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Full Paper

Expression of cdk4 Gene in MNU-Induced Breast Cancer Wister Female Albino Rats Treated with Ethanolic Extract of Peperomia pellucida Aerial Parts

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Abstract

Breast cancer being the most prevalent type of cancer, with 2.26 million cases and primary cause of cancer death in women, thus need for treatment with minimal side effect. To determine the anti- proliferative potential of P. pellucida aerial parts extract on R24 residue of CDK4 n-methyl-n-nitrosourea (MNU) induced breast cancer of Wister female albino rats. Phytochemical screening was performed on ethanolic extract of P. pellucida aerial parts, and fifty (50) Wister female albino rats were grouped into 5 groups based on their body weights. The treatment of animals lasted for 8 weeks. The breast tissues from each rat were harvested and fixed in formalin for histopathology and the expression level of mRNA was determined by Quantitative RT- PCR. Qualitative phytochemical screening revealed the presence of reducing sugar flavonoids, terpenoids, alkaloids, saponins and tannins while the quantitative analysis revealed that reducing sugar has the highest concentration of 63.25 µg/g. The extract shows no signs of toxicity or mortality during the period of observation. The gene expression studies revealed that the target gene was up regulated in the group that received MNU only while the target gene was down regulated in the group that received higher concentration of the extracts. Histological assay shows vascular abnormality in the group that received MNU only, while reduced vascular abnormalities were seen in the groups that received different concentration of the extract. This study shows that P. pellucida aerial possesses antiproliferative potential against MNU-induced breast cancer rats.

Keywords: Gene expression, CDK4, breast cancer, P. pellucida, vascular abnormality, MNU

Introduction

Cancer is defined as a proliferative, invasive, and metastatic illness that develops malignant cells at random due to a buildup of genetic defects [1]. Chemical carcinogens, chronic inflammation, radiation exposure, or a genetic susceptibility can all cause these disorders [2]. It also involves frequent initiation of oncogenes and/or deactivation of tumor suppressor which result in uncontrolled cell cycle progression and inactivation of apoptotic mechanisms. In contrast to benign tumors, malignant cancers build up metastasis [3]. Breast cancer is presently the most prevalent type of cancer worldwide, with 2.26 million cases recorded in 2020 [4]. In 2020, It was the 5th leading cause of cancer deaths with 685,000 deaths in worldwide [4]. Breast cancer is the common cancer among women both in developed and developing countries, and a main cause for public health concern [4], while it occurs all over the world, developed countries have a higher incidence rate and the incidence rate also varies with ethnicity and race [5]. Generally the costs of cancer management,

as well as the diagnosis, are time consuming [6].

Cancers, which include breast cancer, have been managed and treated using radiotherapy, chemotherapy and surgery with harmful side effects [7]. Phytomedicines provide an alternative for different treatment with minimal side effect while shielding patients from its destructive reaction [8]. Phytomedicines which involve the use of herbs has been used to treat several kinds of cancer and this has been proven to be effective and also, confer minimal or no side effects [9]. *Peperomia pellucida* (L.) Kunth (Piperaceae) has a variety of uses, from the usage of its leaves and stems as vegetables [10] to functioning as agents employed in treatment and medications, depending on local customs and geography [10]. Also, several studies showed isolated bioactive phytoconstituents with strong antifungal and anticancer properties from *P. pellucida* [11]. The plant has been utilized culturally for a variety of ailments such as conjunctivitis, convulsions, fatigue, fever, headache, gout, rheumatic pains, skin diseases, to lower blood cholesterol level and breast cancer [12].

A key response of many growth factors in many cell types is the activation of CDK4 by members of the cyclin D family (D1, D2, and D3). CDK4 associate with D-type cyclins and mediate progression through the G1 phase when the cell prepares to initiate DNA synthesis [13]. Oncogenic mutations at the R24 residue of CDK4 results in an inhibition of p16 binding, which in turn results in enhanced kinase activity and increased cell proliferation [14]. Breast cancer can be induced using carcinogenic chemical like but not limited to n-methyl-n-nitrosourea (MNU). The carcinogenicity of MNU originates from its ability to methylate deoxyribonucleic acid (DNA) in aqueous environment (physiological pH) [15]. A single dose of MNU has been shown to induce breast cancer in female Sprague Dawley rats [16]. Also, MNU is also able to induce various cancers in experimental animals including retinal degeneration, esophageal, breast cancer, photoreceptor degeneration, gastric and colorectal malignancies [16].

Materials and Methods

Sample Collection

Fresh plants of *P. pellucida* aerial parts were gotten from University of Lagos state, Nigeria in the month of March and April 2021. The plants were identified and authenticated by Nodza George Botanist in the Department of Botany, University of Lagos. The sample was deposited under the voucher number "LUH 8732".

Preparation of the Ethanolic Extract

P. *pellucida* aerial parts were washed with tap water and then dried at room temperature and grinded into powdering form. 10 g of coarsely powdered of *P. pellucida* aerial parts was put in a stoppered glass container with the 50 ml of solvent (ethanol) to immerse solid matrix and permitted to stand at room temperature for 48 h with repeated perturbation till the soluble matter has broken down. The concoction was then strained, and gauzed by using a mesh of 200 mm, with a muslin cloth and the merged liquids are clarified by filtration or decantation after standing (Sukhdev *et al*., 2008). The concentration of the extract was done using a rotary evaporator at 45 °C, and then the concentrated extract was poured into an evaporating dish to dry completely into a paste. The extract obtained was kept in a cool place for the preparation of the different concentrations to be used in the tests.

Phytochemical Screening

Qualitative phytochemical screening was performed on ethanolic extract of *P. pellucida* aerial parts, using standard method according to Houghton and Raman (Houghton and Raman, 1998). Also, the quantitative analysis of the extracts were determined using standard analytical methods specify by the Association of Official Agricultural Chemists (AOAC).

Animal Material

Fifty female albino rats, 4- 6 weeks of age were obtained from the College of Medicine, Lagos University Teaching Hospital, Idi- Araba, Lagos. Nigeria and left to acclimatise for 2 weeks. The animals were housed in standard clean rat cages at 25 ºC, fed with mash grower and tap water ad libitum. They were maintained under uniform conditions of 12 h light/dark cycle. Experiment was carried out in the animal house of the department of Cell Biology and Genetics, University of Lagos, Lagos. Nigeria in accordance with the rules governing the use of Laboratory Animals as acceptable (WMA, 2008).

Pilot Study

Pilot study was carried out following the protocol of (Chinedu *et al*., 2013).

Preparation of N- Methyl-N-Nitrosourea (MNU)

The carcinogen N- Methyl-N-Nitrosourea (MNU) was purchased from Hangzhou Sage Chemical Company Ltd, Hangzhou, China. It was dissolved shortly before administration in phosphate/citrate-buffered saline at pH 4.2.

Experimental Design

Fifty (50) Wister female albino rats were used for the experiment study. They are separated into 5 groups according to their body weights, after acclimatization. The *P. pellucida* aerial parts extract was dissolved in distilled water to prepare stock solution using dosage as shown in (Table 1). The Extracts were administered orally by gavage using a cannula fitted to a feeding needle daily while the MNU were administered weekly through intraperitoneal injection. The treatment of animals lasted for 8 weeks. The experimental and control animals were carefully checked daily and weight taken weekly. The rats were sacrificed at the end of the eighth week by cervical dislocation. The breast tissues from each rat were harvested and fixed in formalin for histopathology and some breast tissues were collected from each rat for expression studies.

Table 1 : Experimental Design

Group A: MNU + extract 100 mg/kg Group	100 mg/kg of the ethanolic extract + 50 mg/kg MNU per rat
Group B: MNU + extract 300 mg/kg Group	300 mg/kg of the ethanolic extract + 50 mg/kg MNU per rat
Group C: MNU + extract 600 mg/kg Group	600 mg/kg of the ethanolic extract + 50 mg/kg MNU per rat
Group D: MNU Group	50 mg/kg MNU per rat
Group E: Food and Animal feed group	Rat that received distilled water and food only

RNA Extraction and cDNA Synthesis

Expression studies were performed on breast tissues of four (4) randomly chosen rats from each experimental group. Total RNA was extracted using RNA kit (FOWM Biotechnology). The amount of total RNA was determined using a Nanodrops spectrophotometer (Agilent Technologies). Absorbance was measured at wavelength of 260 nm and 280 nm (A_{260} and A_{280}). The absorbance quotient (OD_{260}/OD_{280}) provides an estimate of RNA purity. An absorbance quotient value of >1.8 was considered to be good for purified RNA. The generation of cDNA occurred after treatment with RNase inhibitor through the use of a high-capacity cDNA kit. Briefly, 2 µl of total RNA was treated with RNase I and incubated with kit buffers (30 min at 60 °C) in a thermocycler. The resulting cDNAs were used for quantitative reverse transcriptase polymerase chain reaction (RT- qPCR).

Quantitative Real-time PCR Analysis

Gene expression level was determined by quantitative real-time PCR analysis. Quantitative real-time PCR analysis was performed with SYBR Green PCR Master Mix with Step One Plus (Bio- RAD) under standard conditions: 10 min at 95 °C, 40 qPCR cycles (15 s at 95 °C, 1 min at 55 °C. 15 s at 95 °C), 1 min at 60 °C, and 15 s at 95 °C. The $2^{-\wedge\wedge CT}$ method, was used to quantify mRNA relative to the housekeeping endogenous control gene (GAPDH). Primers for CDK4 and GAPDH and amplification conditions for RT- qPCR are listed below.

Table 2. Primer used in rt- qpcr

Genes	Primer	Bases	Annealing	Cycles
GAPDH	S: 5′ -GGTCGGTGTGAACGGATTT 3′ AS: 5′TGGAAGATGGTGATGGGTTTC-3′	21 19	55 ºC	40
CDK4	S- CAAGTAATGGGACAGTTAAG AS- GAGTTCCCACAGAAGAGAG	20 19	55 ºC	40

Statistical Analysis

Statistical analysis was carried out using GraphPad Prism 9.3.1. Tool and the data were expressed as means ± SD, and the statistical significance of the differences between control and experimental groups was estimated using Kruskal-Wallis test.

Results

The qualitative screening revealed the presence of Reducing sugar, Flavonoids, Terpenoids, Alkaloids, Saponins, Tannins, Glycosides and Steriods, but absence of anthraquinones (Table 3). The quantitative analysis reveals that *P. pellucida* extract has high concentration of Reducing sugar and low concentration of Steriods. The concentration of the phytochemicals in *P. pellucida* is presented in the following order:Reducingsugar> Flavonoids> Terpenoids> Alkaloids> Saponins> Tannins > Glycoside >Steroids as shown in (Table 3).

Table 3: Qualitative and Quantitative Analysis of Aerial parts of *P. pellucida* extract

Phytochemical component	Present/Absent	Quantity (µg/g)
Alkaloid	+	41.38
Saponin	+	32.77
Flavonoid	+	56.93
Tannin	+	29.81
Reducing sugar	+	63.25
Glycoside	+	8.32
Steriod	+	5.13
Terpeniod	+	41.74
Anthraquinone	-	0

The weight of the rats in the group administered 100 mg/kg, 300 mg/kg, 600 mg/kg extract and untreated were significantly ($P<0.05$) higher than that of the control (Rats that received water and animal feed only) (Figure 1).

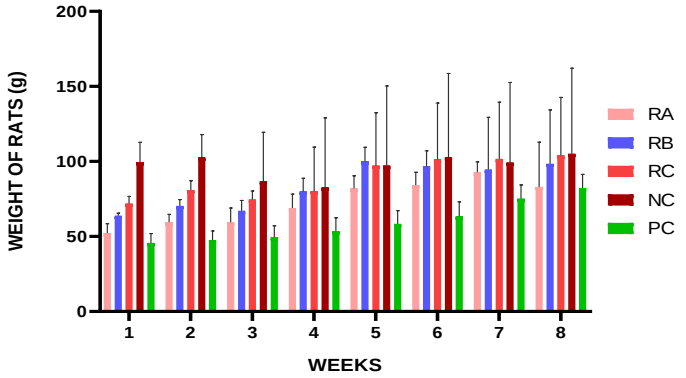

Figure 1: The weight trend of MNU- induced experimental animals treated with different concentrations of *P. pellucida* aerial parts extract.

Values are means of 10 replicates ± standard deviation (SD)

Group RA: Rats that received 50 mg/kg/week of MNU + 100 mg/kg of extract

Group RB: Rats that received 50 mg/kg/week of MNU + 300 mg/kg of Extract

Group RC: Rats that received 50 mg/kg/week of MNU + 600 mg/kg of Extract

Group NC: Rats that received 50 mg/kg/ week MNU only

Group PC: Rats that received water and animal feed only

The mortality rate of the experimental groups treated with different concentrations of *P. pellucida* extract, untreated and control is revealed in (Figure 2). The group that received MNU only (NC) shows the highest mortality rate, but no death was recorded in group that was given water and animal feed (PC).

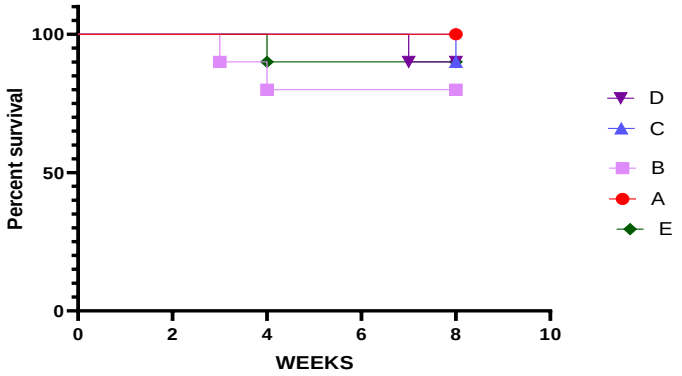

Figure 2: Mortality rate of MNU- induced experimental animals treated with different concentrations of *P. pellucida* extract.

Group A : Rats that received 50 mg/kg/week of MNU + 100 mg/kg of extract
Group B : Rats that received 50 mg/kg/week of MNU + 300 mg/kg of Extract
Group C: Rats that received 50 mg/kg/week of MNU + 600 mg/kg of Extract
Group D : Rats that received 50 mg/kg/ week MNU only
Group E : Rats that received water and animal feed only

Figure 3 shows the histological section of rats that received water and animal feed only (Positive Control). Normal breast cell was observed and few ducts are found in the fibro collagenous stroma. The histological section of the breast tissues of the rats that received 50 mg/kg/week MNU only (NC) is shown in (Figure 3B). This result reveals that mammary breast tissue of NC shows aggregates of many red blood cells congesting the vessel. No malignant cells are seen. However, large vascular abnormality of the breast tissue was observed. Also, Figure 4A shows histological section of the breast tissue of rats that were given 50 mg/kg/week of MNU + 100 mg/kg of the extract (RA). Which shows aggregates of few red blood cells congesting the vessel, but no malignant cells are seen, although, few vascular abnormality of the breast was seen.

More so, the breast tissue of rats that received 50 mg/kg/week of MNU + 300 mg/kg of extract (RB) is shown in (Figure 4B). The result obtained shows aggregates of fewer red blood cells congesting the vessel. Also, no malignant cells are seen, but breast tissue has fewer vascular abnormalities. (Figure 4C) shows histological section of rats that were administered 50 mg/kg/week of MNU + 600 mg/kg of extract (RC). The breast tissue of the rats in this group reveals no or little abnormal vascularization.

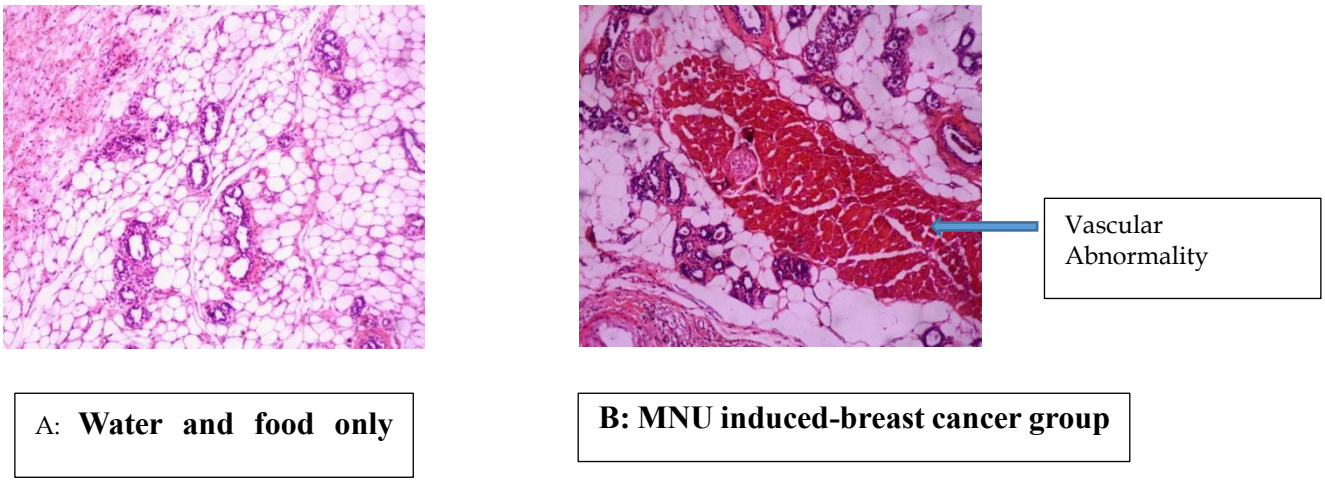

Figure 3: Histological section of the breast tissue of MNU induced- breast cancer in rat that received 50 mg/kg/week of MNU (HE-100X) from NC.

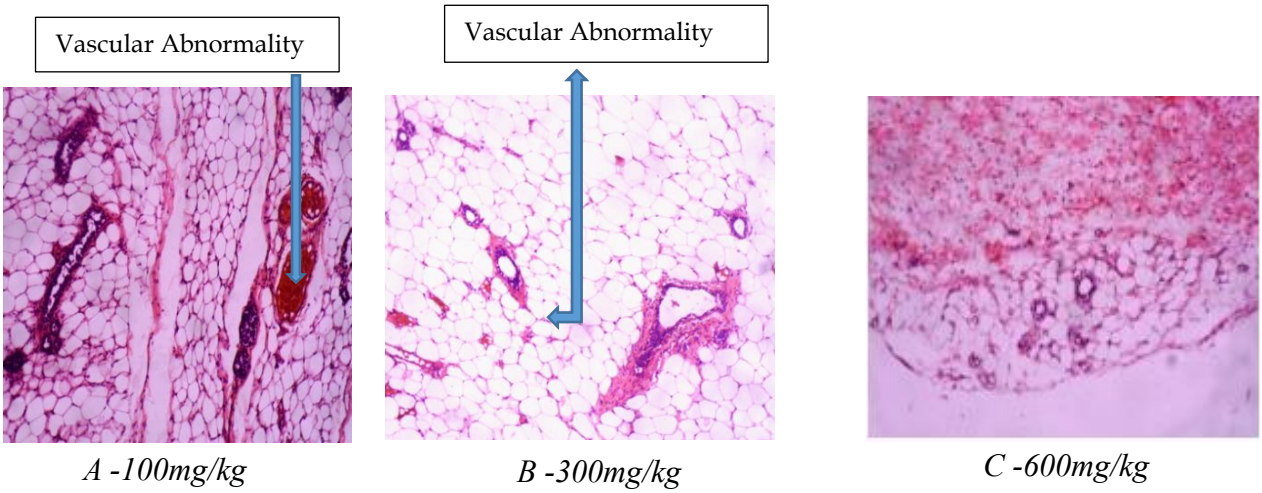

Figure 4: Histological section of the breast tissue of MNU induced- breast cancer in rat and treated with 100, 300 and 600 mg/kg of extract (HE-100X) respective arrows indicated on the figures

Figure 5 shows qPCR assay of CDK4 mRNA expression in MNU induced experimental animals treated with different concentration of *P. pellucida* extract compared to untreated group. Grouped analysis of relative expression of CDK4 mRNA revealed that expression is significantly higher in the MNU induced group only (1.00 ± 0.30) when compared to group that received 50 mg/kg/week of MNU + 600 mg/kg of extract (0.59 ± 0.28) there was significant difference ($p < 0.0398$ Kruskal-Wallis test).

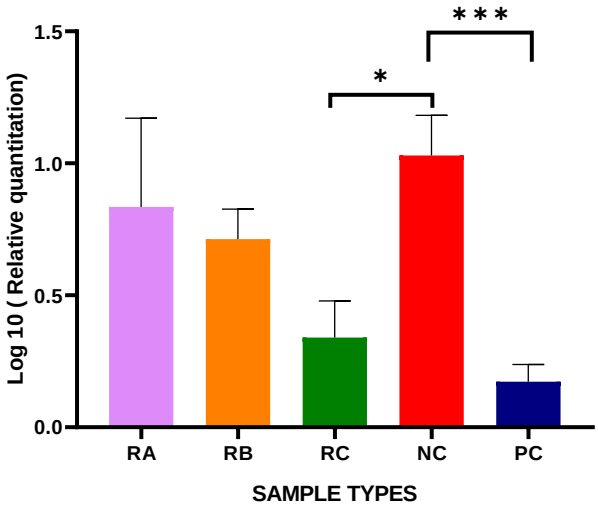

Figure 5: QPCR assay of CDK4 mRNA expression in MNU induced experimental animals treated with different concentration of *P. pellucida* extract compared to untreated group. Bars represent mean ± SEM (n= 4). Statistically significant differences in relation to untreated group: *p= 0.0115; ***p= 0.0010.

Group RA: Rats that received 50 mg/kg/week of MNU + 100 mg/kg of extract
Group RB: Rats that received 50 mg/kg/week of MNU + 300 mg/kg of Extract
Group RC: Rats that received 50 mg/kg/week of MNU + 600 mg/kg of Extract
Group NC: Rats that received 50 mg/kg/week of MNU only
Group PC: Rats that received water and animal feed only

Discussion

In this study, the presence of Alkaloids, Flavonoids, Terpenoids, Alkaloids, Saponins, Tannins, Glycosides, Reducing sugar and Steroids in *P. pellucida* (Table 3) is in agreement with the report of [18]. Also, the quantitative analysis shows that *P. pellucida* have higher concentration of Reducing Sugar, Flavonoids, Terpenoids, Alkaloids, Saponins and Tannins, while the extract showed low concentration of Steroids and Glycosides.

There was significant increase in the body weight of the MNU induced experimental animals (Figure 1). This is against the claim on MNU induced rats [19], who reported that MNU induced rats, had significant reduction in the body weight. Also, it has earlier been reported that fatty acid in the diet of an experimental animals may prevent reduction in the body weight [20].

Also, the higher mortality observed in the group that received MNU only (50 mg/kg) could be as a result of the carcinogenic activity of the MNU [19] where they reported higher death rate in MNU- induced Wister female albino rats. The less mortality rate which was seen in the MNU- induced Wister female albino rats treated with different concentration of *P. pellucida* aerial parts extract is similar to the report on the *P. pellucida* [11] who stated anticancer activity as one of the biological property of *P. pellucida* extract. Also, there was no death in the group of animals given water and animal feed. The pilot study carried out to determine the effect of *P. pellucida* on the rats revealed no mortality. This support the fact, that the death of

the experimental animals were probably caused by the MNU- induced toxicity [21], while the extract was able to reduce the mortality rate in the experimental animals treated with different concentrations of the extract (Figure 2).

This study, also shown from histopathology an enlarge vascular abnormalities in the group administered MNU only, which may be angiosarcoma and this could be an indicative of breast cancer. A finding carried out on the angiosarcoma of the breast [22] revealed that they are tumors that arise from mesenchymal glandular of breast tissue and account for <1% of all known cancers and that tumor vasculature is highly abnormal [23]. This observation can be traced to the carcinogenic property of MNU induced in this group, which is in concordance with the report on the ability of MNU to induced breast cancer [17].The reduced vascular abnormalities seen in the histological section of the breast tissue of MNU induced experimental animals treated with different concentration of the *P. pellucida* extract (Figure 4) could be attributed to the anticancer property of the extract which align with the finding of anticancer property of *P. pellucida* extract [11].

Also, It was observed that vascular abnormality of the breast tissue reduces as the concentrations of the *P. pellucida* extract administered to these experimental animals increase. Also, flavonoids, tannins, terpenoids which are identified in the extract, had earlier been reported to have antioxidants property [24]. Thus, the extract was observed to be more effective on the group that received MNU (50 mg/kg) and treated with *P. pellucida* (600 mg/kg) (Figure 4C) with little or no vascular abnormality compared to other groups that were administered lower concentration of the extract (Figure 4A & 4B).

Furthermore, The relative gene expression obtained from this study revealed that the group that received MNU only (50 mg/kg) has a high expression level (Figure 5); this suggests that the high expression of the target gene in this group could be due to carcinogenic property of the MNU administered to NC group (MNU induced group). This is in concordance with the report that described a single dose of MNU induces tumor growth in female rats within eight to ten weeks after administration [25]. Also, this might leads to mutation on R24 residue of CDK4, according to the findings that showed that oncogenes mutations at the R24 residue of CDK4 could leads to inhibition of p16 binding. As such, it can leads to increased kinase activity, hence rise in cell proliferation [14]. However, group that received MNU (50 mg/kg) and treated with *P. pellucida* (100 mg/kg), (300 mg/kg), (600 mg/kg) showed low relative gene expression levels. The low relative gene expression may be due to the effect of *P. pellucida* extract on these group. Also, the extract might have reduced the mutation at the R24 residue of CDK4, resulting in low gene expression observed in these groups. As such, close proximity observed in the relative gene expression of the group administered MNU + 600 mg/kg and the positive control group could be as a result of the anticancer property of the extract on the group that received MNU + *P. pellucida* (600 mg/kg). Moreover, many plant-derived chemotherapeutic agents have shown to exert their therapeutic effect by directly reacting with DNA or DNA- binding proteins [26].

Conclusions

The present study showed that the ethanolic extract of *P. pellucida* aerial parts can be used as a preventive measure against MNU induced breast cancer proliferation in the breast tissues of female albino rat at a dose of 600 mg/kg. Gene expression study showed that the plant extract prevented MNU induced DNA damage to some extent. Histological assay revealed the presence of vascular abnormality in the breast which

suggests a rare type of breast cancer. Nevertheless, a more detailed investigation is needed to optimize the quality of the extract, effective dose and its specificity on breast cancer susceptibility genes.

Conflicts of interest

The authors declare no conflicts of interest.

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