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Alkaloid fraction of Achyranthes aspera Linn triggers breast cancer apoptosis in mice (*Mus musculus*) model

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ABSTRACT

Background: Breast cancer affects women of various ages, and its recurrence is a significant cause of death. The search for potent anticancer compounds of herbal origin with well-defined mechanisms of action is an essential focus of current research.

Aim: This study aimed to investigate the effects of alkaloids in *Achyranthes aspera* Linn (AAL) leaf extract on necrosis, apoptosis, and related molecular markers, namely, cyclin-dependent kinase 1, Bcl-2 associated X-protein (Bax), rat sarcoma virus (Ras), cytochrome (Cyt) c, and apoptotic activating factor-1 (Apaf-1), in mice models.

Methods: Thirty mice with breast cancer were randomly divided into five groups. The negative control group only received distilled water daily. Mice in the positive control group (PCG) were administered methotrexate (15 mg/Kg) daily. The T1, T2, and T3 groups received oral orally at 75, 100, and 125 mg/Kg body weight daily for 30 days, respectively. On day 31, all mice were euthanized for the preparation of histological specimens of the mammary glands. The negative control group had the lowest number of apoptotic cells, Apaf-1, Cyt C, and Bax expression, and the highest number of viable cancer cells and Ras expression.

Results: The percentages of necrotic cells and breast cancer-expressed CDK-1 were not significantly ($p > 0.05$) different among groups. The percentage of apoptotic cells, Apaf-1, and Cyt c, was highest in T3. Conversely, the percentage of viable cells and breast cancer-expressing Ras was lowest in T3.

Conclusion: Treatment with 125 mg/Kg AAL suppressed cancer cell growth in breast cancer-bearing mice. Further research is necessary to determine the complete signaling mechanism.

Keywords: Herbal medicine, Cancer, Bax, Cyt c, Apaf-1.

Introduction

Breast cancer affects women of various ages, and its recurrence is a significant cause of death (Contiero *et al*., 2023). Cancer treatment has not provided satisfactory results, especially for advanced cancers. However, in addition to killing cancer cells, cancer chemotherapy drugs can also cause the death of normal cells. Breast cancer can be treated with systemic or radiation therapy (Bhattacharyya *et al*., 2020), surgical lumpectomy, or mastectomy, with bilateral mastectomy being the last option (Watkins, 2019). Systemic therapy has side effects, such as fatigue, depression, low concentrations, pain, mucosal changes, skin rashes, and peripheral neuropathy (Haidinger and Bauerfeind, 2019). Systemic cancer therapy aims to increase cancer cell apoptosis. The balance between pro and antiapoptotic gene expression programs determines the progression of cancer cell apoptosis (Guo *et al*., 2018). Apoptosis efficiently removes damaged cells to maintain tissue homeostasis. The inhibition of cyclin-dependent

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kinase 1 (CDK1) attenuates apoptosis (Nie *et al*., 2022), whereas rat sarcoma virus (Ras) is an oncogenic protein involved in malignant transformation (Li *et al*., 2019). An increase in Cyt c stimulates the formation of apoptotic activating factor-1 (Apaf-1) and, followed by the activation of caspase-3, induces an increase in DNase activity, resulting in DNA fragmentation and cell apoptosis (Redza-Dutordoir and Averill-Bates, 2016). The release of Cyt c from the mitochondrial outer membrane is a crucial step in inducing the release of proapoptotic proteins Bcl-2-associated X (Bax) and Bad (Yue and López, 2020); Bax is an inducer of apoptosis (Manne *et al*., 2021). Cyt c released from the mitochondria can also form apoptosomes with Apaf-1 and procaspase-9, leading to the activation of caspases-3/7/9 and resulting in apoptosis (Roberts *et al*., 2022).

The search for potent anticancer compounds of herbal origin with well-defined mechanisms of action is an essential focus of current research. The induction of apoptosis is the main cause of decreased cancer cell proliferation. The regulation of proapoptotic Bax is coupled with the regulation of antiapoptotic Bcl-2 expression (Venkatachalam and Nadumane, 2019).

Achyranthes aspera Linn (AAL) is a traditional medicine in the tropical countries of Asia and Africa. The isolated constituents are mainly flavonoids, tannins, terpenoids, saponins, phytosterols, and phenolic compounds (Nargatti *et al*., 2021). The leaf extract of AAL contains 52.36% alkaloids (Meles *et al*., 2017), and the remaining are saponins, tannins, flavonoids, glycosides, steroids, essential oils, and fatty acids (Raju *et al*., 2022). Phenolic compounds, saponins, and alkaloids are potential cancer drugs that can induce p53 expression and decrease telomere length (Vakili *et al*., 2020). To the best of our knowledge, no study has examined the alkaloid fraction of AAL leaf extract as an antibreast cancer herbal medicine. Therefore, this study aimed to determine the influence of AAL leaf extract on necrosis, apoptosis, and other related molecular markers.

Materials and Methods

Preparation

The extraction of AAL alkaloids was performed using the Indonesian Pharmacopeia method. The alkaloid fractionation process was performed using column chromatography with a silica gel stationary phase and a mixture of ethyl acetate, methanol, and water as the mobile phase. Alkaloids were detected using thin-layer chromatography, and the alkaloid contents of AAL were determined using high-performance liquid chromatography (Song *et al*., 2022).

Experimental animals

Thirty 2-month-old female mice (*Mus musculus*) were subcutaneously administered 10 mg/Kg body weight benzopyrene (Sigma-Aldrich, Darmstadt, Germany) into the mammary glands for 8 weeks to induce breast cancer (Sedeman *et al*., 2022). Breast cancer in mice was evaluated by digital palpation of the mammary glands and histological examination of cell proliferation on mammary gland biopsy under a light microscope (Nikon E200, Tokyo, Japan) at 400× magnification.

Breast cancer-bearing mice were randomly divided into five groups. The negative control group (NCG) received 5 ml of distilled water once daily, whereas the positive control group (PCG) received daily treatment with 15 mg/Kg body weight of methotrexate (PT, OTTO Pharmaceutical Industries, Tangerang, Indonesia). Treatment groups 1, 2, and 3 (T1, T2, and T3) were administered with AAL leaf extract alkaloids at 75, 100, and 125 mg/Kg body weights daily (Meles *et al*., 2017), in 5 ml volume using a gastric probe daily for 30 days. On day 31, all mice were euthanized by cervical dislocation, and mammary glands were collected for histological preparation.

Slide staining

The micro-section was taken on a glass object and stained with hematoxylin and eosin (Feldman and Wolfe, 2014). The stained slides were observed using a light microscope at 400× (Nikon Eclipse E800, Tokyo, Japan). Cell proliferation was indicated by 2–3 nuclei stained with blue purplish (Mirzayans *et al*., 2018). Acridine orange-stained slides were examined under a fluorescence microscope at 100× (Nikon Eclipse E800). Apoptotic cells were yellow to reddish, and necrotic cells were brownish–orange (Liu *et al*., 2015) without bright spots, whereas viable cells appeared green (Byvaltsev *et al*., 2019).

Avidin–biotin complex (ABC) staining

ABC staining was performed to evaluate Apaf-1, Cyt c, Cdk1, Bax, and Ras expression under a light microscope (Nikon E200) at 400x magnification. A negative expression is indicated by a bluish/greenish stain. The positive expression of Apaf-1, Cyt c (Zlobec *et al*., 2006), Cdk1 (Clement *et al*., 2015), Bax (Yuan *et al*., 2022), and Ras (Calaf and Abarca-Quinones, 2016) were indicated by brown stains.

Statistical analysis

Data were analyzed using a one-way analysis of variance, followed by Tukey's honestly significant difference test at a 95% confidence level (IBM SPSS Statistics for Windows version 23, IBM Corp., Armonk, NY, USA).

Ethical approval

The Animal Care and Use Committee of Airlangga University, Surabaya, Indonesia (No. 158/HRECC. FODM/XI/2022). Animal treatment was conducted with minimum pain or discomfort according to the guidelines established by the Institutional Animal Ethics Committee.

Result

Breast cancer was characterized by marked lumps in the mammary glands, and cell proliferation (2–3 nuclei) was observed in mammary gland slides (Fig. 1).

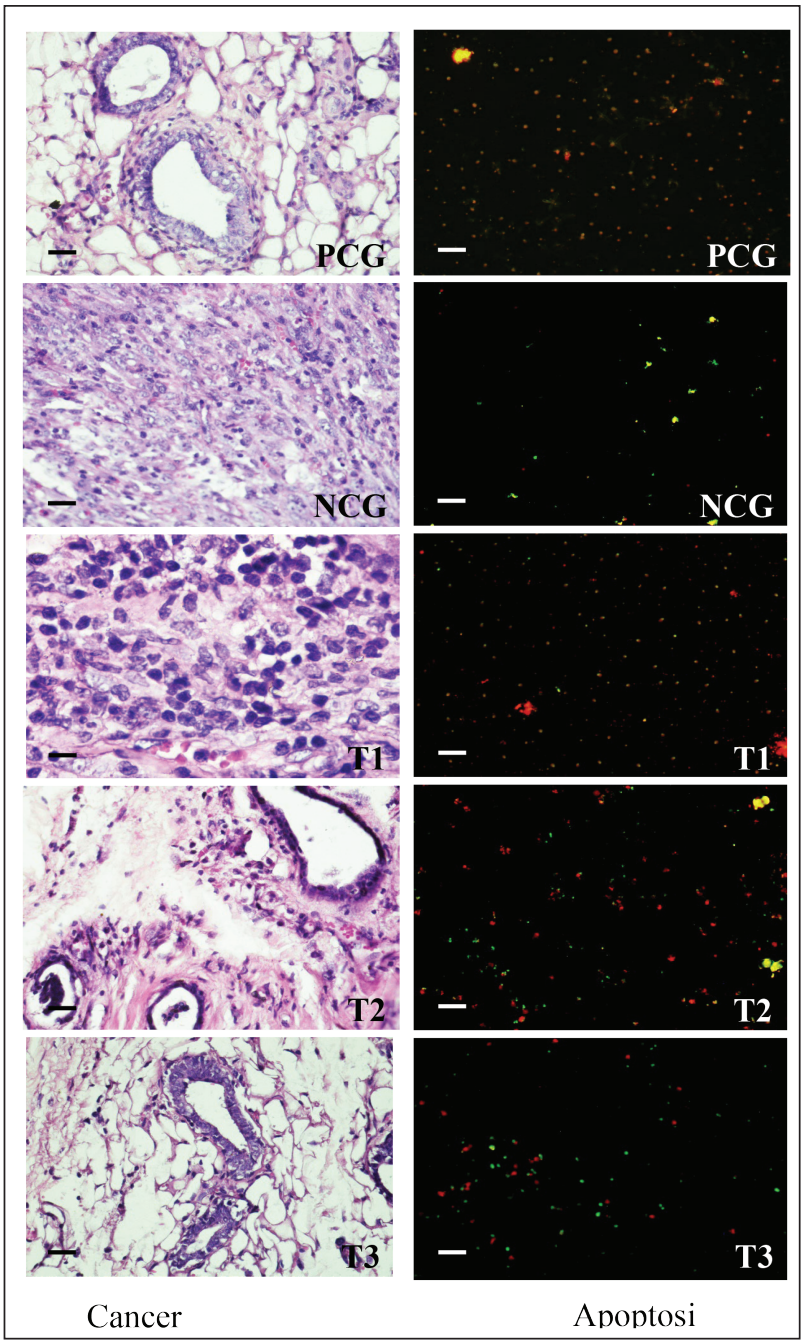

Fig. 1. The proliferation status of breast cancer cells (left) and apoptotic breast cancer (right).

The lowest percentage of necrotic cells ($p < 0.05$) was found in the NCG group, while there was no significant difference between the PCG, T1, T2, and T3 groups ($p > 0.05$). The percentage of apoptotic cells in breast cancer lesions was the highest in T3, even higher than that in the PCG, and the lowest in the NCG. Conversely, the percentage of viable breast cancer cells was the lowest in T3, even lower than that in the PCG, and the highest in the NCG (Fig. 1 and Table 1). The treatment of breast cancer-bearing mice with the alkaloid fraction of AAL increased the expression of Cyt c and Apaf-1, which were higher in the T3 group than in the NCG ($p < 0.05$), but not significantly ($p > 0.05$) different from that in the PCG (Fig. 2 and Table 2). The percentage of breast cancer cells expressing Bax was the highest in T3, even higher than that in the PCG, whereas it

Table 1. Percentage of apoptosis, necrosis, and viable breast cancer cells after treatment with or without the alkaloid fraction of AAL compared with the findings after methotrexate treatment.

Treatment	Necrosis	Apoptosis	Viable cells
PCG	53.50 ± 7.23[a]	15.30 ± 2.67[c]	27.80 ± 2.20[b]
NCG	7.40 ± 2.22[b]	2.30 ± 1.15[d]	90.30 ± 2.27[a]
T1	59.50 ± 5.03[a]	17.9 ± 1.96[c]	23.50 ± 5.01[b]
T2	57.80 ± 3.20[a]	34.40 ± 3.86[b]	7.80 ± 2.14[cd]
T3	54.10 ± 5.56[a]	41.80 ± 5.47[a]	5.10 ± 0.87[d]

Note: NCG: rats only given 5 ml of distilled water once daily; PCG, T1, T2, and T3 groups: daily treatment with 15 mg/Kg body weight of methotrexate; T1, T2, and T3 groups: were followed by given AAL leaf extract alkaloids at 75, 100, and 125 mg/Kg body weight daily for 30 days. The different superscripts in the same column are significantly different ($p < 0.05$).

was significantly ($p < 0.05$) the lowest in the NCG. On the contrary, the percentage of breast cancer cells expressing Ras was the lowest in T3, even lower than that in the PCG; however, it was significantly ($p < 0.05$) the highest in the NCG (Fig. 3 and Table 3).

Discussion

Benzopyrene induces breast cancer cells *in vivo* (Guo *et al*., 2015). Mice with breast cancer were physically and histologically characterized by lumps in the mammary glands and high cell proliferation observed in mammary gland biopsies (Schmitt *et al*., 2017). Cancer treatment aims to suppress as much cell proliferation as possible through apoptotic death (Pfeffer and Singh, 2018). Regulated cell death in cancer can be marked by its signal transduction, including CDK1, Bax, Ras, Cyt c, and Apaf-1, which can be used as indicators of therapeutic progress (Peng *et al*., 2022).

Necrosis is associated with aggressive breast cancer and poor prognosis (Tata *et al*., 2016). Tumor necrosis factor (TNF) induces necrosis in cancer cells (Mercogliano *et al*., 2020). Elevated levels of TNF-α in the serum are correlated with metastasis and poor prognosis of breast cancer (An *et al*., 2020). The treatment of malignant breast cancer by programmed induction of necrosis (necroptosis) can be considered when breast cancer cell apoptosis does not occur as expected (Thakur *et al*., 2019). However, in this study, the percentage of necrotic breast cancer cells was similar among breast cancer-bearing mice treated with placebo, various AAL doses, and methotrexate (Table 1). No comparative data were found on the use of methotrexate in breast cancer mouse models. However, the use of methotrexate on HeLa cancer cell lines was associated with higher (76.4%) viable cancer cells, lower (18.2%) necrotic cancer cells, and lower (5.34%) apoptotic cancer cells (Faraji *et al*., 2022) compared with the rates reported in this study (Table 1).

A close interaction was observed between the metabolic and mitochondrial apoptotic signaling pathways (Daniels *et al*., 2021). In cancer cells, ROS play a role in inhibiting cancer growth, metastasis, and apoptosis; however, they are also involved in the development of cancer, including its malignant transformation (Wang *et al*., 2021). ROS is enhanced by increased metabolic rate, gene mutations, and relative hypoxia in cancer cells. However, ROS also triggers programmed cell death (Perillo *et al*., 2020). Cancer cells cause ROS accumulation, further stimulating cancer cell proliferation, death avoidance, angiogenesis, invasiveness, and metastasis (Hecht *et al*., 2016). ROS induces a decrease in mitochondrial membrane potential, Cyt c release, Bcl-2 dysregulation, and caspase-3 activation, leading to cancer cell apoptosis (Ponraj *et al*., 2018); thereby, ROS has the potential to be a target in breast cancer therapy (Sarmiento-Salinas *et al*., 2019). The extract of *Achyranthes aspera* leaves effectively reduces ROS formation by inhibiting CYP2E1 activity (Deshpande and Une, 2021) and reduces the growth of Dalton's lymphoma via apoptosis through the mitochondrial pathway (Singh *et al*., 2021). The highest percentage of apoptotic breast cancer cells was observed in mice treated with an AAL dose of 125 mg/Kg body weight, which was even higher than that in mice treated with 15 mg/Kg body weight methotrexate (Table 1).

The lowest percentage of breast cancer cells expressed Apaf-1 and Cyt c (Table 2), indicating low apoptosis in breast cancer cells. Apaf-1 expression is low in most cancer cells compared with that in normal cells (Loginov *et al*., 2017). AAL leaf extract effectively inhibits the course of Dalton's lymphoma by attenuating the PKCα signaling pathway and increasing apoptosis through the mitochondrial cascade (Singh *et al*., 2021). The extract from *Achyranthes aspera* inhibited the antiproliferative activity of pancreatic cancer cells by selectively suppressing the transcription of metalloproteases (MMP-1 and -2), MMP inhibitors (TIMP-2), and angiogenic factors (VEGF-A and VEGF-B) (Subbarayan *et al*., 2010). AAL pharmacological properties made it a promising cancer treatment option for thyroid carcinoma in the docking experiments (Alamri *et al*., 2023). Based on the 2,2-diphenylpicrylhydrazyl radical scavenging assess antioxidant activity, AAL leaves exhibit significant promise as an anticancer drug (Bashir *et al*., 2024).

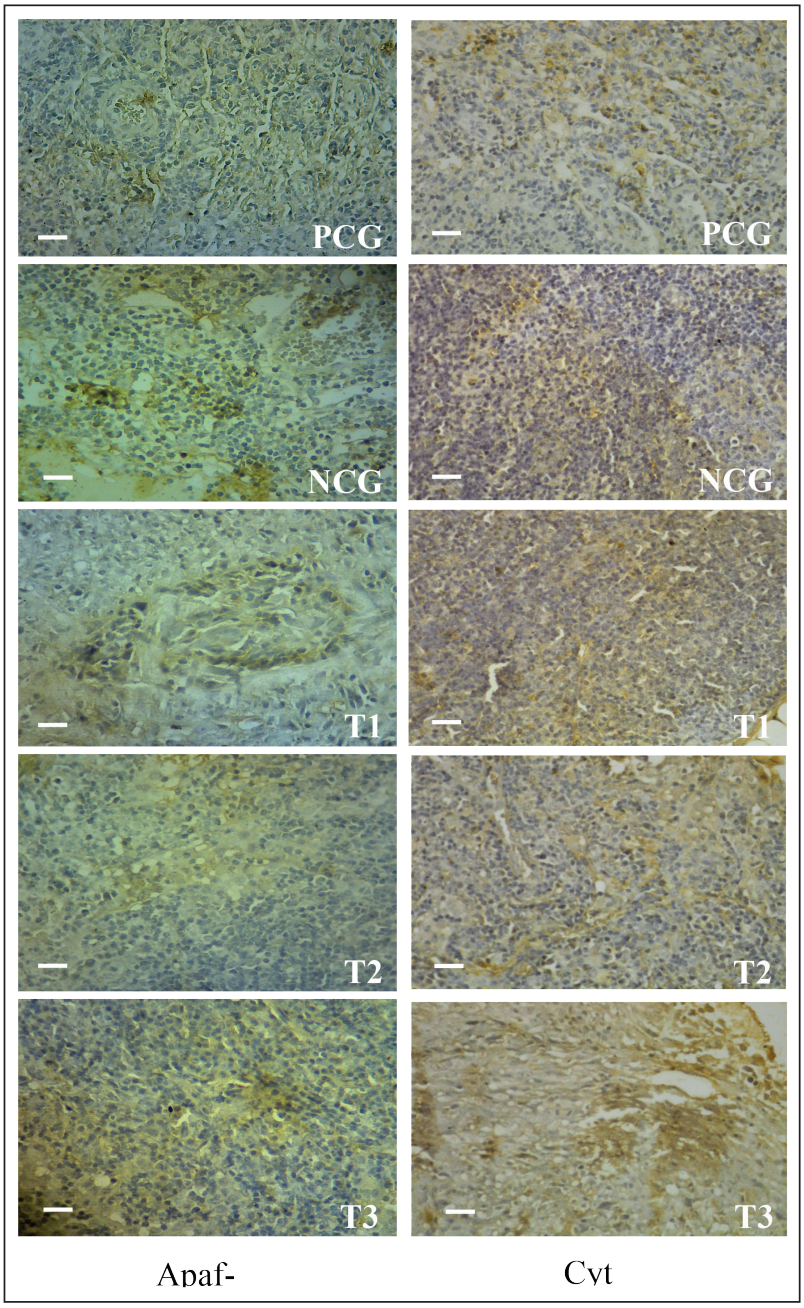

Fig. 2. Apaf-1 (left) and Cyt c (right) expression in breast cancer cells.

Low Apaf-1 expression causes cancer cells to be protected from apoptosis (Bakhshoudeh *et al*., 2021). *APAF-1* plays an essential role in DNA damage-induced apoptosis (Eskandari-Nasab and Hashemi, 2017). Furthermore, Apaf-1 is a crucial molecule that determines whether Cyt c is released from mitochondria to form apoptosomes (Shakeri *et al*., 2017). Competitive binding between Apaf-1 and Cyt c may inhibit apoptosis in breast cancer cells (Jemmerson *et al*., 2021). The 125 mg/Kg body weight dose of AAL alkaloid fraction increased Cyt c and Apaf-1 expression in breast cancer-bearing mice. This effect was comparable to that observed after treatment with 15 mg/Kg body weight of methotrexate (Table 2). High Apaf-1 expression inhibits the viability and colony formation ability and promotes apoptosis of breast cancer cells (Fang *et al*., 2019). Cyt c is oxidized by ROS with Apaf-1 to form apoptosomes (Matsuura *et al*., 2016). In addition, Cyt C acts to transfer electrons in the mitochondrial redox process, activates caspase

Table 2. Breast cancer cells express cytochrome c and Apaf-1 proteins.

Treatment	Apaf-1	Cyt c
PCG	136.33 ± 5.14[a]	124.71 ± 4.32[a]
NCG	78.26 ± 3.19[d]	67.26 ± 4.14[d]
T1	90.82 ± 8.08[c]	79.44 ± 4.97[c]
T2	111.91 ± 9.26[b]	106.47 ± 4.76[b]
T3	140.32 ± 4.31[a]	132.82 ± 3.55[a]

Note: NCG: rats only given 5 ml of distilled water once daily; PCG, T1, T2, and T3 groups: daily treatment with 15 mg/Kg body weight of methotrexate; T1, T2, and T3 groups: were followed by given AAL leaf extract alkaloids at 75, 100, and 125 mg/Kg body weight daily for 30 days. The different superscripts in the same column are significantly different ($p < 0.05$).

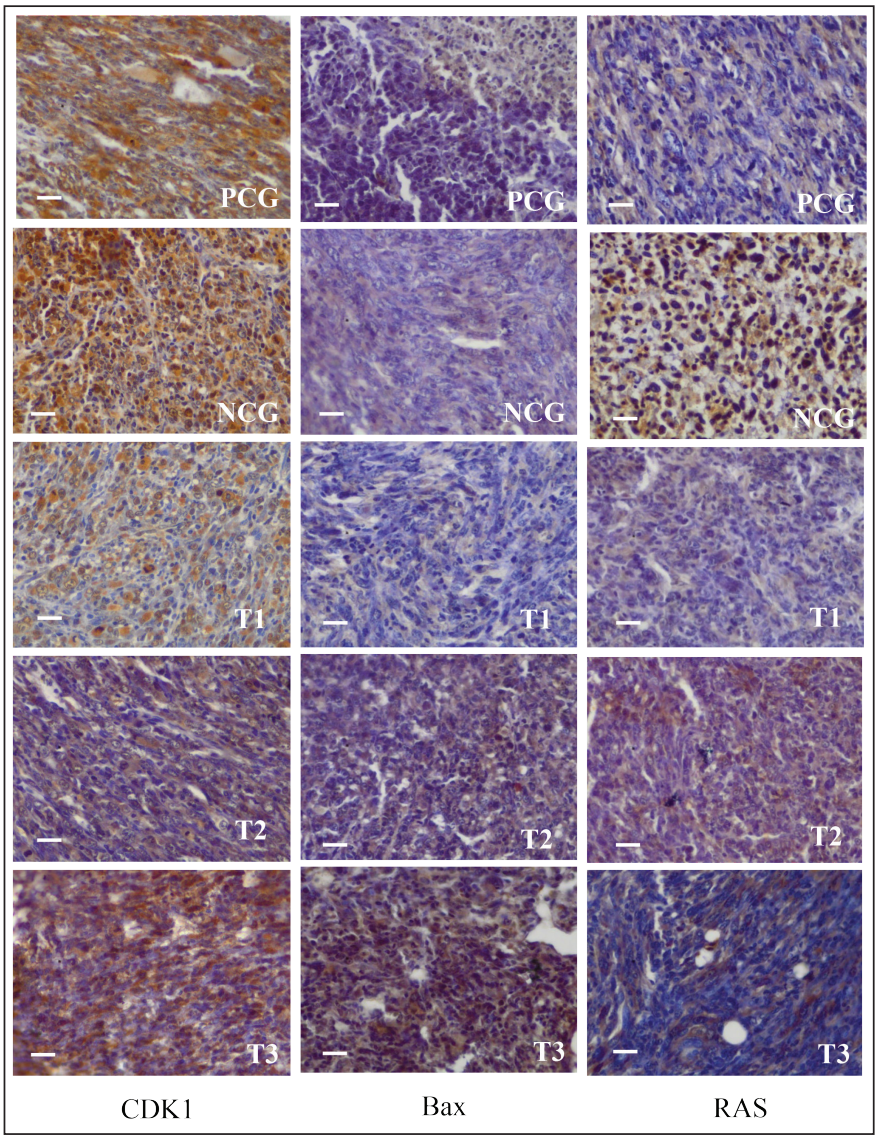

Fig. 3. CDK1 (left), Bax (center), and Ras (right) expression in breast cancer cells.

cascades, and triggers apoptosis in breast cancer cells (Abramczyk *et al*., 2022).

CDK1 is a major cell cycle protein kinase regulator that regulates the normal function of cell mitosis

Table 3. Breast cancer cells expressing CDK1, Bax, and Ras proteins.

Treatment	CDK1	Bax	Ras	Treatment
PCG	512.00 ± 73.96[a]	424.00 ± 66.93[b]	210.00 ± 108.07[b]	PCG
NCG	541.67 ± 166.18[a]	283.33 ± 74.48[d]	305.00 ± 70.92[a]	NCG
T1	591.67 ± 97.45[a]	286.67 ± 102.31[cd]	168.33 ± 56.01[c]	T1
T2	580.00 ± 157.86[a]	311.67 ± 119.40[c]	134.00 ± 23.82[c]	T2
T3	544.00 ± 145.19[a]	488.00 ± 83.49[a]	118.00 ± 61.81[d]	T3

Note: NCG: rats only given 5 ml of distilled water once daily; PCG, T1, T2, and T3 groups: daily treatment with 15 mg/g body weight of methotrexate; T1, T2, and T3 groups: were followed by given AAL leaf extract alkaloids at 75, 100, and 125 mg/Kg body weight daily for 30 days. The different superscripts in the same column are significantly different ($p < 0.05$).

(Diril *et al*., 2012) by combining cell proliferation with protein synthesis (Haneke *et al*., 2020). CDK1 initiates and elevates protein synthesis in cells related to mitotic division and cytokinesis (Kalous *et al*., 2020). In this study, no difference in CDK1 expression was observed after treatment of breast cancer-bearing mice with placebo, methotrexate, or AAL. This indicates that breast cancer cell apoptosis induced by AAL treatment did not involve CDK1 (Table 3). These results are comparable to the results of an attempt to induce apoptosis using solid lipid nanoparticles in tamoxifen without cell cycle arrest in breast cancer (Abbasalipourkabir *et al*., 2016).

BAX is a proapoptotic member of the *Bcl-2* gene family encoding the Bax-alpha protein. The Bax-alpha association forming the Bcl-2 heterodimer functions as an apoptosis activator (Saddam *et al*., 2024) by increasing the opening of mitochondrial voltage-dependent anion channels, followed by decreasing membrane potential and Cyt C release (Grosser *et al*., 2021). Lower Bax protein expression in breast cancer cells may lead to the avoidance of apoptosis of cancer cells. A correlation was reported between the Bax, p53, and caspase-3 proteins and apoptotic mechanisms in breast cancer cells (Pluta *et al*., 2011). Low levelBaxession can be a risk factor for breast cancer malignant tumors (Kholoussi *et al*., 2014). Breast cancer-bearing mice treated with a placebo exhibited the lowest Bax expression. The low expression levels of Bax indicate apoptosis and high breast cancer malignant tumors (Chen *et al*., 2021). The highest percentage of breast cancer cells expressing Bax was noted in breast cancer-bearing mice given the 125 mg/Kg body weight alkaloid fraction of AAL, which was even higher than that in mice receiving the 15 mg/Kg body weight methotrexate treatment (Table 3). These results are comparable to those of capsaicin, which increases Bax protein expression, activates caspase-3, induces apoptosis, and inhibits breast cancer proliferation (Chen *et al*., 2021).

The experimental breast cancer-bearing mice in the placebo group exhibited the highest Ras expression. The percentage of breast cancer cells with the lowest Ras expression was detected in breast cancer-bearing mice given the 125 mg/Kg body weight alkaloid fraction of AAL, which was even lower than that observed in mice treated with 15 mg/Kg body weight methotrexate treatment (Table 3). Ras functions as an ON and OFF switch during signal transduction. Mutations in the Ras regulator cause normal cells to undergo malignant transformation (Simanshu *et al*., 2017). Pathologically, RAS mutations occur in breast cancer because of the overexpression of growth factor receptors. High mitogen-activated protein kinase activity correlates with Ras overexpression in breast cancer (von Lintig *et al*., 2000).

Oncogenic Ras plays a role in the development and spread of metastases and resistance to therapy in breast cancer (Galie, 2019; Gimple and Wang, 2019). Ras oncogene p21, an essential component of the Ras signaling pathway, is hyperactivated in breast cancer, resulting in poor prognosis. Thus, low Ras expression is a target in breast cancer therapy (Banys-Paluchowski *et al*., 2018; Prior *et al*., 2020).

Conclusion

Overall, oral treatment of breast cancer-bearing mice with the 125 mg/Kg body weight alkaloid fraction of AAL extract daily for 30 days resulted in the highest rate of cancer cell apoptosis. This finding was supported by the highest expression of Cyt c, Apaf-1, and Bax and the lowest expression of Ras. However, the mechanism of apoptotic induction by AAL is not known. Therefore, further research into the complete signaling mechanism is necessary.

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Author's contributions

WW, DKM, SM, and IM: conceived the idea and manuscript drafting. ARK, DMSP, JJ, RR, and NS: acquisition, analysis, and interpretation of data. AOA, RZA, SU, FE, and WW: The manuscript was critically read and revised for intellectual content. All authors have read and approved the final manuscript. All

authors have read, reviewed, and approved the final version of the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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Data availability

All data are available in the manuscript.

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