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Anticancer Effect of *Adenopus breviflorus* (Roberty) Extract Fruit on Human Brain Tumour (U-1242) Cells

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Abstract

This study was designed to investigate the effect of ethanol extract of *Adenopus breviflorus* fruit on human brain tumour (U-1242) cells. Cytotoxicity assay was carried out using the Trypan blue dye exclusion assay and Gentian crystal violet assay. Various concentrations of ethanol extract of *Adenopus breviflorus* (EEAB) i.e. 62.5, 125 and 250 µg/ml were prepared from stock solution. The concentration of reference standard #4 (250 µg/ml) was also prepared from stock solution. The effect of EEAB on mean cell count of U-1242 was done using hemocytometer and the percentage cell viability was determined. The physical cytotoxic effect of EEAB on U-1242 cells was done using Gentian crystal violet staining method. Treatment of U-1242 cells with the reference standard #4 and extract caused significant ($p<0.05$) reductions in the mean cell counts and percentage cell viability of viable U-1242 cells relative to the DMSO treated cells. The untreated control U-1242 cells and the DMSO (1.0 µg/ml) treated U-1242 cells showed confluence, while the standard #4 (250 µg/ml) and EEAB (125 and 250 µg/ml) treated U-1242 cells showed great reduction in cell population and cell density. It can therefore be concluded that ethanol extract of *Adenopus breviflorus* fruit probably has cytotoxic effect against human brain tumor (U-1242) cells.

Keywords: *Adenopus breviflorus*, Human brain tumour (U-1242), Cytotoxicity, Mean cell count.

INTRODUCTION

Cancer is a group of diseases involving abnormal cell growth with the potential to invade or spread to other parts of the body [1]. These contrast with benign tumors, which do not spread to other parts of the body. Possible signs and symptoms include a lump, abnormal bleeding, prolonged cough, unexplained weight loss and a change in bowel movements. While these symptoms may indicate cancer, they may have other causes. Over 100 types of cancers affect humans [2].

Among the medicinal plants that are in current use in Nigeria in the treatment of cancer is the fruit of the plant called *Adenopus breviflorus*.

Adenopus breviflorus belongs to the family of Cucurbitaceae. It is commonly called Wild colocynth in English language, "Ogbenwa" in Ibo language and "Tagiri" in Yoruba language [3]. It is a perennial tendril climber. It would usually lie on the ground for want of something to climb and climbs over shrubs and herbs by means of axillary tendrils. The leaves are simple, alternate and palmately veined [4].

Medicinally, the plant is used as a purgative in Tanganyika as well as a vermifuge and cathartic in Nigeria [3]. A decoction from the plant is said to be used in Nigeria for headache [3]. It is used in West Africa for a wide range of gastrointestinal disorders and measles in man. In southern Nigeria, its seed-decoction is reportedly given to pregnant women but the purpose is not stated [5]. It is used as an anticonvulsant, sedative and pain killer [6]. It is used with other medicinal plants as concoctions to aid parturition in humans [7]. Livestock farmers employ the fruit extract of the plant for the treatment of Newcastle disease and coccidiosis in animals [7]. The fruit is also used for money-making charms by Yoruba herbalists of South-Western Nigeria because of the cowrie-like inscriptions on its body [8].

Pharmacologically, it has been reported that the methanol extract of its whole fruit has anti-implantation activity [9] and abortifacient activity [10]. The ethanol extract of its whole fruit has been reported to have a broad spectrum antibacterial activity [11] as well as anti-oxidant and anti-ulcerogenic effects [12]. Its ethanol extract has been reported to have a little toxic and a lot of beneficial effects on the hematological functions and blood chemistry of male Wistar rats [13].

Since *Adenopus breviflorus* fruit has been reported by herbalists to have anticancer activity, this study therefore aims to authenticate the veracity of these claims on human brain tumour
(U-1242) cells.

MATERIALS AND METHODS

Cell culture

Human brain tumor cells (Glioblastoma multiforme) (U-1242) were supplied by the Cancer Research Laboratory, Faculty of Pharmacy, University of Maiduguri, Nigeria. They were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium. The media were supplemented with 10% fetal bovine serum (FBS), glutamine (2 mM), penicillin (100 units/ml) and streptomycin (100 g/ml). The cultures were maintained in a humidified 5% CO_2 incubator at 37 ^{0}C and the cells were sub-cultured every 3–4 days (with a solution of 0.05% trypsin-0.02% EDTA upon reaching 80% confluence) to maintain logarithmic growth and were allowed to grow for 24 hours before use.

Subculture of adherent cell line (U-1242)

Cultures were viewed using an inverted microscope (Amscope, USA) to assess the degree of confluency and the absence of bacterial and fungal contaminants was confirmed. The T-25 flask was placed in laminar flow and the spent cultured medium was discarded with a sterile

Pasteur pipette. The media was washed three times with PBS to remove the serum (because without washing off the serum the trypsin will not be active) by adding 2 ml of PBS to the side of the T-25 flask opposite the cells to avoid dislodging the cells. Then, the cells were rinsed and the rinse was discarded afterwards. A total volume of 2 ml of trypsin-EDTA solution was added to the cells, ensuring the monolayer is completely covered. The T-25 flask was incubated for 2-5 minutes. The cells were examined using an inverted microscope to ensure that all the cells were detached and floated. The cells were aspirated using a micropipette into a centrifuge tube (50 ml) and fresh media was added into the centrifuge tube to inactivate the trypsin. The centrifuge tube containing the cells was centrifuged at 1000 rpm for 5 minutes. About 100-200 µl was removed to perform a cell counting and staining. The required numbers of cells were transferred to a new labelled flask containing pre-warmed media and incubated for the cancer cell line (U-1242).

Plant Material

Fresh samples of *Adenopus breviflorus* fruit were bought in Bodija Market, Ibadan, and were authenticated in the Taxonomy Unit of the Forestry Research Institute of Nigeria (FRIN), Jericho, Ibadan where a voucher specimen (FHI 108336) was deposited in their Herbarium.

Preparation of Crude Ethanol Extract

Large quantity (7.5 kg) of fresh specimens of the whole fruit of *Adenopus breviflorus* were washed free of debris and pulverized using mortar and pestle and air-dried for eight weeks. The resultant dried specimens (300 g) were macerated and extracted with 70 % ethanol for 72 hours at room temperature (26 - 28 ^{0}C). The resulting solution was then filtered using a wire-gauze and a sieve with tiny pores (0.25 mm). The 70 % ethanol was later evaporated using steam bath (40 – 45 ^{0}C) to give a percentage yield of 8.6 % of the starting sample. The dried sample was reconstituted in distilled water to make up test solutions of known concentration.

Experimental Design

Cytotoxicity assay

(a) Cell viability assay (Trypan blue dye exclusion assay)

It is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue, whereas dead cells do not. Trypan blue is a blue acid dye that has two azo chromophores group. The percentage of viable and non-viable cells was determined using trypan blue exclusivity stain. The cell line (U-1242) was grown at 37 °C at humidified 5% CO_2 in RPMI 1640 medium. The RPMI 1640 medium was filtered using 0.45 µm membrane filter. The extract (250 mg) put in 1.5 ml sterile Eppendorf tube was dissolved in 1.0 ml DMSO using a vortex (Thermolyne) and then filtered using a sterile micron filter (0.22 µm). Various concentrations of the extract (EEAB) i.e. 62.5, 125 and 250 µg/ml were then prepared from the stock solution. The concentration (250 µg/ml) of the reference standard #4 was also prepared from the stock solution. Untreated control cancer cells (U-1242) were seeded at a density of 2×10^4 cells/well into three wells (triplet) in a 24-well plate and incubated in culture medium i.e. RPMI 1640 medium only. Treated cancer cells (U-1242) were also seeded at a density of 2×10^4 cells/well into three wells (triplet) in a 24-well plate and they were treated with DMSO (1.0 µl/ml), reference standard #4 (250 µg/ml) and different concentrations of the extract (62.5, 125 and 250 µg/ml) and incubated at 37 °C for 48 hours at 5% CO_2. After 48 hours, cells were washed three times with PBS to remove dead cells, floating cells and serum. The adherent cells were released from the plates with 0.05% w/v trysin-EDTA (30 to 60 µl/well) and incubated for 2 minutes and the trypsin activity was halted by the addition of equal volume (30 to 60 µl/well) of serum-free RPMI 1640 medium. The cancer cells were then transferred into the centrifuge tubes and centrifuged at 1000 rpm for 5 minutes. After centrifuging, PBS (0.25 ml) was added and then 0.4% w/v Trypan blue (0.25 ml) was also added and mixed well using micropipette.

Fix the coverslip on the centre top of the hemocytometer, the notches should be partially exposed. Using a micropipette, 20 µl of the suspended cell/Trypan blue solution was dispensed into the notches of the hemocytometer. The suspended cells/Trypan blue solution would pass under the coverslip by capillary action unless there is an air bubble. Make sure the notches are not overfilled and that the coverslip is not moved once it is place on the grid and the suspended cells/Trypan blue solution is added. The hemocytometer assembly was placed on the stage of a phase contrast inverted microscope and then focus, the power of the microscope was adjusted on the hemocytometer grid until a single counting square fills the field. The observed live cells were clear in colour while the dead cells were blue in colour (Stained cells were the dead cells and unstained cells were counted as viable cells).

There are four main quadrants in haemocytometer and they are subdivided into 16 squares. For each experiment (per well), cells were counted from the four quadrants of the hemocytometer.

The percentage cell viability was determined by using the following formula:

$$\% \text{ Cell Viability} = \frac{\text{Total number of extract treated viable cells} \times 100}{\text{Total number of DMSO treated viable cells}}$$

(b) Crystal violet assay

The cytotoxicity was also evaluated by the crystal violet staining method. The crystal violet assay is designed to obtain quantitative information about the relative density of adherent cells [14]. The crystal violet assay stains the nuclei of viable adherent cells.

After incubating the untreated control cancer cells, the DMSO treated cells, the reference standard #4 treated cells and the extract treated cells for 48 hours in 24-well plates as explained in (a) above, the media were aspirated with a Pasteur pipette and subsequently washed two times gently with distilled water to remove floating dead cells.

Thereafter, 0.25 ml of 5% w/v Gentian crystal violet dye was added to each well for about 30 minutes. The stain was removed and the plates were rinsed using distilled water and air-dried and the cells were subsequently photographed using a digital camera at x100 magnification.

Statistical Analysis
Data were expressed as mean ± S.E.M (Standard Error of Mean). Comparison between the control and experimental groups were done using one - way analysis of variance (ANOVA) with Duncan's multiple range test using SPSS (IBM, Armonk, NY) software (version 23). Differences were considered statistically significant at $p<0.05$.

RESULTS

Effect of Treatment on Mean Cell Count and Cell Viability
Treatment of U-1242 cells with the reference standard #4 and the extract (125 and 250 µg/ml) caused significant ($p<0.05$) reductions in the mean cell count and percentage cell viability of viable U-1242 cells in a concentration-dependent manner relative to the DMSO treated cells (Figures 1 and 2).

Effect of Treatment on Cell Viability
The untreated control U-1242 cells and the DMSO (1.0 µl/ml) treated U-1242 cells show confluent cells (large cell number and high cell density) while the standard #4 (250 µg/ml) and EEAB (125 and 250 µg/ml) treated U-1242 cells show reduction in cell number and cell density (Plates I, II, III and IV).

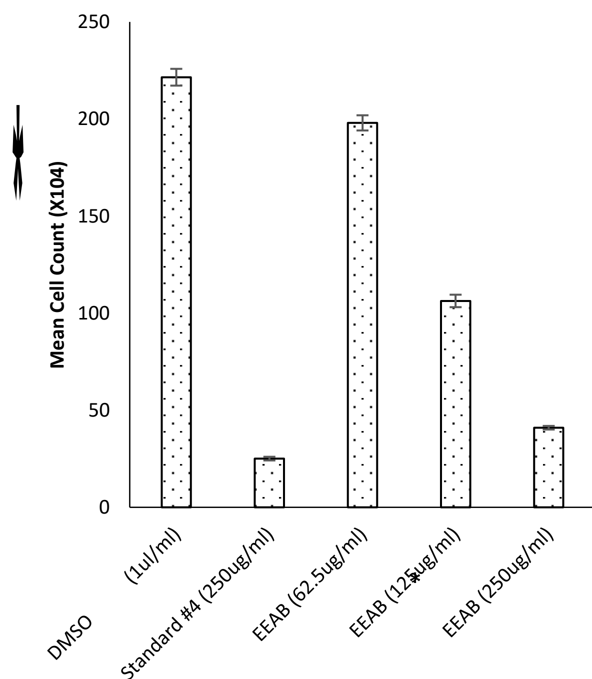

Figure 1: Effect of different concentrations of ethanol extract of *Adenopus breviflorus* (EEAB) on mean cell count of U-1242 cells.
Data presented as mean ± S.E.M. (n=12), *$p<0.05$.

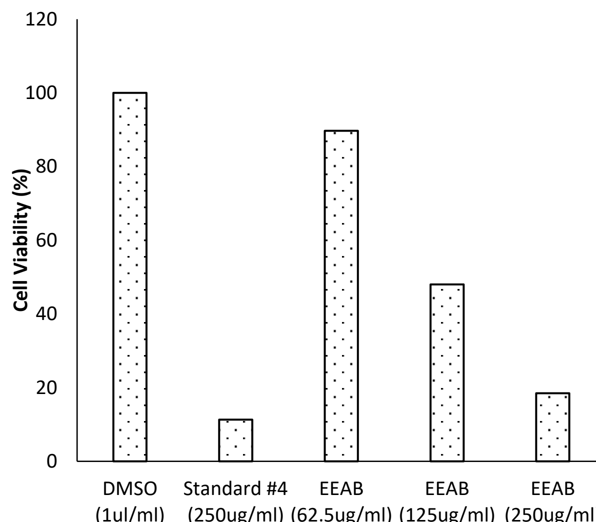

Figure 2: Effect of different concentrations of ethanol extract of *Adenopus breviflorus* (EEAB) on pencentage cell viability of U-1242 cells.

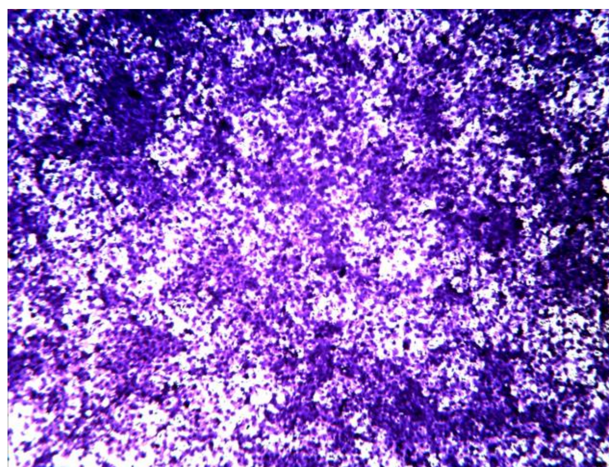

Plate I: Untreated control U-1242 cells stained with Gentian crystal violet dye (x100).
Photomicrograph showing large cell population and high cell density.

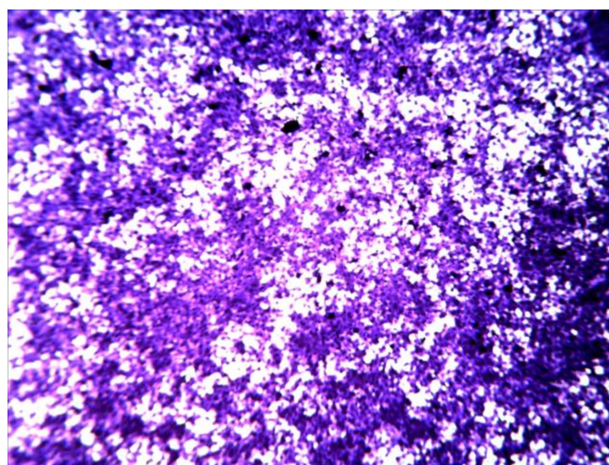

Plate II: DMSO (1.0 µl/ml) treated U-1242 cells stained with Gentian crystal violet dye (x100).
Photomicrograph showing large cell population and high cell density.

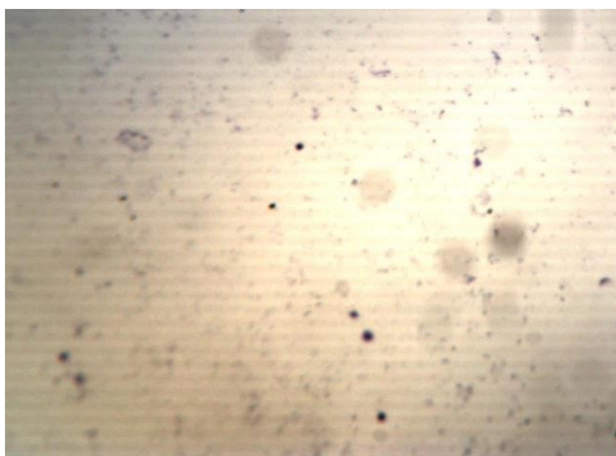

Plate III: Standard #4 (250 µg/ml) treated U-1242 cells stained with Gentian crystal violet dye (x100).
Photomicrograph showing reduction in cell population and low cell density.

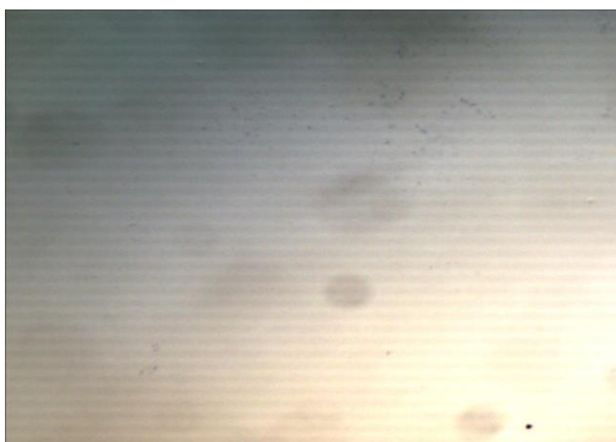

Plate IV: Ethanol extract of *Adenopus breviflorus* (250 µg/ml) treated U-1242 cells stained with Gentian crystal violet dye (x100).
Photomicrograph showing great reduction in cell population.

DISCUSSION

This study was embarked upon to see the anticancer effect of ethanol extract of *Adenopus breviflorus* fruit on human brain tumour (glioblastoma multiforme) (U-1242) cells.
Globally, natural plant compounds have attracted attention as alternative therapeutic strategies in the fight against diseases, primarily because of their low toxicity and high therapeutic index [15]. Many existing and contemporary drugs in clinical use are derived from the natural plants [16]. The resistance of cancer cells to current available chemotherapeutic agents and the toxic side effects of these agents have prompted scientists to further investigate the therapeutic potential of natural agents. Even though many extracts from plants are being investigated for their potential biological activities, it is increasingly evident that the efficacies of these products require their presence in a mixture of complex molecules rather than in a pure single state [17].
Cancer and other chronic diseases share some common pathogenic mechanisms, such as DNA damage, oxidative stress, and chronic inflammation. These diseases can be controlled by resistant to mutagens/carcinogens and/or to inhibit progression of the disease by administering chemopreventive agents [18]. Chemotherapy and surgery are standard methods for treatment of these diseases, although not been fully effective. Most of the anti-tumor drugs currently used in chemotherapy are toxic to normal cells and cause toxicity for immune cells. So, it is important to minimize curing doses to the least amount possible as well as trying to minimize the side effects of these drugs. Therefore, the identification of new anticancer drug with low side effects on the immune system has become an essential goal in many studies of immunoanatomy [18].
Determination of cytotoxicity, commonly used to evaluate the biological activity of natural products, is helpful to confirm whether plant extracts have potential antineoplastic properties [19]. In this study, the ethanol extract of *Adenopus breviflorus* was investigated for its cytotoxic activity against human brain tumor (U-1242) cell line with #4 serving as reference standard using Trypan blue exclusion and Gentian crystal violet assays.
The results have shown that the extract possess a significant cytotoxic activity against the brain tumor (U-1242) cells by significantly reducing the mean cell count and percentage cell viability of brain tumor (U-1242) cells in a concentration-dependent manner. Similar result was reported by [20] in glioblastoma treated with hispolon. Brain tumor (glioblastoma multiforme) is the most common and aggressive primary CNS tumor with a median survival of 15 months and an average incidence rate of 3.19 in every100,000 population [21]. Glioblastoma multiforme cells are highly proliferative and diffusely invade surrounding brain structures, thereby making complete surgical resection practically impossible [22]. Furthermore, majority of glioblastoma multiformes are intrinsically resistant to most forms of radiation and chemotherapy, thus rendering the standard arsenal of anticancer treatments rather ineffective [23]. The failure of the conventional therapies for glioblastoma multiforme to target tumor cells exclusively, make their efficacy limited by non-specific toxicities [24]. Therefore, killing tumor cells efficiently and with less toxic effect is the ultimate target of glioblastoma multiforme chemotherapy and will require the adoption of targeted therapeutic approach as against the deployment of cytotoxic arsenal [25].
Alkaloids, tannins and flavonoids have been reported to possess anticancer activities [26, 27, 28, 29]. It is therefore plausible to suggest that these phytochemical constituents found to be present in the extract [30] may be responsible for its anticancer activity.
The anticancer activity of this extract could also be due to apoptosis since it has been reported that the extracts of *Adenopus breviflorus* are capable of induction of programmed cell death (apoptosis) through the modulation of mitochondrial membrane permeability transition (MMPT) pore signalling pathway [31]. Apoptosis is a hallmark for the appraisal of potential agents for cancer prevention, and a wide variety of natural products have been known to interfere with cell proliferation or induce apoptosis [32]. The morphological characteristics of

apoptosis include membrane blebbing, cell shrinkage, chromatin condensation and apoptotic body formation [33].

The anticancer activity of this extract could be due to DNA damage. DNA fragmentation is a canonical biochemical apoptotic feature. Many plants extracts have been shown to induce DNA damage [34]. DNA damage often involves several signaling pathways, which can also result in cell cycle arrest and eventual apoptosis [35]. DNA damage activates P53 to induce G1 and G2/M phase arrest and apoptosis [36].

The anticancer activity of this extract could be due to increasing intracellular reactive oxygen species (ROS) levels. Excessive amounts of ROS can cause DNA damage, cell cycle arrest and apoptosis [37]. Although all aerobic cells are equipped with protective enzymatic and non-enzymatic antioxidants, increased oxidative stress may overwhelm the protective mechanisms, leading to cell injury [38]. Many natural compounds, such as flavonoids and triterpenoids, have been reported to increase oxidative stress by increasing intracellular ROS levels [39]. Thus, ROS are considered an important factor of natural anticancer agents. Cells are known to thrive in low levels of ROS, but a relative increase in ROS induces cell cycle arrest and apoptosis [40]. ROS-modulating drugs are, however, being proposed as therapeutic strategies to selectively target the destruction of cancer cells [41].

The anticancer activity of this extract could be due to stimulation of an increase in membrane lipid oxidation and induction of phosphorylated-p53 and p21 expression in brain tumor (U-1242) cells. The p53 tumor-suppressor gene does not function properly in most human cancers [42]. In brain cancers, p53 can be inactivated through amino acid-changing mutation in the DNA-binding domain and/or deletion of the $p14^{ARF}$ gene, and in cervical cancers, p53 is inactivated through viral infection [43]. Phosphorylation of p53 by kinases, caused by DNA damage, leads to p53 activation [44]. Phosphorylation of p53 can also occur in response to oxidative stress through the platelet-derived growth factor β receptor (PDGFβ)-mediated ataxia telangiectasia mutated (ATM) kinase activation or direct ATM activation by oxidative stress [45]. One of the first consequences of p53 activation is cell cycle arrest through the p53-dependent expression of $p21^{WAF1/CIP1}$, an inhibitor of cyclin-dependent kinases (CDKs) [46].

Upregulation of p53 and p21 were found to be induced by lipid peroxidation in previous studies: lipid peroxidation increase was associated with p53 mRNA increase in a rat model [47], lipid peroxidation product from increased ferrous iron level in lysosomal compartment triggered upregulation of p53 [48], lipid peroxidation product sensitizes cells to UV-induced killing by inhibiting nucleotide excision repair and forming a peroxide-DNA adduct at codon 249 of the p53 gene [49], and that hydrogen peroxide induced lipid peroxide production increased p21 expression [50].

The anticancer activity of this extract could be through the downregulation of activated phosphorylated-AKT (AKT is also known as protein kinase B, PKB), which causes cell cycle arrest and initiation of apoptosis leading to cancer cell death. The AKT/PKB (protein kinase B) kinases play important roles in signaling pathways that regulate cellular processes controlling cell proliferation, survival, and genome stability [51]. Hyperactivation of the AKT pathway was implicated in many types of human cancer and dominantly inherited cancer syndrome. AKT phosphorylates and inactivates the pro-apoptotic factors BAD (Bcl-2-associated death promoter) and procaspase-9 [51]. In a pro-cell cycle progression mechanism involving p53, AKT promotes the phosphorylation and translocation of Mdm2 into the nucleus, where it downregulates p53, which antagonizes p53-mediated cell cycle checkpoints [52]. AKT directly antagonizes the function of the cell cycle inhibitors $p21^{WAF1}$ and $p27^{Kip1}$ by phosphorylating a site located near the nuclear localization signal to induce cytoplasmic retention of these cell cycle inhibitors [53]. Some investigations have shown elevated AKT activity to be highly prevalent in high grade, late stage and/or metastatic tumors, and several reports have linked AKT activation with reduced patient survival or tumor radio-resistance [54].

In conclusion, these findings have lent some credence to the ethnomedicinal use of the *Adenopus breviflorus* in folklore medicine for the management of cancer. Its therapeutic potential is huge and can be used as alternative to or supplementation for the various therapies currently used in the treatment of brain tumour. On the basis of this study, *Adenopus breviflorus* fruit may serve as an excellent lead for the development of anticancer agent for brain tumor which can be further investigated for pharmaceutical applications and achievement of novel anticancer compounds.

Considering these findings on the effect of ethanol extract of *Adenopus breviflorus* fruit on human brain tumour (glioblastoma multiforme) (U-1242) cells, it is recommended that people suffering from brain tumour could consume *Adenopus breviflorus* fruit as an alternative to or supplementation for the various therapies currently used in the treatments of brain tumour.

Conflict of Interest

We vehemently declare that there is no conflict of interests in this research work.

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