that CAE has ability as anticancer agent by inhibiting cell growth and induceapoptosis through caspase-3 expression on T47D cells. Further study of CAE holds potential for novel therapies of cancer prevention and treatment.

KEYWORDS: *Capsicum annum,* Cytotoxicity, Apoptosis

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INTRODUCTION:

Cancer is defined as the abnormal cells division without control that generally occurs over an extende period of time. Breast cancer is the most frequently diagnosed cancer in females (25%) worldwide. Most breast cancer occur 100 times higher in women than that in men[1]. Breast cancer develops through a multistep process and the pathogenesis of this disease has not yet been elucidated[2]. Breast cancer incidence rates increased among Asian/Pasific women and non-Hispanic black, while were stable among non-Hispanic white, Hispanic and American Indian native women. Breast cancer is a metastatic cancer and can commonly transfer to distant organ such as the bone, brain, lung and liver[3]. Cancer metastasis isresponsible for more than 90% of cancer-related death. Breast cancer is commonly associated with sex, estrogen, unhealthy lifestyle, family history and gene mutation[4].

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Normally, surgery, chemotherapy, and radiation are allowed for breast cancer therapy. However, those

methods have limitation for uncontrolling toxicity for normal cells. Doxorubicin is a chemotherapy drugused in the treatment of several cancers including breast cancer. It acts on cancer cells through intercalation into DNA resulting in the inhibition DNA synthesis and fuction leading to eventual DNA breaks[5]. Although doxorubicin has a lot of beneficial,however the discovery of new drug as alternativeway to cure cancer is highly needed. Plants are reagarded as a prospective sources for cancer treatment due to various therapeutic effects. Over 60% of the currently used antiacncer agents are derived in one way or another from natural sources[6].

Capsicum annum L. commonly known as bell pepper exhibits proven health as well as medicinal significance. It can be consumed either in fresh (salads, salsa, pizza) or processed form as dried powder[7]. *Capsicum annum* belongs to the family of Solaneceae that contain flavonoids, phenolics, caritenoids, alkaloids, and rich source of vitamin C, provitamin A, and calcium. Array of bioactive compunds suggest it a choice for preventing cell demage, cancer insurgence, diabetes prevalence, cataracts, cardiovascular disoders, alzheimer's and parkinson's disease. The principal ingredient present in this species is capsaicin (trans-8-methyl-N- vanillyl-6-non-enamide). The capsaicin content varies from 0,1% to 1 % that recently attracted considerable attention because its anticancer properties toselectively inhibit the growth of tumor cells, both *in vitro* and *in vivo*[8]. Despite the several known effects ofnaringin, this study need more validation as anticancer compound. Recently, we describe that *Capsicum annum* extract (CAE) inhibited tumor growth and apoptosis inductionshown in caspase-3 expression on T47D cells.

MATERIAL AND METHODS:

Ethical Clearance:

All treatment procedures under guided The Medical and Health Research Ethics Committee (MHREC), Faculty of Medicine, Public Health and Nursing, Gadjah Mada University, Indonesia.

Preparation of *Capsicum annum* extract: Capsicum annum L. were obatined from Surabaya,East Java, Indonesia. They were cleaned and chopped into small pieces and shade-dried. They were mashedto powdery form using a mechanical blender and passed through the coarse sieve (0.2 mm). The Capsicum annum L. powder was macerated with ethanol 96% for 72 h at 37 °C. The extract was hematoxylin for 2 min, then wahed with distilledwater.The cells were immersed in absolute etahnol evaporated in waterbath at 60 °C. The residue was stored in a refrigerator at -4 °C.

Cell culture of T47D cells:

T47D cells were obtained from Parasitology Laboratory, Faculty of Medicine, Public Health and Nursing, Gadjah Mada University, Indonesia. Cells were cultured in dulbecco's modified eagle medium (DMEM) media that supplemented with fetal bovine serum 10% (v/v), streptomycin-penicilin 3% and fungizone 1 % then incubated in incubator CO2 5% at 37 °C. Cells were collected after reaching 80% on confluency using trypsine-EDTA 0,25%.

Cytotoxic assay evaluation:

T47D cells 5 x 103 cells/well were implanted into 96-well plate, respectively, then incubated in incubator CO2 5% at 37 °C overnight. Cells were added with five various concentration of Capsicum annum extract (10, 20, 40, 60, 80 µg/mL) for 24 h. MTT reagent (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) 100 mL were addedinto each well. The cells were incubated one more time for 4 h until formazan crystals were formed. SDS-stopper HCL 0,1 N were also added to evaluate the colours for the media. The plate was wrapped in aluminium foil and incubated in dark place overnight. Colour absorbtion were read by ELISA reader at λ 595 nm. The inhibitory concentration 50 (IC50) value were calculated using linear regression of log concentration. Other cells, T47D and Vero were done with same method, respectively.

Caspase-3 staining evaluation:

T47D cells were implanted in six wells 5 x 105 cells/well using steril coverslip as a microplate and incubated in incubator CO2 5% at 37 °C overnight. The two concentration of *Capsicum annum* extract (IC_{50}, $2IC_{50}$) were added in each well for 24 h. The cells were harvested and washed with PBS twice. The cold methanol were used to fixed the cells for 10 min. Then, the cells in coverslip were placed each on a respective slide. The cells were washed with PBS twice. Hydrogen peroxide blocking solution were added to blocked the cells for 10 min. The cells were washed again with PBS pH twice. The primary andibody (caspase-3) was added on cells for 60 min, then washed with PBS twice. Polymer neopoly was added on cells for 30 min, then washed with PBS twice. DAB was added for 3 min, then washed with distilled water for 5 min. Cells were conterstained with

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and in xylol afterward. The protein expression were analyzed under the microscope.

RESULTS AND DISCUSSION

The viability of T47D cells were measured using MTT assay to determine the IC_{50} of *Capsicum annum*extract treatment. The result showed that all various concentrations of CAE inhibited cell growth of T47D cells. The highest viability of T47D cells is 93.16 % at dose of 10 µg/mL. The lowest viability is 38.01 % at dose of 80 µg/mL. The viability of T47D cells from three other concentrations 20 µg/mL, 40 µg/mL and 60 µg/mL are 86.11 %, 75.18 %, and 52,19 %. Doxorubicin also decrease the growth of T47D cells (Figure 1). Meanwhile, the results of viability on Vero cells also decrease at dose-dependent manner, 92.67 %, 88.92 %, 84.88 %, 84.88 %, 81.07 %, respectively. Doxorubicin also decrease Vero cells (Figure 2). The IC_{50} of CAE on T47D cells is 75.81 µg/mL.

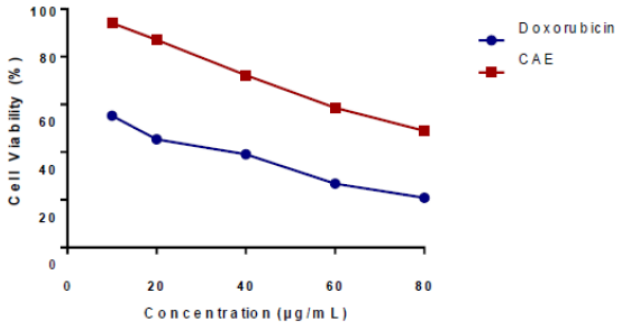

Figure 1. Viability responses of CAE and doxorubicin on T47D cells.

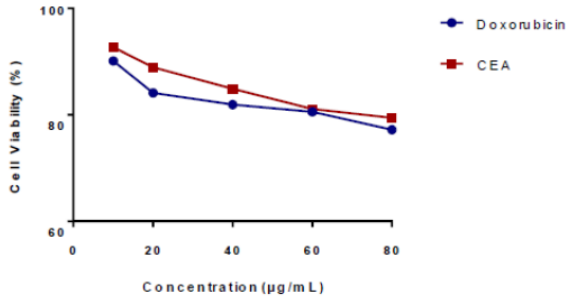

Figure 2. Viability responses of CAE and doxorubicin on Vero cells.

Breast tumor usually start from the ductal hyperproliferation, then develop into benign tumors or carcinoma after constantly by various carcinogenic factors[9]. In this study, the cytotoxic effect of *Capsicum annum* extract (CAE) against T47D cells as mammary tumor cell lines is investigated. CAE inhibit the growth of T47D cells with IC_{50} value 75.81 µg/mL. This result indicate CAE potent to develop as anticancer agent. Cytotoxic activity of CAE bythe content of bioactive compounds that have anticancer effects. Among the bioactive compounds isolated from CAE which has most dominant anticancer activity is capsaicin. Diverse studies have shown that capsaicin has antiproliferative effect on several human cell lines derived from multiple myeloma, pancreatic cancer, prostate cancer,colon cancer, lung cancer and gastric cancer[10,11,12].

As shown in Figure 3, the exposure of T47D cells to IC_{50} and $2IC_{50}$ naringin for 24 h enhanced the number of caspase-3 expression, typical brown colour in cytoplasm of cells. The dose of $2IC_{50}$ (67.16%) is higher than dose of IC_{50} (52.16%). Control cells were not induced apoptosis due to the cells were not treated with CAE.

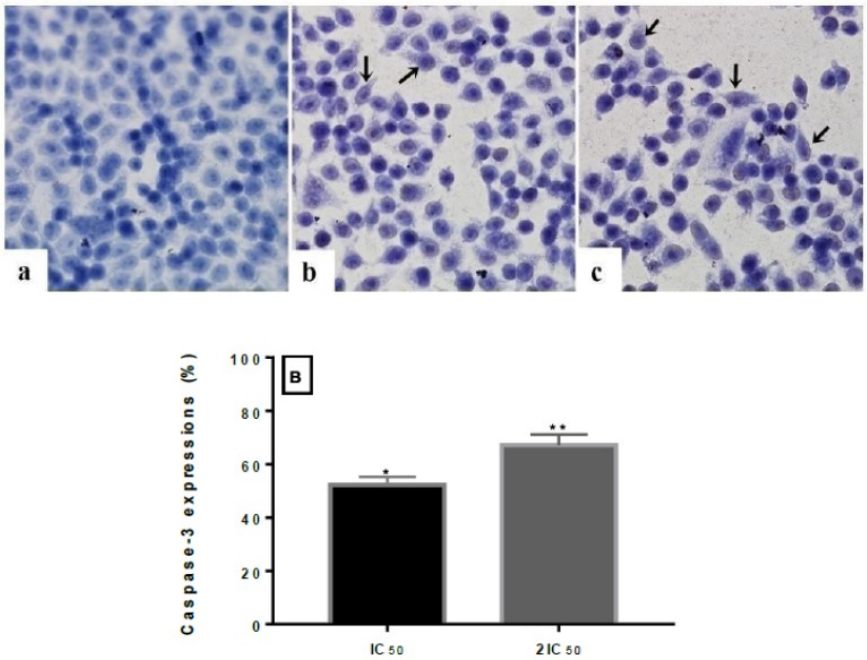

Figure 3. The effects of CAE on T47D cells. The untreated group (a); The caspase-3 expression (black arrow) shown in brown colour in cytoplasm of IC_{50} (b); $2IC_{50}$ (c); B: The bars represent mean ± SD of caspase-3 scores.The data is the mean (n=3) with *p value ≤ 0.05 when compared to IC_{50} value.

A successful anticancer properties should kill cancer cell without causing damages to normal cells. Determining the molecular targets involved in the tumor development process will also provide opportunities to develop cancer–fighting strategy. Caspase-3 is apoptosis marker that leading cell death without making inflamation around the normal cell. This ideal situation is achievable by inducing apoptosis in cancer cells[13,14]. This study showed that the expression of caspase-3 of the CAE treatment$2IC_{50}$ were higher compared to IC_{50}. These data clarify that CAE induced apoptosis through caspase-3expression. Apoptosis is an essential barrier against cancer development and progression and loss of apoptotic signaling is highly associated with malignancy[15]. Previous studies reported that genus of capsicum induce apoptosis through activate caspase-3protein in HepG2 and Hep3B cells[16,17]. Capsaicin may also serve as an antitumorigenic agent in human gastric cancer due to expression of proapoptotic protein such as Bax, caspase-3 and caspase-8[18]. Other studies showed that capsaicin induced apoptosis and cell cycle arrest at G1 phase in A172 human glioblastoma cells, PANC-1 and NPC-TW 039 cells[19,20].

Other compounds also supported CAE as anticancer properties, suh us flavonoids express wide variety of biological effects that may play a role in cancer therapy. It reveal potent antiproliferative, antiangiogenic, induce apoptosis and perturb cell cycle progession[21]. Prior studies demonstrated thatphenolic exhibit anticarcinogenic, induce cell ceycle arrest, inhibit oncogenic signaling cascade controlling cell proliferation , angiogenesis and apoptosis[22]. Carotenoids showed its ability inhibit theproliferation of several types of cancer cells and

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induced apoptosis on the cells[23]. Alkaloids also captures antineoplastic effect on various cancercells[24].

CONCLUSION:

This study revealed that *Capsicum annum* extract (CAE) inhibit cell growth with IC_{50} 75.81 µg/mL and activate caspase-3 expression as an apoptosis marker on T47D cells. It is a interesting natural source to be developed as an anticancer properties. Further study of CAE can be modified in concentration to find the best result of cancer prevention and treatment.

ACKNOWLEDGEMENT:

Authors would like to thank the Departments of Veterinary Basic Medicine, Faculty of Veterinary Medicine, Universitas Airlangga. Special thanks to PMDSU Scholarship by The Ministry of Research, Technology, and Higher Education of the Republic of Indonesia which was awarded to Amaq Fadholly.

CONFLICT OF INTEREST:

The authors declare no conflict of interest.

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- The above mentioned article (s) has been sent to the reviewer of expert comments.
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Thank you for submission of manuscript for publication.

Yours sincerely.
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