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Original Research Article

Cytotoxicity of *Diospyros dichrophylla* extracts on breast and brain cancer cell lines

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Abstract

Purpose: *To examine the cytotoxic properties of different parts of Diospyros dichrophylla on breast and brain cancer cell lines.*
Methods: *Diospyros dichrophylla was identified and extracted with different solvents. The extracts were filtered and concentrated using a rotary evaporator and then lyophilized. Its phytochemical composition was screened according to standard procedures. The cells were cultured according to standard methods, while cytotoxicity studies were performed using MTT assay.*
Results: *Phytochemical screening revealed the presence of flavonoids, glycosides, phytosterols, triterpenoids and phenol in the plant. Aqueous extract of fruits of D. dichrophylla exhibited significant inhibitory effect on breast cancer cells and normal Vero cells ($p < 0.05$). In addition, aqueous extract showed inhibition of > 50 % at 10 and 50 µg/mL concentrations, while dichloromethane (DCM) extracts of dried fruits showed inhibition of 79.87 and 90.97 % at 10 and 50 µg/mL, respectively. Also, DCM extracts showed better inhibition potential than aqueous extract of the fruit. Furthermore, the fresh fruit extract was also cytotoxic to brain cancer cells at 10 and 50 µg/mL. No selectivity of cancer cells was observed.*
Conclusion: *Diospyros dichrophylla is cytotoxic both to normal cells and cancer cells. Its therapeutic potential is limited based on this study but may be further explored.*

Keywords: *Cytotoxicity, Diospyros dichrophylla, Breast cancer cells, Brain cancer cells, Phytochemical analysis, Cell culture*

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INTRODUCTION

Cancer continues to cause a high mortality rate and this is partly due to the non-specificity of therapeutic strategies which become lethal to normal cells. This phenomenon has driven multiple studies targeted at safe and precise drug delivery [1]. An alternative approach in cancer treatment is the use of medicinal plants, which are an effective drug development strategy [2,3]. The continued search and scientific validation of anticancer agents from plants is particularly important because of the toxicity and high cost of synthetic drugs [4]. Metabolites found in

medicinal plants such as amino acids, carbohydrates, alkaloids, flavonoids, saponins and triterpenoids to name a few, are key chemotherapeutic candidates [2].

Diospyros dichrophylla, commonly known as poison star-apple from the *Ebenaceae* family, is a tree shrub, with leaves arranged spirally and small creamy white flowers [5]. The fruit has shiny seeds and appears almost round, like a slightly flattened berry with dense orange-yellow velvety hairs. The plant is found in Eastern Cape, KwaZulu-Natal, Limpopo and Western Cape, South Africa [5]. Although not many pharmacological studies have been done on *D. dichrophylla*, plants such as *Diospyros mespiliformis* belonging to the same family have been shown to have a few phytochemical compounds with antineoplastic history [6,7]. They include alkaloids, tannins, Saponins, glycosides, flavonoids, steroids and terpenoids. Furthermore, studies on *D. mespiliformis,* including other *Diospyros* species, have confirmed the presence of phenols and flavonoids as antioxidants and have also been shown to be useful as anticancer agents [8-10]. Poisonous plants have also been documented to have medicinal potential, inclusive of anti-cancer effects [11]. Previously, the cytotoxic effect of *D. dichrophylla* was observed in a study on brine shrimp. The dichloromethane: methanol (DCM: MeOH) extracts of the inner seeds showed LC_{50} = 29 µg/mL while there was no activity from the whole fruit. Furthermore, isodiospyrin isolated from the plant showed LC_{50} = 0.13 µg/mL [12]. The aim of this study, therefore, was to investigate the cytotoxic potential of *D. dichrophylla* extracts on breast and brain cancer cell lines. Selectivity was tested against the Vero cell line, which is a normal cell line isolated from the kidneys of a monkey. This is an initiative to discover lead compounds that may be useful in anticancer drug development.

EXPERIMENTAL

Plant material

The plant was collected from the Eastern Cape Province of South Africa, in 2016, during the summer season. Specimen identification was confirmed in BLFU Herbarium, University of Free State, Bloemfontein, South Africa.

Preparation of plant extracts

Diospyros dichrophylla leaves and fruits were washed with tap water, air dried and then ground to fine powder. The plant powder was weighed (10 g) and extraction was performed with 48 hours of shaking using water and sequentially using hexane (for removal of lipids), dichloromethane (DCM), DCM: MeOH (1:1 ratio) and methanol (MeOH) according to increasing polarity [13]. The filtrates were then dried under vacuum using a rotary evaporator to obtain extracts. The water extract was freeze-dried [14].

Qualitative phytochemical screening

Phytosterols

To determine the phytosterol presence, 10 mL of chloroform was added to 0.25 g of plant extract and 0.5 mL of the chloroform layer was then taken out, followed by the addition of 1mL sulphuric acid with caution to the side of the test tube. An appearance of a reddish-brown colour in the chloroform layer was indicative of the presence of phytosterols [15].

Tannins

Distilled water (10 mL) was added to 0.25 g of the extract and then boiled. After boiling, the mixture was filtered and filtrate treated with 3 drops of 0.1 % ferric chloride. Presence of tannin was shown by the appearance of a blue-black precipitate [16].

Glycosides

One milliliter (1 mL) of acetic acid was added to 0.25 g of extract. The mixture was then treated with 1 drop of 0.1% ferric chloride. Thereafter, 1 mL of concentrated sulphuric acid was added with caution to the mixture. The presence of glycosides was indicated by the appearance of a brown ring [15].

Triterpenoids

Triterpenoids were tested by adding 1 mL of chloroform to 2 mg of each respective plant extract. Concentrated sulphuric acid (3 mL) was added with caution to the mixture. Formation of an interface with a reddish-brown colour was indicative of the presence of triterpenoids [15].

Saponins

Saponins were tested by mixing 0.25 g of plant extract and 2.5 mL of distilled water, which was then boiled and then filtered. Subsequently, 2 mL of distilled water was added and then the mixture was shaken vigorously to attain a frosting/foam-like structure, which was an indication of the presence of saponins [16].

Flavonoids

The presence of flavonoids was determined by adding 5 mL of ethyl acetate to 0.25 g of plant extract and heated for 3 minutes, allowed to cool and then filtered. Dilute ammonia solution (0.25 mL) was added to 2.5 mL of filtrate. After shaking the mixture, a yellow precipitate was an indication of the presence of flavonoids [15].

Alkaloids

One percent (1 %) of hydrochloric acid (2 mL) was added to 0.2 g of plant extract. Following that, 1 mL of Meyer's reagent was added to the mixture, followed by the addition of 1 mL of Drangendorff reagent. Appearance of an organic precipitate was an indication of the presence of alkaloids [15].

Quantitative phytochemical analysis

Total phenolic content

Total phenolic content was determined according to the method earlier described by Phuyal and colleagues [17]. Briefly, 1 mg/mL garlic acid solution was prepared as the standard solution (5 mg garlic acid in 5 mL methanol). As a positive control, the garlic acid solution was made into 25, 50, 75, 100, 125 and 150 µg/mL dilutions while methanol was used as blank. To each concentration (500 µL), 2.5 mL of 10 % Folin-Ciocalteu reagent was added and incubated for 5 minutes at room temperature. Seven percent (7 %) sodium carbonate (2 mL) was subsequently added and then incubated at 40 ℃ in a water bath for 30 minutes. The same procedure was followed with the plant extracts and then transferred to 96 well plates with each concentration in triplicates. Absorbance was read at 760 nm with a microplate reader (Thermofischer Scientific, RSA). Total phenolic content of the extracts was calculated from the regression equation of calibration curve y = 00031x + 00116 R^2 = 0.9713 and expressed as mg gallic acid equivalents (GAE) per gram of sample in dry weight (mg/g). The formula Y = mX+C was used to determine the X (TPC) of the extract. Where Y is the absorbance of the extract, m and C were taken from the standard curve.

Total flavonoid

Total flavonoid content was determined according to the method of Yadav and Agarwala (with slight modification)[18]. Quercetin solution (1 mg/mL) was prepared as standard solution (5 mg quercetin in 5 ml methanol). As a positive control, quercetin solution was made into 25, 50, 75, 100, 125 and 150 µg/mL dilutions and methanol was used as a blank during the test. Ten percent (10 %) of Aluminium chloride (100 µL) was added to 500 µL of each concentration. To the mixture, 100 µL of 1 M potassium acetate was added and made up to 5 mL with distilled water. The mixture was then incubated for 30 minutes at room temperature. The same procedure was followed for plant extracts and then transferred to 96 well plates with each concentration of blank and control as well as extracts in triplicates. Absorbance was read at 420 nm with a microplate reader (Thermofischer Scientific, RSA). The total flavonoid content of the extracts was calculated from the regression equation of the calibration curve (y = 0.001x + 0.0144 R^2 = 0.9729) and expressed as mg quercetin equivalents (QE) per gram of sample in dry weight (mg/g). The formula Y = mX+ C was used to determine the X (TPC) of the extract. Where Y is the absorbance of the extract, m and C were taken from the standard curve above.

Cell culture

Cell lines of breast cancer MCF-7, MDA-MB-231, glioma U87 cells and Vero cells were obtained from Cellonex, South Africa. The respective cells were cultured in Dulbecco's Modified Eagles medium (DMEM) and Eagle's Minimum Essential Medium (EMEM) + 4.5 g/L D-Glucose L-Glutamine Pyruvate (Thermofischer Scientific, RSA), supplemented with 10 % Fetal Bovine Serum (FBS, Thermofisher Scientific, RSA) and 0.6 % Penicillin (Thermofischer Scientific, RSA). The cells were then incubated at 37 ^oC in 5 % CO_2 until they reached 80 % confluence and were confirmed microscopically.

Cell viability assay

Cell viability was evaluated using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Different concentrations (100, 50, 10, 5, 1 and 0.1 µg/mL) of the extracts were introduced to the cells in a 96-well plate in triplicates. One row with no extracts served as the control, while another row served as blank and plates were then incubated for 48 hours. Twenty-five microlitres of MTT reagent (5 mg/mL in PBS) was added to each well, incubated for 4 hours at 37 ^oC, and shaken for 10 minutes thereafter. The supernatant was aspirated and $DMSO_4$ (100 µL) was added to the wells to dissolve the formazan crystal. The plates were gently shaken on a shaker for 5 minutes and then read immediately on a microtitre plate reader at 540 nm.

Statistical analysis

The MTT assay was performed in triplicates and the results were analyzed using MS Excel. Other data were analyzed using one-way analysis of variance (ANOVA) to compare experimental groups. Using GraphPad Prism 6 software, a post hoc test (Bonferroni) was used to confirm the level of significance between groups (version 4; GraphPad Software, La Jolla). Values of *p* less than 0.05 were considered significant.

RESULTS

Qualitative phytochemical screening

The qualitative phytochemical determination showed the presence of phytosterols, glycosides, triterpenoids, saponins and flavonoids, as shown in Table 1. However, the tests showed negative results for alkaloids and tannins.

Total phenolic content

Methanol extracts of the leaves contain a significantly high amounts of phenols (78.38 mg (GAE)/g), with the dried fruit extracts having 51.04 mg (GAE)/g phenol content. Water and DCM extracts on the other hand had less than 50 mg (GAE)/g of phenols (Figure 1). The qualitative test revealed the presence of phenols in the *D. dichriphylla* plant, showing that methanol extracted phenols better than the other solvents.

Total flavonoid content

The results show that dichloromethane (DCM) extracts of both leaves and dried fruits showed the highest flavonoid content of 314.65 mg QE/g and 634.27 mg QE/g, respectively. On the other hand, the water extract of the dried fruit contained 72.2727 mg QE/g, while methanol extracts showed less than 50 mg QE/g flavonoids (Figure 2).

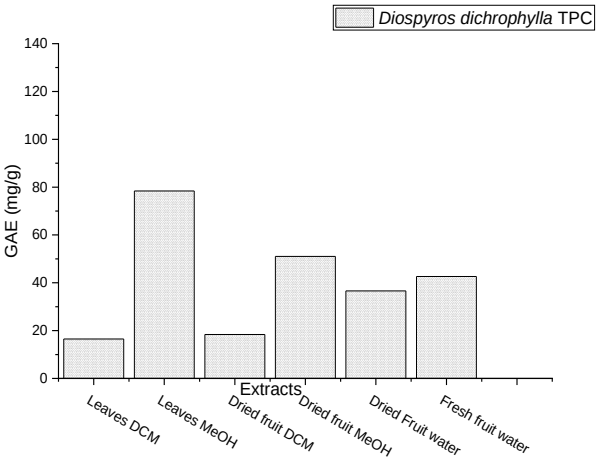

Figure 1: Total phenolic content of *D. dichrophylla* in comparison to garlic acid, expressed as gallic acid equivalent (GAE). NB: The calculated standard deviation was too small in value to draw out visible error bars

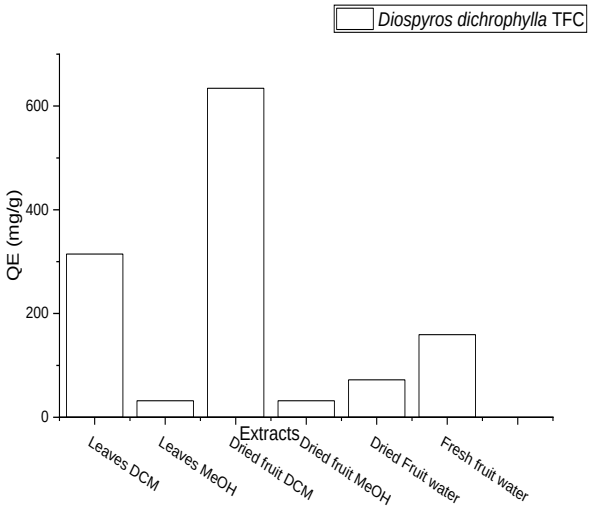

Figure 2: Total flavonoid content of *D. dichrophylla* in comparison to quercetin expressed as quercetin equivalent (QE). NB: The Standard deviation was too small in value to draw out visible error bars

Table 1: Qualitative phytochemical analysis results

Plant extract	Alkaloids	Flavonoids	Saponins	Glycosides	Tannins	Phytosterols	Triterpenoids
Leaves DCM	-	+	-	+	-	-	-
DCM: MeOH	-	+	+	+	-	-	-
MeOH	-	-	+	+	-	-	-
Dried fruit DCM	-	-	-	+	-	+	+
DCM: MeOH	-	-	+	+	-	-	-
MeOH	-	-	+	+	-	-	-
Water	-	-	+	+	-	+	+
Fresh fruit water	-	+	+	+	-	-	-

+: Detected; -: Not detected

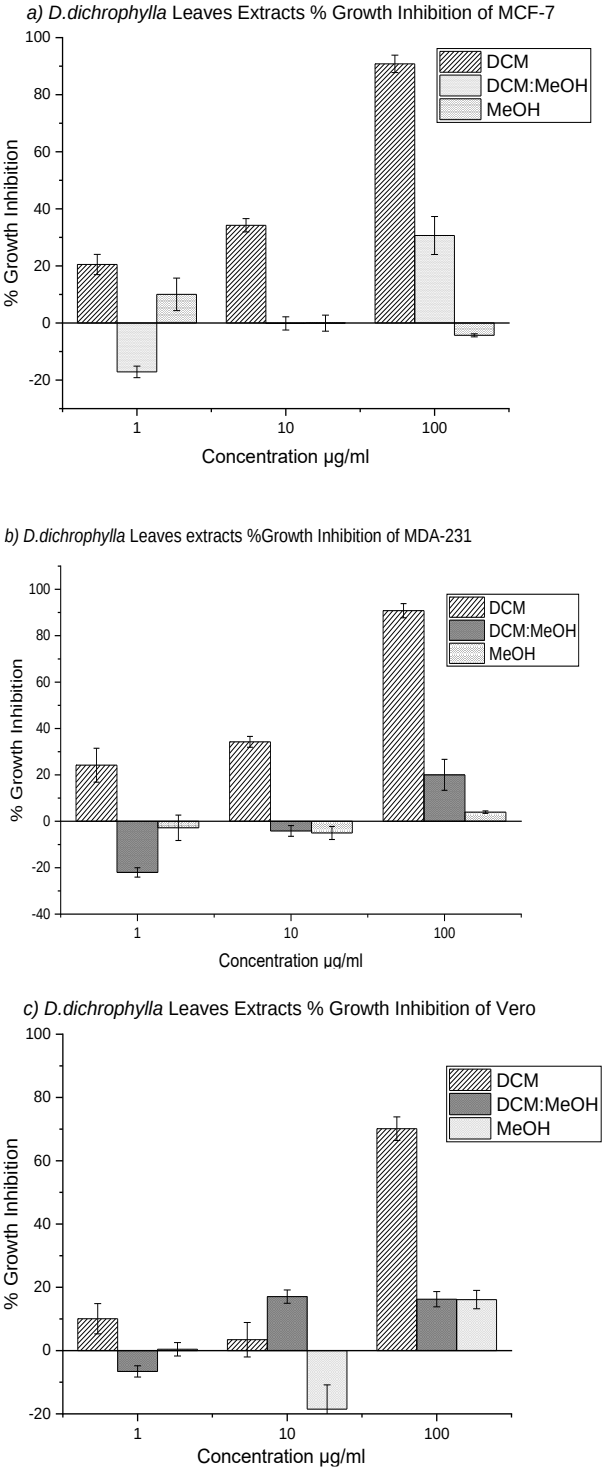

Figure 3: Comparison of percentage growth inhibition of *D.dichrophylla* leaf extracts on MCF-7 (a), MDA-231 (b) and Vero (c) cell lines, which are presented as percentage inhibition against concentration

Cytotoxicity test

Results presented in Figure 3 indicate that DCM and MeOH extracts of the leaves have no notable inhibitory effect on MCF-7, MDA-231 and Vero cell lines (Figure 3a-c). The DCM extract of the leaves showed > 95 %, 90 % and > 65 % growth inhibition at 100 µg/mL concentration against MCF-7, MDA-231 and Vero cell lines, respectively.

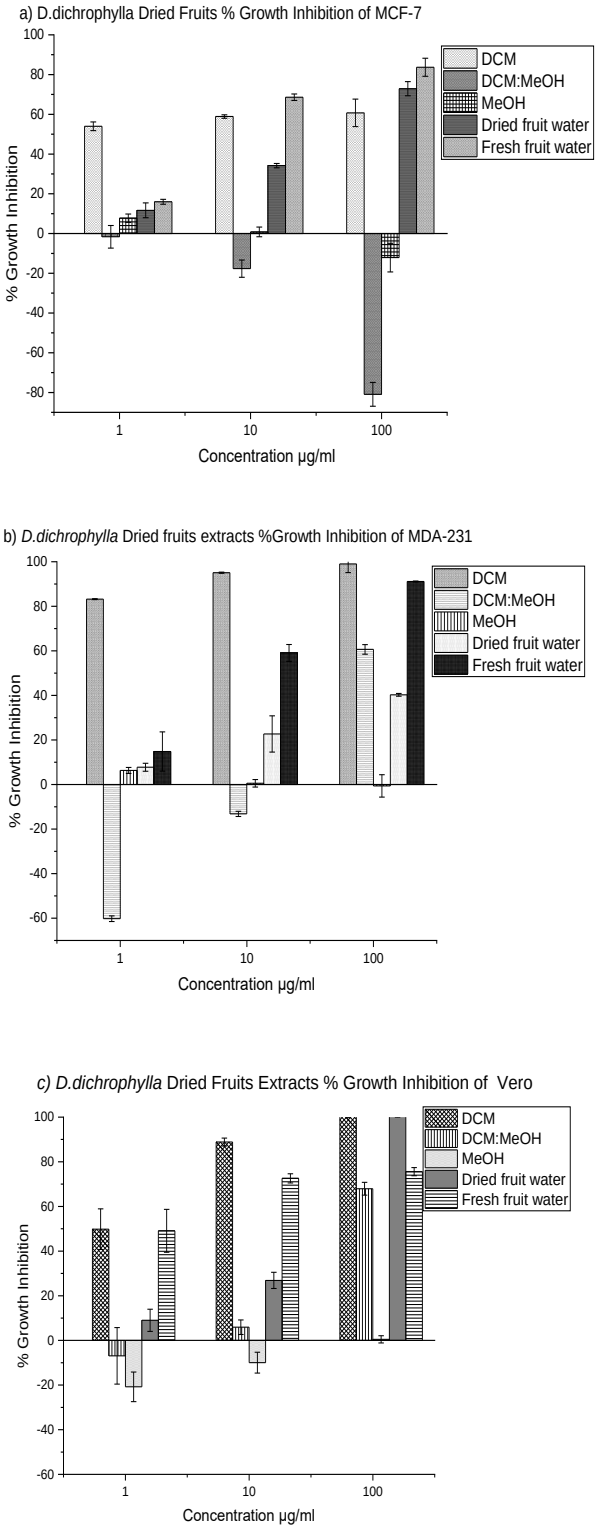

Figure 4: Comparison of percentage growth inhibition of *D. dichrophylla* fruit extracts on MCF-7 (a), MDA-231 (b) and Vero (c) cell lines. The results are presented as percentage growth inhibition against the concentration of the extracts

The results presented in Figure 4 a to c showed no significant difference ($p > 0.05$) in growth inhibition of MCF-7, MDA-231 and Vero cell lines. The fruit extracts were observed to have the same effect on normal cell lines and breast cancer cell lines. The DCM extracts had a very good inhibitory effect at all concentrations in all three cell lines. It is observed that at a concentration of 1µg/mL, fresh fruit water extracts had a proliferative effect on normal cells (Figure 4 c) with very little effect on MCF-7 and MDA-231 cell lines (Figure 4 a and b). Proliferation was observed at 100 µg/mL of DCM: MeOH extract on the MCF-7 cell line (Figure 4 a).

Furthermore, the fresh fruit extract showed inhibition of > 50 % for 10 and 50 µg/mL concentrations on U87cells while dried fruit DCM extracts showed inhibition of 59.37 %, 76.05, 79.87 and 90.97 % at 5, 10, 50, 100 µg/mL, respectively (Figure 5).

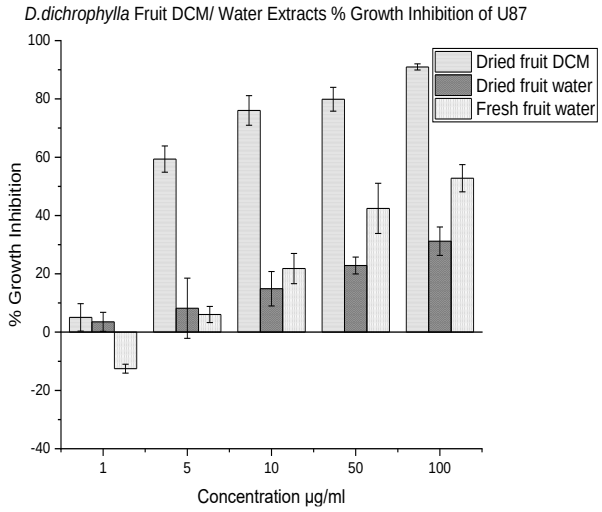

Figure 5: Percentage growth inhibition of U87 with *D. dichrophylla* fruits water extracts and DCM extracts

DISCUSSION

Poisonous plants have been shown to have anti-cancer potential, which depends on the mode of administration and dosage [16]. *Diospyros dichrophylla* is reported to be a toxic plant to both animals and humans. However, there is very little information in the literature recorded on its medicinal and phytochemical constituents. From this study, the plant is observed to contain flavonoids, glycosides, phytosterols as well as triterpenoids. Furthermore, there is good phenolic content found in the plant. These phytochemicals play a significant role in medicine, as studies have reported [19].

Qualitative phytochemical analysis revealed that DCM fruits have glycosides, flavonoids, phytosterols and triterpenoids. The water extracts also contain glycosides, phytosterols, triterpenoids as well as saponins. Presence of these phytochemicals in these extracts is potentially the reason for the inhibitory activity observed in the cytotoxicity evaluation of the plant [20,21]. Majority of phytochemical molecules were not found in other extracts, possibly the reason why other extracts did not show any significant activities on selected cancer cell lines. Also, *D. dichrophylla* fruits were observed to have a potential breast cancer cell line inhibitory effect. Both fruits DCM and water extracts showed a significant inhibitory effect on all breast cancer cell lines screened. These are interesting results because from ancient times, traditional healers have mainly used water for folk medicine [22].

Consequently, MCF-7 and MDA-231 cell results encouraged further investigation of *D. dichrophylla* water extracts on another (U87 brain cancer) cancer cell line. Only extracts that showed growth inhibition on MCF-7 and MDA-231 (fruits DCM and water extracts) were tested against U87. This was done to further investigate the antineoplastic potential of *D. dichrophylla* on a larger spectrum of cancer cells. The result revealed that DCM extracts have better inhibition potential than the water extracts. Fresh fruits also possess an inhibitory effect on the U87 cell line only at 10 and 50 µg/mL, while higher concentrations of fresh fruits promoted the growth of cells. This result could potentially lead to investigating the plant for wound healing properties. The water extracts of dried fruits also encouraged proliferation of U87 cells rather than inhibition as was observed with MCF-7 cell line. It is however notable that there is no selectivity of extract against normal Vero cell lines because results show similar activities both on breast cancer cell lines and Vero cell lines.

A study by Cantrell and colleagues revealed that cytotoxicity activity observed in brine shrimps could be reflective of the bioactivity of the plant. Their study revealed that seeds DCM: MeOH extracts are inhibitory at 29 µg/mL, while isolated compounds showed inhibition at 0.13 µg/mL [12]. However, no activity was observed from DCM: MeOH extracts of fruits in this study. Growth inhibition activity was observed in DCM extracts in both MCF-7 and U87 cell lines. It is worth investigating in subsequent studies whether seed extracts (without the whole fruit) would rather be more active as Cantrell *et al*'s study indicated.

Limitations of this study

Although studies have shown the presence of antioxidant and anticancer potential of plants in the Ebeneceae family, especially *Diospyros* species, these studies have not made it clear as to whether the plant's compounds are selective against normal cells. This continues to leave a loophole in the investigation because a drug is safe to use if it does not harm normal cells. In this study, it was observed that *D. dichrophylla* is also toxic to normal cells. It would be interesting to find out from other studies on other species whether the plant extracts/ fractions are toxic or not to normal cells.

CONCLUSION

Diospyros dichrophylla is toxic both to normal cells and cancer cells. The therapeutic potential of this plant is very limited despite the presence of vital phytochemicals. This study further confirms that the phytochemicals of this plant play an important role in medicinal drug development. It is thus important that these phytochemical compounds are thoroughly investigated, isolated and tested for further drug discovery. Future studies could investigate the effect of the seeds alone, especially compounds that are isolated from seed extracts.

DECLARATIONS

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Ethical approval

This study was approved by the University of the Free State Health Science Research Ethics Committee and issued an approval number HSREC 132/2017 (UFS-HSD 2017/0806).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Mahlomola Alice Mokhatla, Ayodeji Mathias Adegoke, and Paballo Direko carried out the phytochemical and cytotoxicity investigations. Mamello Patience Sekhoacha and Samson Mashele conceptualized the project and provided supervision of the work, funding and infrastructural support. All the authors contributed to the writing of the manuscript and approved the manuscript for publication.

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