The Effect of Galing Plant Extract (*Cayratia trifolia L.*) on *Rattus norvegicus*, Wistar Strain White Rats with Breast Cancer through the Analysis of Cyclin D1 and Cyclooxygenase-2

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Abstract

Every year, cancer patients has increased. Disease recovery methods, such as by surgery, radiotherapy and/or chemotherapy sometimes do not provide satisfactory results and sometimes provoke negative side effects. Therefore, it is necessary to alternate ways to overcome it by utilizing medicinal plants, including galing plants (*Cayratia trifolia* L.). This study was conducted to determine the declination of the expression of cyclin D1 and cyclooxygenase-2 (COX-2) cells occurred after the galing plant extracts administration to adult female white rats (*Rattus norvegicus*). This study used true experimental design research method, employing a posttest design with a control group (i.e., the posttest-only control design group). Rats were divided into three groups (8 rats each): a normal group; a control group (breast cancer-conditioned rats that treated by carboxymethyl cellulose for four weeks); and the galing group (breast cancer-conditioned rats treated by *Cayratia trifolia* L. extract at a dose of 200 mg/kg of body weight every day for four weeks). This study obtained that the growth of cancer cells in the galing group was slower than that in the control group. Likewise, the expression of the COX-2 protein and cyclin D1 in the galing group was smaller

in comparison with both the normal group and the control group. As such, it can be confirmed that there was a decrease in COX-2 and cyclin D1 protein expression in galling-group breast cancer cells versus normal- and control-group ones. We concluded that *Cayratia trifolia* L. can be applied as new alternative herbal-based medicine for cancer treatment.

Keywords: breast cancer, cyclin D1, COX-2, galing plant (*Cayratia trifolia* L.).

Resumen

Cada año, los pacientes con cáncer ha aumentado. Los métodos de recuperación de la enfermedad, como la cirugía, la radioterapia y / o la quimioterapia a veces no proporcionan resultados satisfactorios y, a veces, provocan efectos secundarios negativos. Por lo tanto, es necesario alternar formas de superarlo utilizando plantas medicinales, incluidas las plantas de galing (Cayratia trifolia L.). Este estudio se realizó para determinar la declinación de la expresión de ciclina D1 y ciclooxigenasa 2 (COX-2) después de la administración de extractos de plantas de galing a ratas blancas adultas (Rattus norvegicus). Este estudio utilizó un verdadero método de investigación de diseño experimental, empleando un diseño de prueba posterior con un grupo de control (es decir, el grupo de diseño de control solo de prueba posterior). Las ratas se dividieron en tres grupos (8 ratas cada una): un grupo normal; un grupo de control (ratas condicionadas con cáncer de mama que se trataron con carboximetilcelulosa durante cuatro semanas); y el grupo de galing (ratas condicionadas con cáncer de mama tratadas con extracto de Cayratia trifolia L. en una dosis de 200 mg / kg de peso corporal todos los días durante cuatro semanas). Este estudio logró que el crecimiento de células cancerosas en el grupo de Galing fuera más lento que en el grupo de control. Del mismo modo, la expresión de la proteína COX-2 y la ciclina D1 en el grupo de galing fue menor en comparación con el grupo normal y el grupo de control. Como tal, se puede

confirmar que hubo una disminución en la expresión de la proteína COX-2 y ciclina D1 en las células de cáncer de mama del grupo de galling versus las de los grupos de control y normal. Concluimos que Cayratia trifolia L. se puede aplicar como una nueva medicina alternativa a base de hierbas para el tratamiento del cáncer.

Palabras clave: cáncer de mama, cyclin D1, COX-2, planta galing (*Cayratia trifolia L.*).

INTRODUCTION

The incidence of cancer every year seems to be increasing. Based on available data for the incidence of malignancy, breast cancer ranks highest, with 2,762,000 new cases annually, followed by colorectal cancer at 1,140,000 cases, endometrial cancer at 590,000 cases, and cervical cancer at 248,000 cases, respectively (SEER, 2012). Currently, the methods used to treat cancer include surgery and the administration of radiotherapy and/or chemotherapy, but these methods largely do not provide satisfactory results and often have side effects that are detrimental to the affected patients. Therefore, it is necessary to think about how to overcome the limitations of the current scenario, namely to provide medicinal ingredients that can kill cancer cells and provoke the least possible number of side effects. One effort to overcome cancer is underway by utilizing medicinal plants including galing plants (*Cayratia trifolia* L.). This research concept was developed by observing how existing cancer treatments led many cancer sufferers to stop therapy due to experiencing side effects. The hope is that the use of herbal medicine can reduce side effects and inhibit cancer growth. It was reported by Kumar in 2011 that *Cayratia trifolia* L. contains a flavonoid component called stilbene that acts in a manner similar to piseid, kaempferol, and resveratrol.

The discovery of anti-angiogenesis is necessary to inhibit the growth and metastasis of cancer cells. The reduction of metastasis in cancer cells can be performed by administering

compounds such as flavonoids. The chemical compounds contained in the plant *Cayratia trifolia* L. (Domin) include the active compounds resveratrol, delphinidin, malvidin, and quercetin (Tapaset al., 2008; Road et al., 2008; Singh et al., 2012).

This study employed galing plants (*Cayratia trifolia* L.), which are wild plants that propagate and are mostly found in Southeast Asia, including Indonesia. This plant has been widely used as a traditional treatment for various types of diseases. Almost all parts of *Cayratia trifolia* L. have been incorporated in traditional medicine. The chemicals contained by *Cayratia trifolia* are stilbenes (e.g., piceid, resveratrol, viniferin, ampelopsin), kaempferol, and quercetin (Kumar, 2011). All parts of *Cayratia trifolia* L. are reported to have secondary metabolites of alkaloids, steroids, terpenoids, flavonoids, and tannins (Singh, 2012).

Kaempferol is a chemical already used as an anti-angiogenesis agent in ovarian cancer therapy (Luo H, 2009). According to Harborne (2000), some derivative plants of flavonoids have been found to function as disease-prevention agents and therapeutic agents in traditional medicine in Asia for thousands of years. In vivo studies suggest that certain foods containing flavonoids have antitumor activity. The hydroxylation patterns in ring B of flavones and flavonols such as luteolin and quercetin seem to affect the activity of cancer cells, especially within the context of inhibiting the activity of protein kinase and antiproliferation. Flavonol and flavones target surface enzyme signal transduction cells such as tyrosine proteins and focal kinase adhesion and the concept of angiogenesis inhibition seem to be a promising option as an anticancer protocol.

Besides cyclooxygenase-2 (COX-2) inhibition, flavonoids also inhibit other proteins such as protein kinase B (Akt) and nuclear factor kappa B (Kandaswani et al., 2005). Flavonoids have an important role as chemopreventive agents and have been proven to inhibit angiogenesis, the proliferation of tumor cells, and endothelial cells in vitro. According to Kim (2003), COX-2 has very little normal tissue and, although the active time is short, due

to having an intermediate–early gene response, it will increase 20-fold the expression of growth factors, cytokines, promoter tumors, and oncogenic mutations. COX-2 is not found in significant quantities, however, if there is no stimulation or stimulation (Bertagnolli, 2008).

Amplification of the cyclin D1 gene causes overexpression or dysregulation of cyclin D1 proteins. This condition can result in abnormal cell cycle progression, which leads to the development of cancers including breast cancer (Arnold and Papanikolaou, 2005). Thus, the overexpression of cyclin D1 is associated with cancer development, and cyclin D1 deregulation is responsible for increasing levels of cyclin D1 in cancers such as breast cancer (Alao, 2007). Recent findings have identified a new mechanism for the stability regulation of cyclin D1. A number of therapeutic agents have been shown to induce the degradation of cyclin D1. Cyclin D1 ablation therapy is suggested to be useful for cancer prevention and treatment (Alao, 2007).

The present research aimed to explain the mechanism behind the provision of *Cayratia trifolia* L. extract to overcome the growth of breast cancer cells and to analyze the decrease in the number of cells expressing COX-2 and cyclin D1 in breast cancer tissue following the oral administration of *Cayratia trifolia* L. in a female rat model.

MATERIALS AND METHODS

Breast cancer animal model

This study employed 24 female white rats (*Rattus novergicus*, adult Wistar strain; age: 10 weeks) with a weight of 200 to 300 g. They were deemed healthy based on a certificate of production of experimental animals. To induce breast cancer, the experimental animals were dosed with 20 mg/kg body weight of 7,12-dimethylbenzanthracene (DMBA) orally for four weeks (two times a week). Following a maturation period of nine weeks, the animals were palpated the breast glands. Palpation was performed every week during DMBA

induction and, generally, the identified breast tumors were ± 1 cm (according to the protection of breast tumor palpation). Subsequently, the rats were divided into three groups: a normal group, a control group that included rats given carboxymethyl cellulose through an intragastric probe for four weeks, and a treatment group that included rats with breast cancer who were given *Cayratia trifolia* L. extract at a dose of 200 mg/kg body weight every day for four weeks.

The processing of tissue into paraffin blocks to make microscopic slides and the application of hematoxylin and eosin (H&E) to visualize microscopic images of breast carcinoma were carried out. This study relied on a light microscope (M= 400×). Calculations were performed using light microscope with a graticule calculated from 100 cancer cells placed on the ocular lens.

Measurement of cancer volume

The volume of breast cancer was the volume of 10% formalin transferred to the measuring cup after the breast cancer tissue was inserted. The technique for measuring breast cancer volume used is as follows: (1) the breast cancer was separated from the surrounding fat tissue and put into a measuring cup containing 10% formalin and (2) the volume of breast cancer was measured using a volume of 10% formalin, which was transferred by breast cancer tissue in cc units.

Immunohistochemical analysis

Immunohistochemical examination with primary antibodies in the form of COX-2 antibodies and cyclin D1 reactive to rat tissue was carried out by the labeled streptavidin-biotin technique, using the enzyme horseradish peroxidase and the di-amino benzidine chromosome. Interpretation of the results of the immunohistochemical examinations was

carried out by counting the number of positive brown cells. Calculations were performed using light microscope (M= 400×) with a graticule calculated from 100 cancer cells placed on the ocular lens.

COX-2 analysis

COX-2 expression was designated as the percentage of rat breast cancer cells expressing COX-2, which was calculated based on brown color on the nucleus and/or cytoplasm of cancer cells by immunohistochemical examination method and examined by microscope (M= 400×) (IHC World, 2003).

Cyclin D1 analysis

The expression of cyclin D1 was established as the percentage of rat breast cancer cells that express cyclin D1, which was calculated based on brown color on the nucleus and/or the cytoplasm of cancer cells by immunohistochemical microscope examination(M= 400×) (IHC World, 2003).

RESULTS AND DISCUSSION

The experimental model of breast cancer was obtained by providing carcinogenic DMBA or methylnitroso-urea (MNU). Yang et al. (1999) used MNU carcinogens to obtain a breast cancer model, while Barros et al. (2004) employed DMBA carcinogens. In this study, we used DMBA carcinogens for reasons of feasibility: the use of MNU requires a low temperature during delivery to reception, which we thought would be difficult to implement.
The induction of breast cancer in the rat subjects was carried out by the Faculty of Veterinary Medicine, University of Surabaya using a carcinogenic DMBA dosage of 20 mg/kg body weight given twice a week for four weeks, with subsequent waiting until the breast was

palpable, which generally occurred at the interval of nine weeks from the time of the last DMBA administration. Following palpation of the nodules in the breasts of the rats, *Cayratia trifolia* L. extract was given to see if there was an inhibition of breast cancer growth.

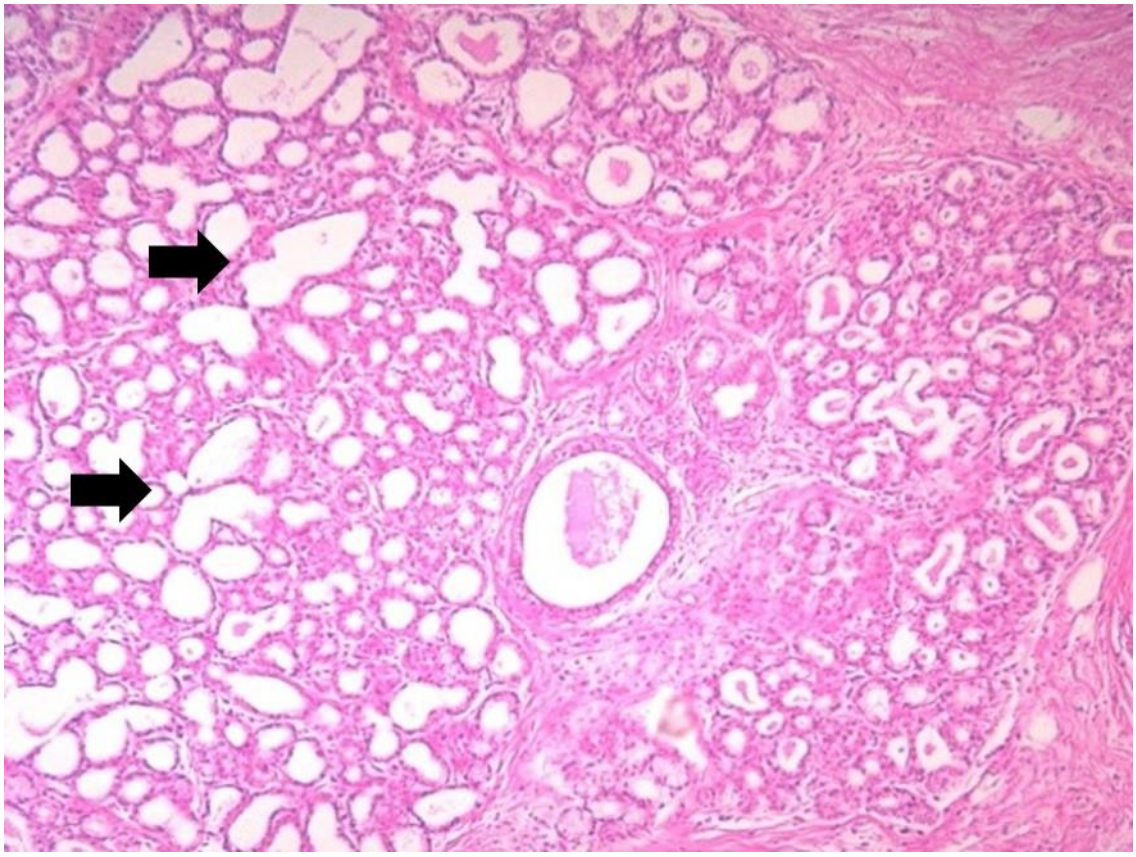

Figure 1. Histopathological picture of the breast with H&E staining. The arrows indicate normal (healthy) rat breast glands (400x magnification).

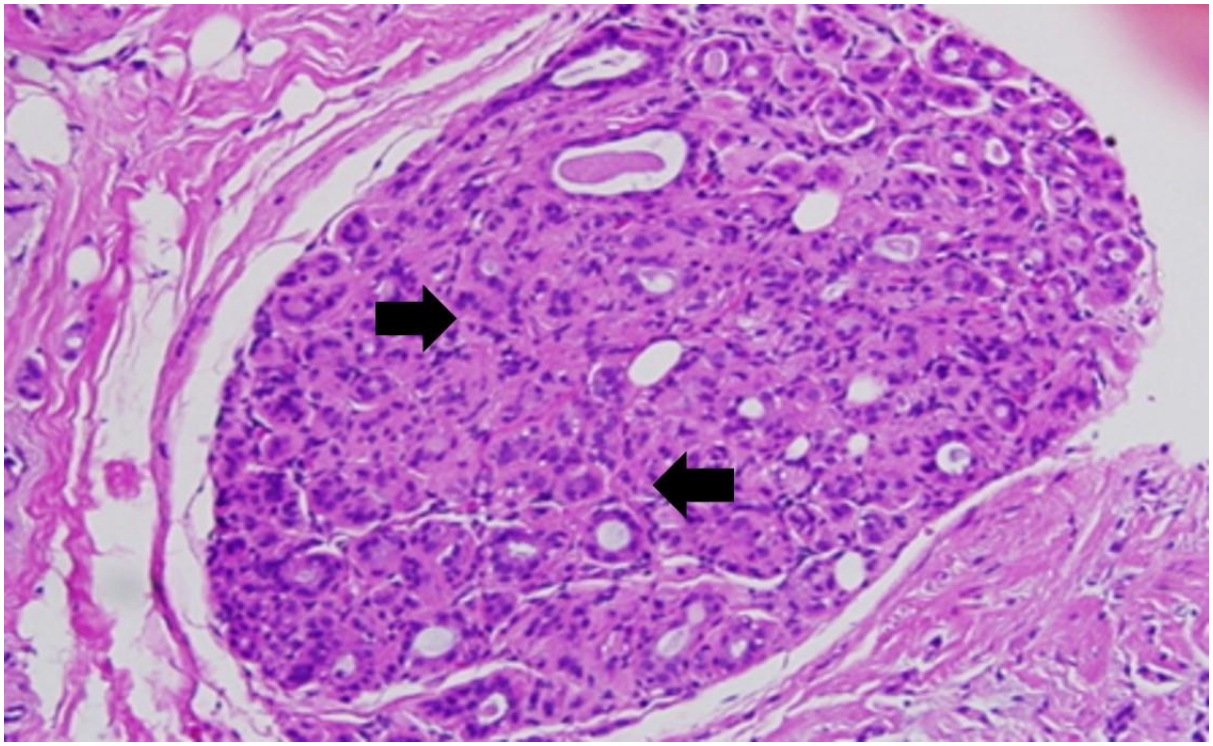

Figure 2. Rat breast cancer can be observed (arrows) following H&E staining in rats treated with DMBA and Galing plants (400x magnification).

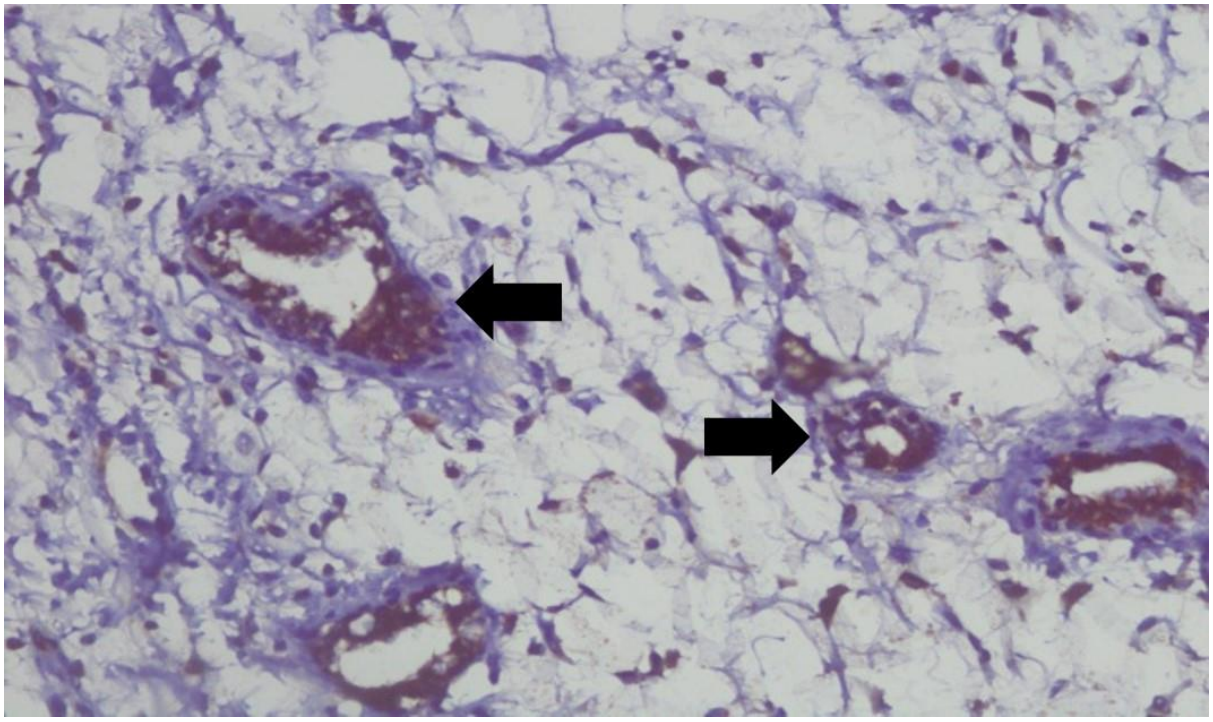

Figure 3. Expression of COX-2 in ductal epithelial cells. Positive expression is characterized by the observance of brown ductal cytoplasm of epithelium cells.

The results of observations of cyclin D1 expressions

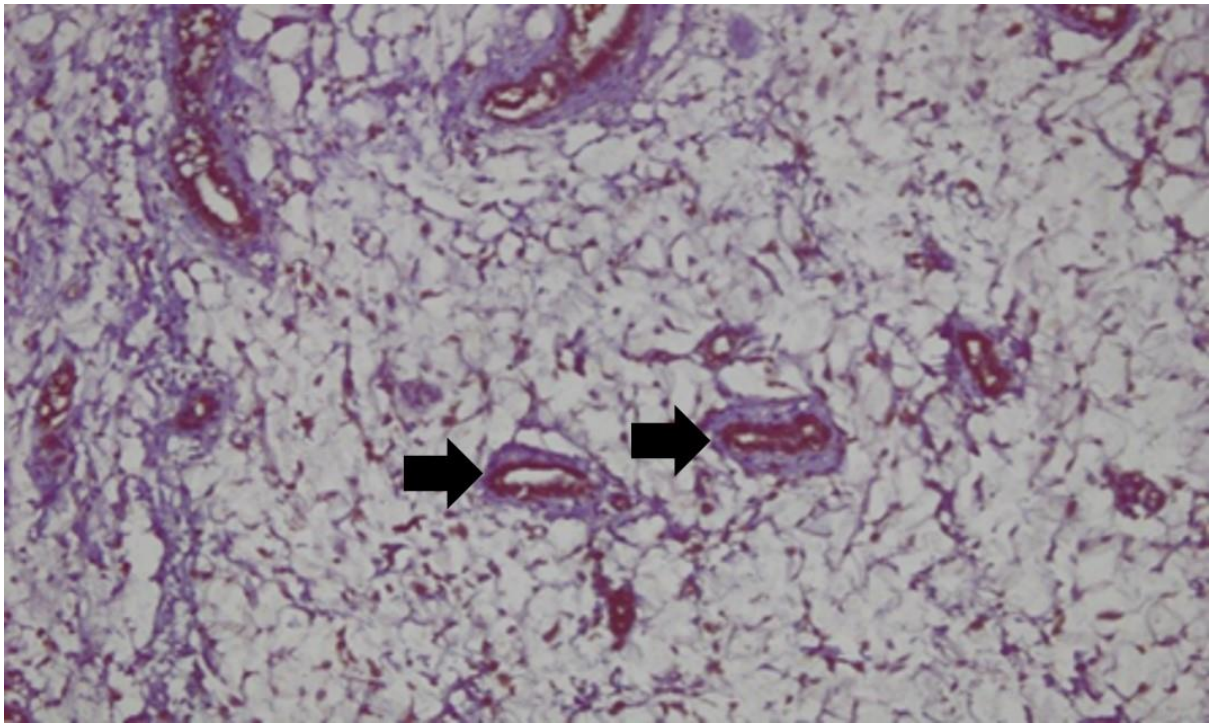

Figure 4. Cyclin D1 expression in ductal epithelial cells. Positive expression is characterized by the observance of brown ductal cytoplasm of epithelium cell.

Characteristics of Rat Breast Cancer

Results for the normal distribution of cancer volume data were obtained via the Shapiro–Wilk test using the Statistical Package for the Social Sciences version 23 software program (IBM Corp., Armonk, NY, USA).

Table 1. The results of normal distribution according to the Shapiro–Wilk test

Group	N	p-value	
		COX-2	Cyclin D1
Normal	8	0.390	0.257
Control	8	0.466	0.915
Galing	8	0.403	0.237

The data normality test results revealed that all data had a normal distribution ($p > 0.05$). To find out the differences between COX-2 and cyclin D1 levels between the three groups, variance analysis can be used.

Table 2. COX-2 test results between groups with variance analysis.

Group	N	Average ± standard deviation	p-value
Normal	8	0.78 ± 0.012^{a}	
Control	8	0.85 ± 0.008^{b}	< 0.001
Galing	8	0.62 ± 0.006^{c}	

Note: different letter sign indicates the values has statistically significant different

The results of an analysis of variance (ANOVA) showed a p-value of < 0.001, meaning that there were significant differences in COX-2 level among the three groups. Of note, the ANOVA outcomes and further findings obtained using the LSD test for the three

groups differed significantly. As can be seen in Table 2, the expression the COX-2 protein in the experimental group is lower when compared with those of the normal and control groups. This indicates that the administration of *Cayratia trifolia* L. in rats with breast cancer will inhibit COX-2 protein expression.

COX is a catalytic enzyme that converts arachidonic acid into prostaglandins. This enzyme can be inhibited by nonsteroidal anti-inflammatory drugs. Prostaglandin is an important mediator in signal transduction pathways that play a role in cell growth and differentiation. The COX enzyme has two isoforms, namely COX-1, which is normally expressed in many tissues, and COX-2, which is expressed when induced by certain stimuli such as mitogen, cytokines, and growth factors. In various cancers such as colon, prostate, and breast cancer, there is an increase in the expression of COX-2 enzymes.

Table 3. Results of the cyclin D1 test between groups with variance analysis

Group	N	Average ± standard deviation	p-value
Normal	8	0.78 ± 0.011^{a}	
Control	8	0.85 ± 0.006^{b}	< 0.001
Galing	8	0.62 ± 0.007^{c}	

Note: different letter sign indicates the values has statistically significant different

The ANOVA results indicated a p-value of < 0.001, indicating that there were significant differences in cyclin D1 levels among the three groups. The LSD test was conducted to find out which groups were different, with the normal, control and experimental group results all being significantly different. In Table 3, the expression of the cyclin D1 protein in the experimental group was lower when compared within the normal and control groups.

Cyclin D1 protein importantly involves in regulating the progress of a cell during the G1 phase of the cell cycle. The cyclin D1 gene, CCND1, is amplified in approximately 20% of mammary carcinomas, and the protein is overexpressed in approximately 50% of cases. This has led to an intensive study to ascertain whether cyclin D1 is a biological marker in breast cancer; however, the clinical work has produced unexpected results.

The role of the cyclin D1–CDK4/6-RB pathway in breast cancer cells, including crosstalk with other oncogenic signaling pathways, has been examined. Mitogenic forces including ER transcriptional activity and signaling through ERBB2/PI3K/AKT/mTOR increase cyclin D1 levels, activating CDK4/6 and promoting cellular progression to the S phase. There is extensive crosstalk between the PI3K and CDK4/6 pathways: not only does PI3K pathway activity increase cyclin D1 levels but also the cyclin D–CDK4/6 complex can modulate TSC2 phosphorylation and hence mTORC1 activity. The combined inhibition of CDK4/6 and nodes in the PI3K pathway can thus maximally suppress mTORC1 activity as well as RB phosphorylation, inhibiting two promoters of S-phase progression.

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