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RESEARCH ARTICLE

# Proapoptotic Effect *Annona muricata* Leaf Extract on HT29 Colon Cancer Cell

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## Abstract

The occurrence of colon cancer has rapidly increased across the globe and currently ranks among the third highest disease difficult to treat with drugs. Therefore, there is need for the use of natural compound in its treatment. Graviola (*Annona muricata*) leaf has several anti-apoptosis and proliferation active compounds which make the colon cancer induced by constitutive activation of NF-KB. This experiment therefore, utilized leaves of *Annona muricata* extracts obtained from Materia Medica in Batu. The leaves were found to inhibit cell proliferation by MTT assay and also induced apoptosis by Annexin V and Flowcytometry with the ability to inhibit NF-KB expression present in nucleus cells. The crude extract of Graviola leaves inhibits cell proliferation and increases apoptosis and through the inhibition of NF-KB pathway. It also increases the apoptosis of HT29 cell.

**Keywords:** *Apoptosis, Graviola, NF-KB, HT29 cell.*

## Introduction

Colorectal Cancer (CRC) is the second most common cause of death across the globe. The rapid increase in the cancer incidence in several populations was previously considered to be at low risk [1]. CRC is an emerging public health problem in Indonesia and currently ranks among the third highest. Its age-standardized incidence rates per 100.000 populations in Indonesia were 19.1 for men and 15.6 for women [2]. Most CRC patients in Indonesia are sporadic with the country ranking fourth most populated in the world. Some food substances such as meat and other supplements also increase its risk.

The etiology on risk factor shows that there many factors responsible for the growth of cancer, such as infectious agents, lifestyle, diet, environment, and reproduction. Furthermore, inflammation tends also to play important roles in Indonesian CRC patients [3]. The external factors are radiation, free radicals, ultraviolet rays, viruses, infections, cigarettes, and chemicals from food. While the internal factors are congenital, hormonal, psychological, and immunity factors [4]. The bimolecular path of CRC consists of proliferation and apoptosis mechanisms [5]. Cell growth in individuals is regulated by a balance system, namely apoptosis and proliferation [6]. NF-KB activation might be involved in the development of colitis-associated cancer, and sporadic CRC. NF-KB activation is associated with hallmarks, while constitutive NF-KB activation is frequently observed in CRC and is also associated with angiogenesis and is resistance to chemotherapy.

Furthermore, several NF-KB inhibitors have been proven to be useful, with intervention opportunities which exists from the cell surface, and propagated through the cytoplasm, to the transcription factor binding the DNA in the nucleus. Identifying a therapeutic channel in tumors, is, however, challenged by the ubiquitous nature of NF-KB in normal tissues. The specificity of the agent to its intended and selected target is critical to killing the cancerous cells and sparing of non-cancer tissues [7]. Nuclear factor kappa B is the most essential

component of the inflammatory signaling pathway capable of promoting the tumor genesis. It is a central transcription factor activated by inflammatory signals in response to infectious agents, cytokines, and necrotic cellular remnants. Activation of NF-KB leads to the overexpression of cell cycle genes, apoptosis inhibitor, and protease [2].

Cancer-causing mutations in the NF-KB pathway leads to its constitutive activation, deregulating expression of genes that propagate cell proliferation and protects it from entering apoptosis. Its clear molecular drivers have been established in lymphoma [6]. However, its numerous regulated cytokines are a growth factor for tumor cells such as EGF.

NF-KB transcription factor regulates multiple aspects of innate and adaptive immune functions and serves as a pivotal mediator of inflammatory responses. Furthermore, NF-KB induces the expression of various pro-inflammatory genes, including those encoding cytokines and chemokine's, and also participates in inflammatory regulation. Also, it plays a critical role in regulating the survival, activation, and differentiation of innate immune cells and inflammatory T cells [9]. Consequently, deregulated NF-KB activation contributes to the pathogenic processes of various diseases.

In this review, the activation and function of NF-KB in association with inflammatory diseases is highlighted with the development of therapeutic strategies based on NF-KB inhibition [4].Nuclear transcription factor was initially discovered as a factor in the nucleus of B cells, which binds to the enhancer of the kappa light chain of immunoglobulin. This has since been shown to be expressed ubiquitously in the cytoplasm of all cell types, conserved from Drosophila to man.

It translocates to the nucleus only when activated, and where it regulates the expression of over 200 genes that control the immune system, growth, and inflammation [10]. The deregulation of NF-KB is capable of meditating a wide variety of diseases, including cancer. However, the strategy used to determine if its activation is beneficial or harmful is controversial. The development of novel therapeutics targeting NF-KB requires a full understanding of its role in pathology and physiology.

The current review is an attempt to describe two sides of the NF-KB coin as a friend or foe [8].

There are numerous herbs with properties such as antioxidants used to reduce NF-KB pathway such as Graviola leaves extract (Acetogenin) which is easy to get and capable of killing cancerous cells contained in the womb without causing nausea, weight loss, and hair loss, associated with chemotherapy. Acetogenin that comes into the body will stick to cell wall receptors and function by damaging ATP in mitochondrial walls[5,13] Its key role is very important because phytochemical studies reveal that annonaceous Acetogenins are the major constituents of Graviola leaves [11,12].

In this research, the researchers analyze the effect of leaves extract on colorectal cancer, and will elucidate the controversy whether SLE increases Apoptosis, decreases proliferation, or inhibits NFKB by HT29 cell line. The general objective is to prove that Graviola leaves inhibit cell proliferation in colon cancer, analyze the increase in apoptosis associated with colon cancer and its capability to increase NFkB in colon cancer.

## Research Methods

### Research Procedures

This research is the experimental design research in the laboratory using in vitro model. We also design this study with randomized posttest controlled group design. The HT-29 (human colon cancer cells) were purchased from the American Type Cell Collection. graviola leaf extract take from Materia Medical in Batu-Indonesia.

Cancer cells were cultured in DMEM media. Culture medium consist of: the base medium for this cell line is ATCC-formulated McCoy's 5a medium Modified, Catalog no. 30-20007 to make the components to the base medium: fetal bovine serum to a final concentration of 10% buffer solution, flowcytometry, FITC Annexin V Apoptosis Detection Kit with PI (Biolegend, 640914) and Anti human NF-KB PE kit (Santacruz).

### Data Analysis

The homogeneity test is a test of whether or not the variances are equal to two or more distributions. The homogeneity test to be discussed in this paper is the Homogeneity

 

Test of Variance and Bartlett Test. Homogeneity test is done to find out whether the data in variables independent and dependent are homogeneous or not (Gujarati, 2016). Data from the cell line study were reported as the means ± standard error of n per group. The experimental data were analyzed with One-Way analysis of variance by Graphad Prism 7.0C. The treatment was 4 times replication.

*In vitr*o results were presented as the means ± standard error of the mean from three independent experiments. In order to make relationship between independent variables and dependent variable, in this study will used analysis or Pearson correlation and regression.

## Results and Discussion

### Annona Muricata Leaf Extract Decrease the Number of HT29 Cell

MTT assay was also conducted in three consecutive treatment phases in terms of the length of time taken in hours of 24, 48, and 72, respectively. The treatment included the four different dose of Graviola leaf extract administered, which comprised of 50 µg, 100 µg, 200 µg, and 400 µg. Each amount of treatment time was 24 hours, which indicates the visible amount of cells dependent on the dose is given.

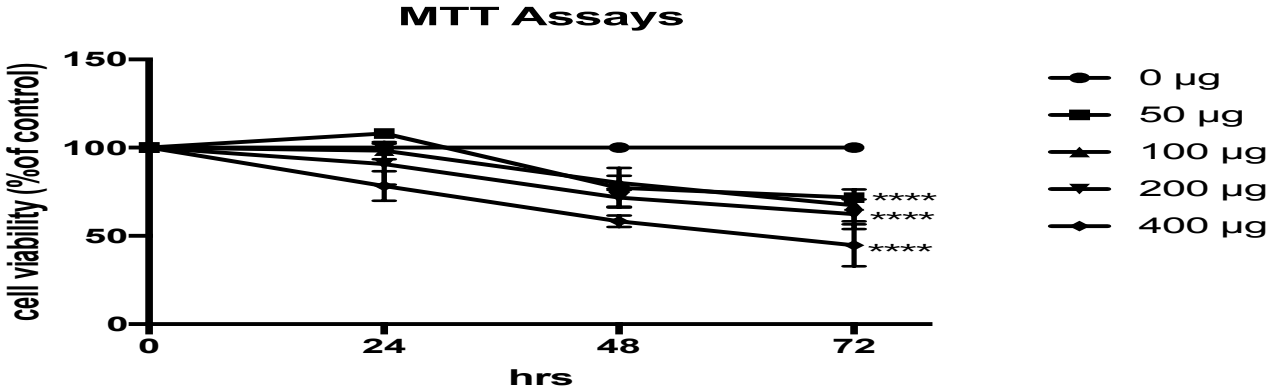


**Figure 1: MTT Assay treatment 24, 48, 72 hours**

From the above chart, the longer the treatment of MTT assay conducted, the greater the number of dead cells die, thereby causing induce apoptosis. In this research thereforethe treatment which meets the requirement for the further conduct the NFkB and Annexin V investigation as a follow-through is the 24 hour- treatment using 100 µm, 200 µm, and 400 µm because cancerous cells are retained alive. Inhibitory effect of Graviola leaf extract on the growth of HT29 cells is seen from the effect of cytotoxicity of the leaf extract on the HT29 cells detected by MTT assay.

The results indicate that the increased concentration showed the inhibition of Cytotoxicity on the HT29 cells in accordance with the dose and time-dependent manner of the Graviola leaf extract concentration by comprising 50, 100, 200, 400 mg/ml for 24, 48,72 h in vitro ($P<0.05$; Table I and Fig. 2).SLE - induced G0/G1 cell cycle arrest. Both untreated and treated cells with Graviola leaf extract for the indicated concentrations (50, 100,200 or 400 ug/ml) and the cell proliferation distribution were analyzed using FCM, thereby, making it ready for flow cytometry.

### Annona Muricata Leaf Extract Induce the Apoptosis and Inhibit NF-KB

Flow cytometry using the FITC Annexin V Apoptosis Detection Kit with PI was conducted on 16 cells of HT-29 culture cells comprising of 4 groups with each subjected to 4 replications. Furthermore, cell harvesting was carried out 24 hours after SLE administration. The cells undergoing apoptosis was computed with the results obtained using the flow cytometry examination.

The analysis of each treatment group obtained a value of $p< 0.0001$ for the ANOVA test with F value 58.74. This means that there is a significant difference between apoptosis using the dosage of 0mg, 100mg, 200 mg, and 400 mg. Also, the value of R Square is 0.9362, which indicates a strong relationship between the groups, and a significant difference in the result of apoptosis when SLE dose is administered.

 

The next step is to process the existing data using the post hoc test method of multiple comparisons as well as the Brown-Forsythe test to reveal Apoptosis (total %) between each treatment group. The Apoptosis (% total) in the control group was shown at 0.3420, which is > 0.05, which means that there is no significant difference.

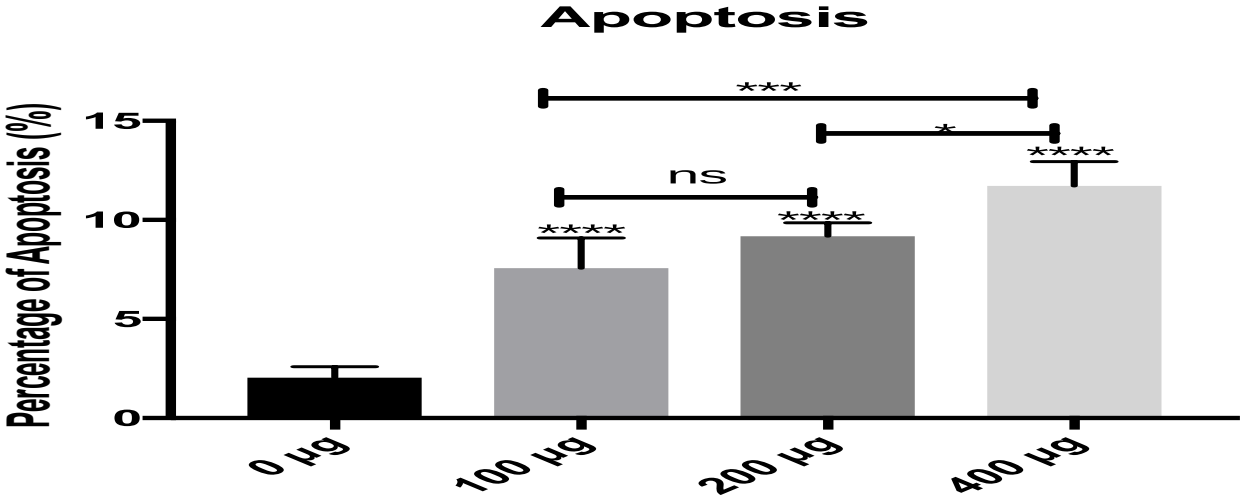


**Figure 2: The comparison of the average of Apoptosis between Groups**

The administration of a dose of 400 ug/ml decreases apoptosis (% total 6.70 ±5.25) which shows higher average than the dose of 200 ug/ml (9.78±0.99), therefore, the dose of 400 ug/mlis more effective in decreasing apoptosis (% total) compared to 200 ug/ml. Furthermore, the provision of Graviola leaves extract at a dose of 200 ug/ml decreases a greater amount of apoptosis (% total) than 100 ug/ ml (9.84±2.29).

Therefore, Graviola leaf extract of 200 ug /ml was is more effective in the reduction of apoptosis (% total) than the extract at dose 100 ug /ml. The results obtained from a comparison between each treatment using multiple Brown-Forsythe Test showed that the total NFKB in the control group was significantly different, with 0.4979 > 0.05. Meanwhile, Barlett's test showed p-value < 0.0001, which indicates that there is a significant difference between groups. The Turkey multiple comparisons test also showed an insignificant p-value, mean difference of 0.05748 (dosage 0mg vs 100mg), insignificant mean difference of 0.0596 (dosage 0mg vs 200mg), significant mean differences of 0.137 (dosage 0mg vs 400 mg), mean differences of 0.002125 for 10mg vs 200 mg, mean differences of 0.0795 for dosage 100mg vs 400 mg, and mean differences of 0.07737 for dosage 200mg vs 400 mg.

Plot response (main effect) on the graph indicates the magnitude of the effect of each treatment in comparison to the average NFKB (% total). Based on the response plots, a sequence of the treatment effect is established from the highest to lowest average as follows.

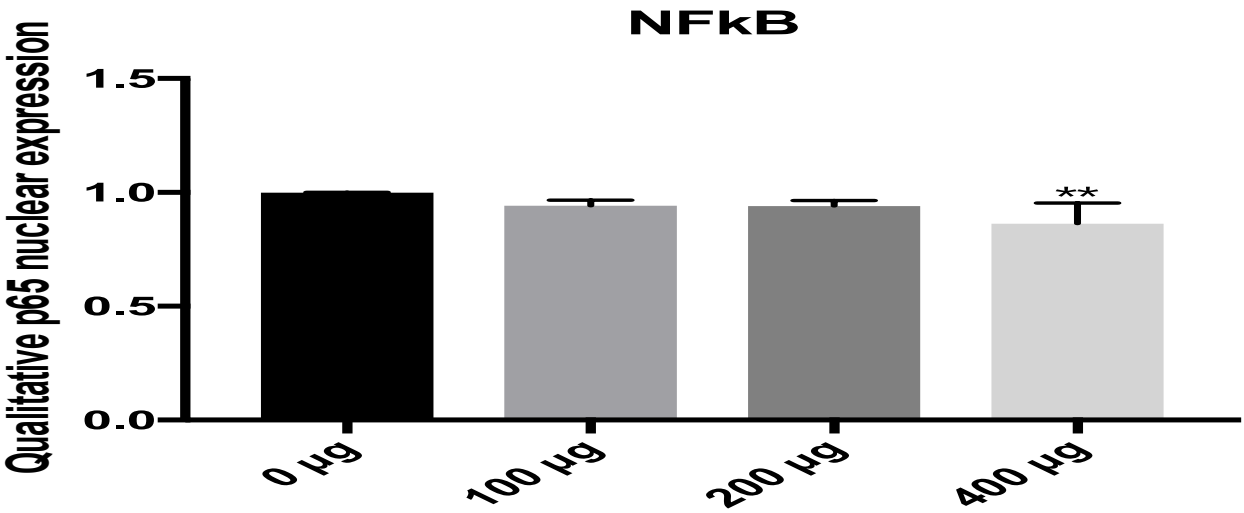


**Figure 3: The comparison of the average of NF-kB between Groups**

The difference in average NFKB (% total) shows the effect of each treatment dose and the different degree of Graviola leaf extract, which is revealed by the dose of 100 ug /ml. Furthermore, the total number of NFKB increases after the treatment dose increases from 200 ugs/ ml to 400 ug /ml. The administration of the dose of 400 ug/ml increases the value of NFKB (% total (40.13±2.92) which reveals higher average than a lower dose which is 200ug/ml (25.94±3.54).

Therefore, it proves to be more effective. However, the provision of Graviola leaves extract at a dose 200 ug/ml is capable of increasing the average NFKB (% total) more effectively than 100 ug/ ml (24.53±3.45), and is, therefore, more effective and greater when compared to the control group (45.23±3.38).

The relationship between NF-KB and Apoptosis in SLE HT-29 cell culture was tested using correlation and regression analysis. The correlation result reveals the Pearson correlation -0.653 with p-value < 0.05. This means that there is a negative relationship between NF-KB and Apoptosis in SLE HT-29 cell culture.

Meanwhile, the result of the regression analysis is significant, as evidenced by the p-value 0.002 < 0.05. Therefore, it is concluded that there is a significant effect of NF-KB and Apoptosis in SLE HT-29 cell culture. From the result, of MTT Assay data, the best timeframe is 24 hours because according to the HT 29 cell culture of colon cancer using alternative experiment with values of 100µm, 200 µm, 400 µm, a lot of cancerous cells remains alive after 48 and 72 hours.

After conducting flow cytometry and Annexin V, it was analyzed that the longer experiment conducted, the higher the dosage given, thereby resulting in a higher amount of dead cancer cells. Acetogenin compounds in Graviola leaves function similar to a chemotherapy drug known as Adriamycin (a trading name), which is popular owing to its ability to effectively treat leukemia and cancer in the lungs, breast, and thyroid.

Adriamycin contains anticancer compounds doxorubicinable to interfere in the DNA division activity of cancer cells [15, 16]. In summary, it is administered through injection or infusion and functions similar to acetogenin on Graviola leaves. It is able to inhibit ATP energy production, resulting in cell division and impaired cancer cells [17, 18, 19].Acetogenins only kill cancerous cells in the body. However, the normal body cells will not be attacked and will continue growing. Chemotherapy leads to nausea, weight, and hair loss, but the use of Acetogenins has no adverse effects [20, 21]. It protects the immune system and prevents deadly infections. Furthermore, its usage makes cancer patients feel stronger and healthier during the nursing process, with an improved physical appearance [23].

When cancer cells are resistant to chemotherapy, it is advisable to utilize Acetogenins. This phenomenon is called multi-drug resistance (MDR) or chemotherapy drugs. One way the cancer cells fight chemotherapy drugs is creating an inter-cell pumps able to push anticancer agents out of the cell before killing cancer [24].

Annonaceous Acetogenins does not only effectively kill cancer cells, but it also strengthens and exceeds the effectiveness of Adriamycin (medicine chemotherapy). Below are compounds Imia chemistry members capable of destroying various types of cancer cells [25].Most chemotherapeutic and targeted cancer therapies kill tumor cells through the generation of pro-death signaling that initiates the intrinsic apoptotic pathway of programmed dead cells [26].

The rebound point in the apoptotic cascade is mitochondrial outer membrane permeabilization (MOMP), leading to the formation of an apoptosome, which facilitates caspase activation and subsequently triggers hallmarks of apoptotic cell death.

The cellular decision to initiate MOMP is controlled by a delicate balance between the pro- and anti-apoptotic molecules of the BCL-2 family [27].One of the reasons for chemotherapy resistance is the failure of tumor cells to animate into apoptosis due to defects in its intrinsic pathway (e.g., changes in p53).

Despite significant improvements in treatment, its cure rates for many cancers remain suboptimal. The rise of cytotoxic chemotherapy has led to curative therapy for a subset of cancers, though it is difficult to

 

predict the intrinsic treatment resistance for individual patients. The wave of molecularly targeted therapies has focused on drug gable-activating mutations and is thereby, limited to specific subsets of patients. The intrinsic mitochondrial pathway of apoptosis represents one promising target for new therapies and successful targets [22].

The loss of function in p53 is frequently present in the later stages of colorectal tumor genesis, located in the chromosome. Its mutation is one of the key steps in colorectal carcinogenesis and stimulates high proliferative activity through the loss of cell cycle control and apoptosis. When p53 is mutated, the protective role of WAF-1 is not expressed. Furthermore, it regulates energy balance, through activation of the AMPK pathway [28, 29].

Morikawa and other researchers further explored its role in energy balance and analyzed that among non-obese patients, p53 positivity was associated with reducing cancer-specific survival while its adverse effect of obesity on CRC patient mortality was observed in p53 negative subjects [30].

During its pathogenesis progression, mutations in different cyclin-dependent kinases (CDKs) are also involved. P53, through the AMPK pathway, up-regulates the CDK 1A inhibitor, involved in regulating the cell cycle (energy balance status, cellular senescence, and stem cell aging). Ogino and colleagues observed the p21 loss of function in 79% of CRC and found it to be significantly associated with p53 expression.

COX-2-positive tumors were found to be associated with increased cancer-specific mortality regardless of p53 status, which indicates that it could be an independent prognostic factor of colorectal cancers [31]. Another result showed a negative relationship between NF-KB and apoptosis owing to the decrease in linear regression.

According to the hypothesis, "The crude extract of Graviola leaves inhibits cell proliferation and increases apoptosis through inhibition NF-KB pathway of accepted HT29". This means that the crude extract of Graviola leaves inhibits cell proliferation of NF-KB in HT29 cell, and increases its Apoptosis. The $R^2$ is 0.8865, which indicates that 88.65% of the NF-KB affects the apoptosis in HT-29 cell culture. This is big, with a strong relationship and effect.

The relationship between NFKB and cell proliferation in SLE HT 29 Cell culture is tested using ANOVA with a significant result because of the p-value $0.000 < 0.05$. This means that there are differences between the control groups of 100µg, 200 µg, and 400 µg. NF-KB is a family of transcription factors with a broad spectrum of work, which induces cell defense, proliferation, plays a role in the regulation of the immune system and inflammatory response [32].

Its normal cells will activate several genes involved in cell death suppression through mitochondrial pathways and death receptors. Furthermore, it induces the expression of apoptosis inhibitors (IAP) and some members of the Bcl2 anti-apoptosis family. It is one of the proteins involved in the disorder apoptosis of a nuclear factor-kappa activation pathway. NFKB is a family of transcription factors with a broad spectrum of work; it induces cell defense, proliferation, and role in the regulation of the immune system and inflammatory response [33]. Its activation leads to the resistance of chemotherapy.

Constitutively activated NF-KB is often seen in various cancer cells, cell lines, xenograft animal models, or clinical sites. While for CRC, 66% cell lines and 40% of human were activated. Other reports found 60 to 80% activated NF-KB in almost all CRCs and activated NF-KB promotes the proliferation of cancer cells and rescues it from death.

When the constitutive activation of NF-KB was inhibited by knocking down IKKg with siRNA (KD cells), greater apoptosis was induced in KD cells than in control cells by stimulation with TNF-aor 5-FU [34].NF-KB is present in a remarkable number of patients with CRC, many who showed resistance to chemotherapy could account for the constitutive activation of NF-KB.

Furthermore, it induces the expression of inhibitors of apoptosis (IAP) and some members of the Bcl2 anti-apoptosis family. Therefore, anti-NF-KB therapy is likely to rescue many cases of CRC. NFKB also interferes with transcriptional activity of p53 through increase anti-apoptosis genes and suppression of p53. Therefore, it inhibits apoptotic processes induced by p53.

 

Also, it promotes progression cell cycle through gene regulation such as cyclin D1, D2, D3, and E, as well as c-myc and c-much. NFKB is thought to be related to pRb activity via cyclin D1, with a rise in mechanism anti-apoptosis NFKB general concept that NFKB activation contributes to resistance apoptosis [35].The result of this study is also supported by the previous study done by Li et al. (2011) which showed that the inhibition of NFKB increases the sensitivity of cancer cells to apoptotic reactions in chemotherapy and radiotherapy.

The activation role of NFKB is associated with post-treatment response to cancer radiation, down to the locally advanced cervical stage, is still developed. NF-KB activity in the cancer cells is affected by the number of bonds with the inhibitor KB (IkB), limiting its activity. However, the differences with this result research are found in Graviola leaves extract used to replace chemotherapy and radiotherapy because Acetogenin in the Graviola leaves extract (SLE) functions as a powerful anti-cancer tool[36].Activation of NF-KB is due to the transduction of P65 into the nudes and nucleus. In the cells after treatment by Annona muricata (Graviola), the researchers found that after treating the leaves with P65, the expression decreased compared with control, however, it wasn't related to the dosage of R factors.

## Conclusion

The crude extract of Graviola leaves inhibits cell proliferation and increases apoptosis through the inhibition of NF-KB pathway of HT29. It also increases the Apoptosis of HT29 cell.

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