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Screening Of Bismillah Leaf *(Vernonia Amygdalina)* Extraction For Antiproliferative Activies In Human Glioblastoma Brain Cancer Cell Lines

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ABSTRACT

Vernonia amygdalina is a member of the *Asteraceae* family. It is a shrub or small tree that can reach up to 10 m in height and with a trunk diameter reaching up to 40 cm. The plant is a vegetable in its native land and is put through a detoxification process before it is cooked as food. The process involves boiling the leaves several times while changing the water after each cycle until the bitterness disappears. This study was carried out to screen the ethanol, methanol and ethyl acetate extracts of *Vernonia Amygdalina* as an anti-proliferative agent that inhibits the growth of human glioblastoma brain cancer cell lines (U-87). The anti-proliferative study indicated that only ethyl acetate extracts from *Vernonia amygdalina* were found to be cytotoxic towards U-87 cancer cell lines with an IC_{50} value of 18.80 ± 1.11 µg/ml. There was no IC_{50} value for ethanol and methanol extracts from *Vernonia amygdalina.* Thus, ethyl acetate extracts from *Vernonia amygdalina* could be envisaged for further exploration in efforts to develop newer and safer cytotoxic compounds.
Keywords: *Vernonia amygdalina,* Antiproliferative, Cancer cell, Ethyl acetate, U87 cell line

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INTRODUCTION

Cancer is the second leading cause of death and is becoming the leading cause among those in the old age group. It is estimated that by 2030 the number of new cancer cases will increase by 70% worldwide due to demographic changes alone [1]. The process of cancer development is a consequence of genetic and epigenetic alterations that lead to disruption of basic biological functions [2]. Among all the possible strategies, prevention appears to be the most practical approach for reducing cancer incidence and burden. Vegetable and fruit consumption is inversely associated with cancer incidence and mortality [3]. Currently, interest in a number of fruits high in polyphenolic compounds has been raised due to their reported chemo-preventive and/or chemo-therapeutic potential [2,4].

Glioblastoma multiforme (GBM) is one of the most devastating cancers with a mean survival rate of twelve to fifteen months [5]. Current therapy includes a combination of radiation, surgery and chemotherapy. In addition, the rate of relapse after surgical resection of the tumour is very high [5]. The main chemotherapy available is *temolozolamide*, which is quite expensive and has a high level of toxicity. Thus, the scarcity of therapeutic options and the need to improve the quality of life of patients requires that alternative or complementary forms of therapy are explored [6, 7]. Natural products from plants have from time immemorial been adopted for their pharmacological activity against various diseases, including cancers [8, 9, and 10].

Available literature indicates that antioxidant compounds from plants have the potential to be employed in the treatment of GBM by scavenging free radicals and normalising the oxidative state in the body [11, 12 and 13]. Interestingly, polyphenols have demonstrated promising results in chemoprevention and management of cardiovascular diseases. It has also shown a potential in neurodegenerative diseases and other brain related diseases including brain tumours [14]. The role of polyphenols in mitigating the potential damage of oxidative stress has also been demonstrated. Due to their ability to modulate the activity of multiple targets involved in carcinogenesis through direct interaction or modulation of gene expression, polyphenols can be employed to inhibit the growth of cancer cells [15, 16].

Vernonia amygdalina is a member of the *Asteraceae* family. It is categorised as a shrub or small tree that can reach up to 10 m in height with trunk diameter reaching up to 40 cm [17]. It was recently introduced into the Malaysian herbal armament and there has been quite a good following on the use of the plant to treat diabetes, hypertension and hypercholesterolemia. The plant is a vegetable in its native land and is put through a detoxification process before being cooked as food. The process involves boiling the leaves several times while changing the water after each cycle until the bitterness disappears [18]. Extracts from the leaves have been reported to possess hypolipidemic and antihyperlipidemic properties [19, 20 and 21]. In folklore medicine, it is used to cure many diseases including eczema, measles and anemia [22].

Although innumerable studies have been carried out in regards to antioxidants and polyphenols in *Vernonia amygdalina*, several studies exhibited a close relationship between antioxidant activities and total phenolic content [23, 24 and 25]. The mechanism of action in anticancer activity of phenolics could be by disrupting cellular division during mitosis at the telophase stage [26]. Since the elimination of cancer in the early stages is an integral part of chemoprevention, measuring anti-proliferative properties against cancer cells using common assay, such as the MTT assay, could provide useful insights on the chemo-protective potential of natural extracts. Thus, this study aims to investigate the anti-proliferative activity of *Vernonia amygdalina* extracts obtained from ethanol, methanol and ethyl acetate on the brain cancer cell line, U87.

MATERIAL AND METHODS

Extraction Procedure

The method used by Mohd Adzim Khalili *et al.* [27, 28] with slight modifications was adopted in this study. The leaves of *Vernonia amygdalina* were collected from their natural habitat around the state of Terengganu in Malaysia. The leaves were air-dried under shade at room temperature for 24-hours followed by the sequential soaking process (1:10; w/v) using ethanol, methanol and ethyl acetate for 24-hours at room temperature (24-25^{o}C).The supernatants for each solvent were filtered through Whatmann®No. 41 filter paper (pore size 20-25 µm) and were then concentrated under reduced pressure at 40^{o}C. Finally, all the extracts were store at -20^{o}C until they were used during the analysis.

Preparation of extracts

The method employed by Mohd Adzim Khalili *et al.* [29] with slight modifications was used in this study. To screen the extracts obtained from leaves of *Vernonia amygdalina,* 10 mg/mL of the stock solution from each sample extract was prepared by dissolving 10 mg of extract in 1 mL of DMSO. All extract solutions were kept at a temperature of 4^{o}C throughout the experiment. Stock solutions were further diluted in RPMI-1640 (Sigma MO, USA) medium with serum to obtain a final concentration of 60, 30, 15, 7.5, 3.25 1.875 and 0.9375 µg/mL.

Cell culture condition

Human *glioblastoma multiforme* cell lines (U-87) was obtained from the cell bank in the Department of Biotechnology, Universiti Sultan Zainal Abidin. The cancer cells were grown and maintained at a temperature of 37^{o}C in an incubator humidified with 5% CO_2 in a RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) at 95% relative humidity. The medium was then changed twice a week.

Cell growth inhibition assay

The anti-proliferative activity of leaf extracts of *Vernonia amygdalina* were obtained using ethanol, methanol and ethyl acetate to inhibit the proliferation of U-87 cell lines using the micro-titration colometric method of tetrazolium salt reduction by Mosmann [30] A total of 100 µL cell suspensions were dispersed in triplicates into 96 well cultured plates at optimised concentrations of 2.0 x10^5 cells/mL of U-87. After a 24 hour recovery period, the extract solutions that were diluted to a final concentration of 1000 µg/mL using RPMI-1640 medium were further serially diluted to obtain concentrations of 60, 30, 15, 7.5, 3.25 1.875 and 0.9375 µg/mL. The prepared concentrations were then plated out in triplicates. Each plate included untreated cell controls and a blank cell free control. After 48 hours of incubation, MTT (20 µg/mL) was added to each well and re-incubated for further 4 hours. The medium was then removed and DMSO was added into each well to solubilise the formazan crystals. Finally, the absorbance was read at wavelengths of 570 nm using a flourometer micro-plate reader (TECAN, INFINITE M200) and the percentage of cell viability was calculated with the appropriate controls taken into account. The relative viability of the treated cells was expressed as % cytoviability, using the formula;

$$\% \text{ cytoviability} = [A_{490} \text{ of treated cells}] / [A_{490} \text{ of control cells}] \times 100\%$$

The inhibition concentration (IC_{50}) was then determined by non-linear regression analysis of the corresponding dose response curve.

RESULTS AND DISCUSSION

The usefulness of plants to humankind is not only as a source of raw materials for industries, but also as a source of food and medicine. From the earliest times, plants have provided humankind with a source of healing. Many parts of plants such as fruits, seeds, bark, root, and flowers have been used as medicaments to cure various diseases that afflict humankind and animals [31]. Plants have been known to contain or possess abundant phytochemicals, antimicrobials and pharmacological components that include anthraquinones, flavonoids, saponins, polyphenols, tannins and alkaloids [32]. One such plant suspected of possessing medicinal value is the bitter leaf *(Vernonia amygdalina)*. Many herbalists and naturopathic doctors recommend aqueous extracts of bitter leaf for their patients for emesis, loss of appetite-induced ambrosia, dysentery and other gastrointestinal tract problems [33].

Studies have shown that polyphenols have high antioxidant activity and are protective against tumours. *Vernonia amygdalina* extracts are rich in antioxidant polyphenols and have been studied for their anticancer activity against various tumours [34, 35]. Previously, the antioxidant activities of *Vernonia amygdalina* leaf extracts were measured using the ferric thiocyanate (FTC) method. Mohd Adzim Khalili *et al.* [27, 28] found that *Vernonia amygdalina* extract in ethyl acetate displayed the highest phenolic content (2.693 mg/g), followed by chloroform extract and methanol extract using the FTC method. Based on the analysis, the phenolic content had increased due to the concentration of the solution, which clearly shows that there is a positive correlation between total phenolic content and antioxidant activities [36].

On the other hand, Mohd Adzim Khalili *et al.* [29] had revealed that *Vernonia amygdalina* was able to inhibit the growth of MCF-7 and HT-29 cells in a dose-dependent manner. The cytotoxic activity extracts were obtained by using the MTT assay. In this study, the cytotoxic activity that inhibits the growth of human *glioblastoma multiforme* cell line (U-87) according to different solvents of ethanol, methanol and ethyl acetate of *Vernonia amygdalina* was also obtained by using the MTT assay. The MTT is reduced from an insoluble purple formazan into an insoluble colored formazan product by mitochondrial dehydrogenase activity of viable tumor cells, which can be measured spectrophotometrically after dissolution. Various extractions were used to determine the relationship between the number of cells and the amount of MTT formazan generated and between the amount of MTT formazan generated and the duration of cell incubation with MTT [30].

The cytotoxic activity of ethanol, methanol and ethyl acetate extracts of *Vernonia amygdalina* on the growth of U87 cancer cell lines in vitro was shown in Table 1. According to the National Cancer Institute standards, criteria crude extracts possessing an IC_{50} value of less than 20 µg/mL were considered active against the tested cancer cells [37, 38]. In this study, ethanol, methanol and ethyl acetate extracts of *Vernonia amygdalina* were evaluated for their cytotoxicity toward U-87 cancer cell lines. The degree of cytotoxicity was defined as a concentration that reduced the cell number to 50% as compared to the untreated specimen (IC_{50}). The IC_{50} value was determined quantitatively after staining the cells with crystal violet and using the MTT assay [28].

Table 1: Cytotoxic activity of U-87 cell lines according to ethanol, methanol and ethyl acetate extracts of *Vernonia amygdalina*.

Extracts	IC_{50} values (ug/ml)
	U87 cancer cell lines
Ethanol	nil
Methanol	nil
Ethyl acetate	18.80 ± 1.11 µg/ml.

In this study, only ethyl acetate extracts of *Vernonia amygdalina* were shown to be cytotoxic towards U-87 cancer cell lines with an IC_{50} value of 18.80 ± 1.11 µg/ml. Meanwhile, ethanol and methanol extracts of *Vernonia amygdalina* did not show any cytotoxic activity in different concentrations (IC_{50} value above 50% of cell viability). Although several studies have reported the anti-proliferative activity of ethanol and methanol extracts of *Vernonia amygdalina* due to high phenolic content on several types of cancers, it is possible that the activity exhibited by ethanol and methanol extracts do affect the human *glioblastoma multiforme* cell line (U-87) in its ability to scavenge free radicals and normalise the oxidative state in the body [39, 40 and 41]. From this study, we can conclude that only ethyl acetate extracts of *Vernonia amygdalina* have the potential to be used as a new cancer therapeutic agent.

CONCLUSION

The primary aim of this study as described was carried out to screen the ethanol, methanol and ethyl acetate extracts of *Vernonia Amygdalina* as antiproliferative agents in inhibiting the growth of human *glioblastoma multiforme* cell lines (U-87). *Vernonia amygdalina* extracts known to rich in antioxidant polyphenols and have been studied for their anticancer activity against various tumors [34, 35].

Previous studies had suggested that *Vernonia amygdalina* content high in phenolic compound and antioxidant capacity in ethyl acetate extracts as compared to methanol and chloroform [28, 29]. Furthermore, extracts were shown to be cytotoxic towards MCF-7 and HT-29 cancer cell lines tested. Thus, it was concluded that *Vernonia amygdalina* extracts have a good potential to be used as a new cancer therapeutic agent.

However, in this study done with three extracts used, only ethyl acetate extracts of *Vernonia amygdalina* possess ability to inhibit human *glioblastoma multiforme* cell line (U-87) proliferation based on dose dependent activities compared to ethanol and methanol extracts. This activity could be attributed to the highest phenolic content of *Vernonia amygdalina* extract in ethyl acetate in previous studies. Meanwhile, those two extracts showed fluctuations result analysis above IC_{50} graph for their dose dependent activities.

This may occur because of extract's effects with cell line tested for the ability to scavenge free radicals and normalizing oxidative state. Thus, ethyl acetate extracts of *Vernonia amygdalina* were considered for further exploration towards the development of newer and safer cytotoxic compounds. Although this study was done to show an alternative way to cure cancer, but still the trials should be further conducted on a larger scale to assess the efficacy of the proposed treatment.

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