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Pistia stratiotes LINNAEUS POTENTIAL TO PREVENT DAMAGE OF BRCA2 GENE IN METHYLNITROSOUREA (MNU) INDUCED FEMALE WISTAR ALBINO RATS

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ABSTRACT

Background: Breast cancer-associated gene 2 (BRCA2) is an anti-oncogene for breast cancer risk. Mutation of the BRCA2 gene can cause down-regulation of the cell cycle checkpoint which can result in uncontrolled cell growth leading to breast cancer.
Objectives: This study aimed to evaluate the status of the BRCA2 gene in the presence of Pistia stratiotes Linn (water lettuce) in Methyl Nitrosourea (MNU) induced female Wistar albino rats.
Methods: *P. stratiotes* plant, 25 female albino rats and methyl nitrosourea were used for this study. A Crude extraction protocol was employed in the preparation of the aqueous extract of *P. stratiotes*. Qualitative and quantitative phytochemical screening of the extract of *P. stratiotes* was carried out using a standard protocol. Agarose gel electrophoresis was used to analyze the BRCA2 gene of the deoxyribonucleic acid (DNA) extracted from the breast tissues of the experimental rats and amplified using Polymerase Chain Reaction (PCR).
Results: The qualitative screening revealed the presence of flavonoids, alkaloids, saponin, tannin, reducing sugars, glycosides, steroids, and terpenoids, while the quantitative screening showed that terpenoid was present in the highest amount (9.05 in 25 ug/ml of extract) while tannin was found to be the lowest (1.09 in 25 ug/ml of extract). The gel electrophoresis of the PCR-amplified BRCA2 gene revealed similarities in the band of the group that received 0.15 mg/ml/day of *P. stratiotes* extract when compared with the band of the positive control group.
Conclusions: This study shows that *P. stratiotes* has the potential to prevent damage to BRAC2 gene in MNU-induced female albino rats.

Keywords: Breast cancer, BRCA2, *Pistia stratiotes*, phytochemical, methyl nitrosourea

INTRODUCTION

Breast cancer remains one of the leading causes of cancer-related deaths and a global challenge to date. Over the years, new therapeutic developments and strategies have been made to combat breast cancer. Breast cancer is a type of cancer that develops in the breast tissues. Breast cancer can develop in the ducts or lobules and have the ability to metastasize to other parts of the body. Breast cancer was among the most common cancer in women worldwide in 2020 (World Cancer Research Fund International, 2022) . In 2020, more than 2.3 million women were diagnosed with breast cancer worldwide and 685,000 deaths were recorded (Breast Cancer Research Foundation, 2020). Breast cancers can be classified into various types based on their location in the breast; common ones include ductal carcinoma in situ (DCIS) (which begins in the lining of the milk ducts), invasive lobular carcinoma (which begins in the lobules of the breast), while others like phyllodes tumors (which starts in the connective tissue of the breast) and angiosarcoma (forms in the lymph lining or blood vessel) are less common. Breast cancers also have subtypes with are classified based on the receptors they express. Different genes are associated with breast cancer, and they include; BRCA1 and 2, ATM, TP53, CHEK2, PTEN, CDH1, STK11 and PALB2. These genes regulate activities in the body; mutation in any of them can potentially lead to breast malignancy. Treatment of breast cancer

depends on how far advanced the cancer stage is and what type of cancer is present. The treatment options for breast cancer include surgery, radiation, chemotherapy, hormonal therapy and gene therapy. Some of these treatments have complications and shortcomings associated with them such as pain and scarring, nerve damage, lymphedema (Pierce, 2019), hair loss and cost of treatment. Due to the shortcomings of these treatment options for breast cancer, alternative methods which involve the use of medicinal plants and herbs, have now been adopted for the potential treatment and management of breast cancer since they are known to have minimal side effects. Medicinal plants and herbs possess a large variety of active phytochemicals like carotenoids, flavonoids, ligands, polyphenolics, terpenoids, sulfides, lignans and plant sterols. Recent studies have shown that these secondary metabolites present in the extract of certain plant species can suppress cancer cells through DNA damage and initiation of apoptosis-inducing enzymes (Khan et al, 2019). However, according to Bhattachary et al., 2021, at imprecise concentrations these phytochemicals could be toxic and elicit a negative effect (Bhattacharya, et al., 2021) on normal cells. *Pistia stratiotes* commonly known as water lettuce are of medicinal value. A phytochemical screening done on the leaves of *P. Stratiotes* showed the presence of secondary metabolites which are of anticancerous, antimicrobial, antioxidant, antidandruff, antiproliferative and anti-inflammatory properties (Tyagi and Mala, 2017). This study evaluates the status of the BRCA2 gene in the presence of *Pistia stratiotes* in female wistar albino rats induced with methyl nitrosourea (MNU). BRCA2 is a tumor suppressor gene and mutation in this gene can increase the risk of breast cancer developing in women (Sanghamitra, et al., 2018). In this research, methyl nitrosourea used is known to be an alkylating agent with direct carcinogenic effect (Minari and Okeke, 2018) capable of damaging DNA and causing cancer. This study was carried out to reveal the potential of *Pistia stratiotes* in preventing damage to the BRAC2 gene caused by methyl nitrosourea induced in female albino rats.

Methods

Collection of plant material and authentication

Leaves of *Pistia stratiotes* were collected from the lagoon front of the University of Lagos, Lagos State (Figure 1). The leaves were identified and confirmed taxonomically at the Department of Botany, University of Lagos, Nigeria and voucher number of 8945 was allocated to the plant.

Plant preparation and extraction

10g of coarsely powdered *P. stratiotes* was placed in a stoppered glass container with a solvent and allowed to sit at room temperature for 48 hours with frequent agitation until the soluble matter dissolved. The mixture was then strained, and filtered by using a mesh of 200mm; the marc (the damp solid material) was pressed with a muslin cloth. The combined liquids were clarified by filtration or decantation after standing. The concentrated extract was formed using a rotary evaporator at 45 °C, which after was poured into an evaporating dish to dry completely into a paste.

Animal purchase, experimental design grouping and treatment

In this study, 25 female wistar rats 21 days old weighing between 30-60g, were divided into five groups namely, A, B, C, D and E. Each groups had a total of 5 rats. Group A (negative control) was induced with MNU only, Group E (positive control) were neither induced with MNU nor treated with extract, other groups B, C and D were induced with MNU and administered different concentrations of the plant extract. Groups A, B, C and D underwent acclimatization for 2 weeks before 6 weeks of their induction and respective treatment.

Group A (Negative Control): Rats received 0.1 mg/ml/week of MNU only
Group B: Rats received 0.1 mg/kg/week of MNU + 0.2 mg/ml/day of extract
Group C: Rats received 0.1 mg/kg/week of MNU + 0.1 mg/ml/day of exract
Group D: Rats received 0.1 mg/kg/week of MNU + 0.15 mg/ml/day of extract
Group E (Positive Control): Rats received food(pellet) and tap water only

N-Methyl-N-Nitrosourea (MNU) was administered at the beginning of the sexual maturity of the experimental animals (42-84 days of age). The MNU was administered at the intraperitoneal region (lower abdomen) of the rats.

Figure 1 *Pistia stratiotes*

Source Tyagi and Mala, 2017

Qualitative and quantitative phytochemical screening of *Pistia stratiotes* leaves

Test for Alkaloids: A quantity (3 ml) of the concentrated extract was placed in a test tube and 1 ml HCl was added to it. The mixture was gently heated for 20 min, cooled and filtered. The filtrate was then analyzed using Wagner test**.** 1 ml of the extract was treated with Wagner's reagent; the formation of brown-reddish precipitate indicated the presence of alkaloids. The alkaloid content was calculated and expressed as a percentage of the weight of the sample analyzed.

Test for Saponins: 2 ml extract was mixed with 2 ml of distilled water and then agitated in a test tube for 10 minutes. The formation of foam indicated the presence of saponin. The saponin content was calculated and expressed as in percentage.

Test for Steroids: 1 ml extract was dissolved in 1 ml of chloroform and an equal volume of concentrated H_2SO_4 acid was added from the side of the test tube, the upper layer turns red and the H_2SO_4 layer showed yellow with green fluorescence. This indicated the presence of steroids. The percentage of steroids was evaluated.

Test for Flavonoid: 0.5g of an extract with 3 ml of water was boiled, and filtered. The filtrate was treated with 10 % NaOH solution. The formation of intense yellow colour indicated the presence of flavonoid, which turned colourless on the addition of dilute HCl. The residue is weighed and the flavonoid content is calculated and expressed in percentage.

Test for reducing sugars: 0.5g of an extract with 3 ml of water was boiled, and filtered. The filtrate was treated with 2 ml of Fehling's solutions A and B. The formation of a red precipitate indicated the presence of reducing sugar. The percentage of reducing sugar was evaluated.

Test for Tannins: 0.5g of the extract with 3 ml of water was boiled, and filtered. The filtrate was treated with 2 drops of 10% $FeCl_3$. The Formation of green, brown, or blue-black precipitate indicated that tannin is present. The tannin content was calculated and expressed as in percentage.

Test for Glycosides: Using Keller-Killani test, 1 ml of aqueous extract was added with 1 ml glacial acetic acid containing a drop of $FeCl_3$ and 1 ml of concentrated H_2SO_4. A brown colour ring at the interface indicated the presence of glycosides. The percentage of glycosides was evaluated.

Test for Terpenoid: 1 ml extract was dissolved in 1 ml of chloroform and an equal volume of concentrated H_2SO_4 acid was added from the side of the test tube, upper layer turned greenish and the H_2SO_4 layer showed yellow with green fluorescence. This shows terpenoid was present. The terpenoid content was calculated and expressed in percentages.

Test for Anthraquinones: 5 ml of extract was hydrolyzed with dilute H_2SO_4, then 1 ml of benzene and 1 ml of NH_3 were added. The formation of rose pink colour suggested the presence of anthraquinone. The percentage of anthraquinones was evaluated.

DNA extraction and purification

The total DNA from the female albino rats in the different groups was extracted using Quick-DNA™ Miniprep Plus Kit (Inqaba Biotec, Ibadan, Nigeria). A NanoDrop spectrophotometer was used to check for the purity and concentration of the DNA samples extracted. At 260/280 ratio of absorbance, the purity of the DNA samples was measured.

Polymerase chain reaction (PCR) and amplification of the BRCA2 gene

The BRCA2 gene was amplified using a designed BRCA2 primer, which comprised a forward and reverse primer. A PCR master mix consisting of PCR buffer, dNTPs, $MgCl_2$ and DNA polymerase was added with the primers and DNA samples to catalyze the amplification. The sample mixture for the PCR was then loaded into a Thermocycler. The following parameters were used for the amplification of the BRCA 2 gene; first denaturation step at 95˚C for 5 min, followed by 40 cycles of denaturation at 95˚C for 30 sec, annealing at 60˚C for 30 sec, extension at 72˚C for 30 sec and final extension was accomplished at 72˚C for 5 min.

Oligonucleotide primer

The primer of interest used in this study is the BRCA2 gene primer. The forward sequence of the primer is (5'-CAAAGAATTAGGGAATGTTGTTGC-3') and the reverse sequence (5'-CACTTCAACTCCCAGACTTTCA-3') (Amir et al, 2019).

Agarose gel electrophoresis

After PCR the amplicons were analyzed using gel electrophoresis with 2% agarose at 70 V for 1 hour. The bands formed at the end of the electrophoresis process were viewed using an ultraviolent light (UV) in a trans-illuminator and a photograph of the illuminated gel was taken.

Results and discussion

Qualitative and quantitative phytochemical screening of *Pistia stratiotes* leaves

The qualitative screening of *Pistia stratiotes* leaves revealed the presence of alkaloids, saponins, flavonoids, tannins, reducing sugars, glycosides, steroids, terpenoids and the absence of anthraquinone (Table 1). In the quantitative phytochemical analysis, terpenoid was found to be present in the highest amount (9.05 in 25 ug/ml of extract) while tannin was found to be the lowest (1.09 in 25 ug/ml of extract) (Table 1). The presence of alkaloid, saponin, flavonoid, and glycoside in the result of the phytochemical screening of *Pistia stratiotes* of this study (Table 1) agrees with the findings of Hossan, Khan and Uddin, 2018 (Hossain et al, 2018) who reported their presence as phytochemicals of *Pistia stratiotes* plant. The presence of tannin and steroid corresponds with the findings of Tyagi, 2017 (Tyagi and Mala, 2017).

Effect of Methyl Nitrosourea (MNU) on the female albino

A tumor on the neck region of a female albino rat from group A (Negative control) was seen on the eighth week of induction with the carcinogen, MNU (Figure 2). The inflamed growth seen on the neck of one of the albino rats of group A (negative control rats that received 0.1 mg/kg/week of MNU only) in Figure 2 appears to look like a tumor. The inflamed growth is most likely a result of the methyl nitrourea (MNU) this group was induced with since MNU is known to possess carcinogenic properties (Minari and Okeke, 2018) capable of causing tumor and aberrant growth.

Polymerase Chain Reaction (PCR) amplification of the BRCA2 gene

The BRCA2 gene amplified from group A DNA samples (negative control group that received 0.1mg/kg/week of MNU only) appeared smaller in band compared to the BRCA2 gene of group E (positive control group that received food and tap water only) (Figure 3). The BRCA 2 gene band of the DNA samples from each groups treated with different concentrations of *Pistia stratiotes* extract in group B, group C, and group D appeared wider and slightly curved when compared with the bands of the negative control group (group A) (Figure 3). However one band from group D, sample D3 (the group that received 0.1 mg/kg/week of

MNU + 0.15 mg/ml/day of extract) appeared similar to the BRCA2 gene bands in the positive control group (group E) (Figure 3).

Table 1 Qualitative and quantitative phytochemical screening of *Pistia stratiotes* leaves

Test	Qualitative	Quantitative (25 µg/ml)
Alkaloid	+	4.07
Saponin	+	8.19
Flavonoid	+	1.21
Tannin	+	1.09
Reducing sugar	+	4.73
Glycoside	+	7.47
Steroid	+	6.18
Terpenoids	+	9.05
Anthraquinone	-	0.00

Key (+) presence of phytochemical (-) absence of phytochemical

Figure 2 Inflamated growth on the neck region of mnu-induced albino rat from group A (negative control group)

Group A (Negative Control): Rats received 0.1 mg/kg/week of MNU only

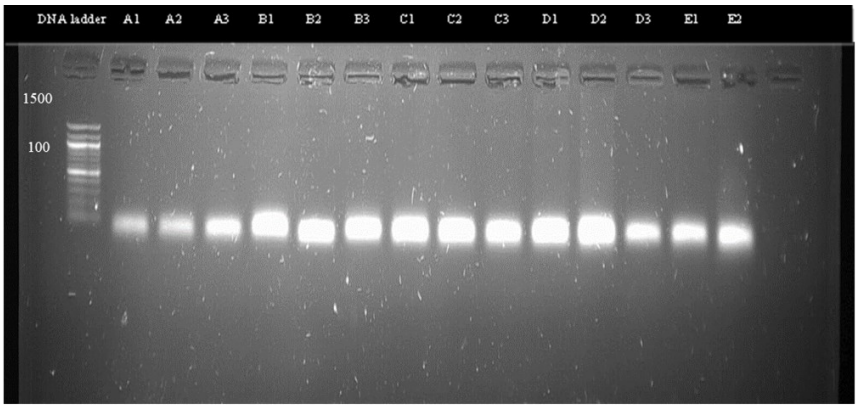

Figure 3 Gel electrophoresis pattern of BRCA2 gene in DNA sample of the female albino rats in each group amplified through PCR

Group A (Negative Control): Rats received 0.1 mg/ml/week of MNU only

Group B: Rats received 0.1 mg/kg/week of MNU + 0.2 mg/ml/day of extract

Group C: Rats received 0.1 mg/kg/week of MNU + 0.1 mg/ml/day of extract

Group D: Rats received 0.1 mg/kg/week of MNU + 0.15 mg/ml/day of extract

Group E (Positive Control): Rats received food(pellet) and tap water only

The gel electrophoresis pattern of the BRCA2 gene in the DNA samples of the female albino rats in each group amplified through PCR in Figure 3 confirms the amplification of the BRCA2 gene in the samples. The dissimilarities in the appearance of the bands of the BRCA2 gene amplified from group A DNA samples (negative control group that received 0.1 mg/kg/week of MNU only) when compared with the BRCA2 gene bands of group E (positive control group that received food and tap water only) implies that there may have been damage on the BRCA2 gene of group A (negative control) caused by methyl nitrosourea (MNU) that it was induced with since MNU is a powerful carcinogenic agent (Minari and Okeke, 2018) capable of causing DNA damage (Figure 3). The BRCA2 gene band of the DNA samples from the groups induced with MNU and treated with different concentrations of *P. stratiotes* extract, group B, group C, and group D differed in size and shape when compared with the bands of the negative and positive control group, except one band from group D, sample D3 (the group that received 0.1 mg/kg/week of MNU + 0.15 mg/ml/day of extract), which appeared similar to the positive control BRCA2 gene band (Figure 3). These similiarities between D3 and the positive control BRCA2 gene band could have resulted from the secondary metabolites present in the aqueous extract of *P. stratiotes* such as flavonoids, alkaloids, glycosides and tannins which are known to target cancer at apoptotic sites, genetic mutations and DNA damage through well-regulated and intricate pathways (Akhtar et al., 2020). This suggests that DNA damage was completely prevented in D3 as compared to the other groups receiving different concentration of the plant extract. Also, the concentration of the plant extract given to group D being in an optimum amount may have been a contributing factor. The phytochemical screening in this study revealed that the mentioned phytochemicals were present in the aqueous extract of the plant (Table 1).

Conclusion

The present study showed that an aqueous extract of *Pistia stratiotes* (water lettuce) has the potential to prevent damage to the BRCA2 gene in MNU-induced breast cancer female albino rats. However, further

studies must be carried to evaluate the active compounds present in *P. stratiotes* extract responsible for its preventive potential against damages to the BRCA2 gene and the optimum dosage that the extract should be administered at in future therapeutics against breast cancer and other forms of cancer using medicinal plants.

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