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Cytotoxic Activity jof *Dialium Guineense* Wild (Fabaceae) Fruit and Stem Bark Methanol Extracts and Fractions

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A – Research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of article.

Abstract

Background: Cancer is a disease characterized by uncontrollable multiplication and spread of abnormal forms of the body's own cells. Due to the challenges of orthodox drugs other treatment options are being investigated. *Dialium guineense* has found ethnomedicinal application in the management of various ailments including cancer.
Objectives: To evaluate the cytotoxic activity of *D. guineense* fruit and stem bark extracts using preliminary and confirmatory methods.
Material and method: Preliminary screening was carried out on the extracts using bench-top assay methods for cytotoxicity involving the use of tadpoles of *Raniceps ranninus* (20-200 µg/mL) and growth inhibition with radicle of *Sorghum bicolor* seeds (1-30 mg/mL). The extracts were further tested on a breast cancer cell line (AU 565) at 50 µg/mL using 3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay. Solvent partitioning of the stem bark extract was carried out using chloroform and the resulting fractions subjected to biological testing.
Results: The stem bark extract showed 100 % mortality at 40 µg/mL in the tadpole lethality assay while the fruit showed no mortality. The aqueous fraction of the stem bark extract was also more active than the chloroform fraction with an LC_{50} of 32.4 µg/mL. A concentration dependent effect was observed in the growth inhibitory test using *S. bicolor* with 67.92 and 49.06 % reductions obtained for the stem bark and fruit extracts respectively. The chloroform fraction produced the highest anticancer effect with 46.55 % inhibition against AU 565 cell line.
Conclusion: *D. guineense* stem bark has cytotoxic potential and is a good candidate for further *in vivo* anticancer studies.

Keywords: *Dialium guineense*, Growth Inhibitory, Cytotoxic, MTT

INTRODUCTION

The search for new drugs from medicinal plants used in the treatment of various ailments has witnessed a rising trend. Discovery of effective and less toxic drugs for the treatment of tumor-related diseases is no exception.
Anticancer drugs derived from plants may act through a direct cytotoxic effect on tumor or cancer cells (Aarthi *et al*., 2016). Different approaches for detection of plants with likely anti-tumor properties have been used by researchers. Other simple bench-top assays which are predictive of anticancer effect can be used where cancer cell lines are not readily available. They include cytotoxicity on certain zoological organisms like the nauplii of *Artemia salina* (McLaughlin *et al*., 1991) mosquito larvae and tadpoles (Obuotor and Onajobi, 2000) as well as

growth inhibitory effect on *Sorghum bicolor* seed radicles (Ayinde and Agbakwuru, 2010).

The ability of plant extracts to impart cytotoxicity on these organisms has been regarded as a measure of their ability to inhibit growth of tumor producing cells (McLaughlin *et al*., 1991).

Dialium guineense from the family Fabaceae is commonly called velvet tamarind or black velvet in English. It is known as "Icheku" in Igbo language of South east Nigeria, "Awin" in Yoruba language of south west Nigeria and as "Tsamiyar kurm" among the Hausas in the Northern part of Nigeria (Ezeja *et al*., 2011). Different parts of the plant have ethnomedicinal properties, which are used against various diseases. Its fruit is eaten among some women in South-east Nigeria to improve lactation and check genital infections (Nwosu, 2000) while the bark and leaves are used to cure pulmonary troubles, malnutrition, malaria, fever, jaundice, ulcer, eye, diarrhea, and heart problems (Bero *et al*., 2009; Okerulu *et al*., 2015). Bero *et al*. in 2009 also reported their use in treating wound, hemorrhoids, severe cough and stomach ache.

The bark has also been used in folklore medicine for treating cancer, headache, and pains (Besong *et al*., 2016). Idu *et al*., (2009) reported the usefulness of the bark for oral hygiene and stomach ache among the Esan tribe of Edo state.

The methanolic leaf extract of *D. guineense* has been reported to possess *in vitro* antioxidant activity and antimicrobial effect on 6 bacterial species (*Staphylococcus aureus, Streptococcus mutans, Escherichia coli, Bacillus cerus, Pseudomonas aeruginosa, Klebsiella pneumonia*), and 4 fungal species (*Candida albicans, Microsporum gypseum, Trichophyton mentagrophytes* and *Trichophyton rubrum*) (Gideon *et al*., 2013). The methanolic stem bark extract has also been cited to possess analgesic properties (Ezeja *et al*., 2011).

The ability of *D. guineense* pulp extract to protect against aflatoxinB1-induced hepatotoxicity and oxidative stress was demonstrated by Abdulwasiu *et al*. in 2014. It has also been shown to have a moderate anti-plasmodial effect on *Plasmodium falciparum* (Bero *et al.,* 2009) as well as antiulcer effect (Balogun *et al.,* 2013).

D. guineense has been indicated in the management of cancer ailments therefore this work was aimed at examining the anticancer effects of its fruit and stem bark methanol extracts using both predictive and confirmatory methods.

METHODOLOGY

Collection and identification of plant material

Dialium guineense fruit and stem bark were collected in October, 2017 in Benin City, Edo State, Nigeria. Voucher number UBHD 368 was assigned after identification at the Department of Plant Biology and Biotechnology, University of Benin by Dr Henry Akinnibosun.

Extraction of plant material

About 1 kg of each of the dried and powdered fruit pulp and stem bark of *D. guineense* were extracted with the aid of a soxhlet apparatus using methanol and the resulting extracts kept in the refrigerator at 4ºC until needed.

Source and identification of the tadpoles of *Raniceps ranninus*

Tadpoles (5-6 days old) were obtained from small water settlements around the Faculty of Pharmacy, University of Benin and identified as *Raniceps ranninus* tadpoles by Professor M. Aisien, Animal Parasitologist, Department of Animal and Environmental Biology, Faculty of Life Science, University of Benin, Benin City, Nigeria.

Source and preparation of the guinea corn (*Sorghum bicolor*)

Sorghum bicolor seeds were obtained from a local market in Benin city, Edo state and were rinsed with absolute alcohol to remove the preservative. About 100 mL of water was added, stirred with the seeds and immediately decanted to remove the compromised seeds floating at the top of the water. The viable seeds which remained submerged were dried on filter papers and used for the experiment.

Determination of the cytotoxic effects of the extracts on tadpoles (*Raniceps ranninus*)

Tadpoles (10) were placed in 50 mL capacity beakers containing 15 mL of water from the water settlements of the tadpoles and this was made up to 49 ml with distilled water. 1 ml of the different concentrations of the extracts (1, 2, 5 and 10 mg/mL) was then added to make a total volume of 50 mL. A final concentration of 20, 40, 100 and 200 µg/mL respectively was obtained. The experiment was done in triplicates for each concentration. The mortality rate of the tadpoles was observed for 24 h (Ayinde and Agbakuru, 2010).

Determination of the growth inhibitory effects of the extracts on guinea corn (*Sorghum bicolor*)

Concentrations of *D. guineense* fruit and stem bark extracts (1, 2, 5, 10, and 20 mg/mL) were prepared. Following the method described by Ayinde and Agbakuru (2010), each concentration (10 mL) was poured into 9-cm-wide glass Petri dishes under laid with cotton wool and filter paper (Whatman No 1). Twenty (20) viable seeds were spread on each plate and kept in a dark environment. The length (mm) of the seed radicles was measured at 24, 48, 72 and 96 h. The control seeds were treated with 10 mL of distilled water. The experiment was carried out in triplicates.

Solvent partitioning of the methanol extract of *D. guineense* stem bark

The methanol extract of *D. guineense* (25g) was re-dissolved in water and partitioned exhaustively in three aliquots with chloroform in a separating funnel. The resulting chloroform and aqueous fractions were concentrated to dryness over a water bath at 50ºC. These fractions were subjected to cytotoxic test using *R. ranninus* tadpoles at 20 – 200 µg/mL and growth inhibitory tests using *S. bicolor* seeds at 1-30 mg/mL concentration following earlier stated methods.

Determination of cytotoxic activity using cancer cell lines

The cytotoxic activity was performed using MTT assay on human breast cancer (AU 565) cell line. The cancer cells were obtained from the molecular bank of the International Center for Chemical and Biological Sciences (ICCBS) at the University of Karachi, Pakistan. The cancer cells were placed in 96-well plates at a density of 10,000 cells/well/100 µL and allowed to incubate for 24 h in ATCC modified complete RPMI medium (supplemented with 90% FBS, 1 % penicillin, 1 % streptomycin) at 37 °C and 5% CO_2 for the healthy growth of the cells. Solutions of the extracts and fractions (50 µg/mL) were prepared in sterile DMSO and tested against the cells. Doxoubicin at 50 µM was used as the positive standard. After treatment, the cells culture were allowed to incubate for 48 hours at 37 °C and in humidified atmosphere of 5 % CO_2 after which 200 µL of MTT (0.5 mM) dye was added in each well and then incubated for another 3-4 h. The resulting formazan crystals were dissolved in 100 µL of DMSO. The absorbance of the resulting solution was measured at 570 nm. (Puig *et. al*., 2011; Kritsanawong *et. al*., 2016). The experiments were performed in triplicates.

Statistical analysis

All data obtained were expressed as mean ± SEM and analyzed with One-way Analysis of Variance (ANOVA) using SPSS 21. $P< 0.05$ was regarded as significant.

RESULTS

Cytotoxic effects of the extracts and fractions on *R. ranninus* tadpoles

At the end of 24 h period, the stem bark extract produced 100 % mortality at 40 µg/mL concentration but no mortality was observed with 20 µg/mL. The fruit extract produced no mortality at both concentrations (Figure 1). The aqueous fraction obtained from the stem bark showed a higher cytotoxic effect with 100 % mortality at 40 µg/mL compared to the chloroform fraction which gave 36.67 % mortality at the same concentration. An LC_{50} of 32.4 µg/mL was obtained for both the stem bark extract and aqueous fraction while the chloroform fraction had LC_{50} of 128 µg/mL.

Growth inhibitory effect of the extracts and fractions on *S. bicolor* seed radicles

A general increase in the lengths of the seed radicles was observed throughout the incubation period. Those of the control seeds increased progressively but the seeds treated with the methanol extracts of *D. guineense* stem and fruit were observed to elicit concentration-dependent reductions in the length of the radicles that emerged. The average length of the radicles at 24 h for the control was 5.6 ± 0.16 mm, while the seeds treated with 10 mg/mL of the stem bark extract showed an average length of 3.27 ± 0.05 mm, indicating 41.61% growth reduction. After 96 h, the control seeds had an average length of 74.43 ± 0.31 mm, whereas the seeds treated with 10 mg/mL of the stem bark extract produced an average length of 23.88 ± 0.15mm indicating 67.92% reduction in length (Figure 2). The fruit extract demonstrated reduced growth inhibitory effect with 49.06 % reduction after 96 h (Figure 3).

The chloroform and aqueous fractions of the stem bark were equally observed to inhibit the growth of the radicles of the seeds. An average length of 4.72 ± 2 mm in the controls at 24 h was reduced to 1.93 ± 0.44 mm with 10 mg/mL concentration of the aqueous fraction showing 59.11 % reduction, which was further increased to 76.23 % reduction at 96 h (Figure 4). The chloroform fraction produced higher percentage growth reductions of 80.1% at 96 h (Figure 5). The variations in length were found to be significant at $P<0.05$.

Cytotoxic activity of *D. guineense* extracts and fractions on AU 565 cell line

The stem bark extract producing 38 % inhibition at 50 µg/ml was observed to be more active than the fruit extract. Generally, the extracts showed moderate activities against the breast cancer cell line with the chloroform fraction relatively demonstrating the highest inhibition of 46.55 % (Table 1).

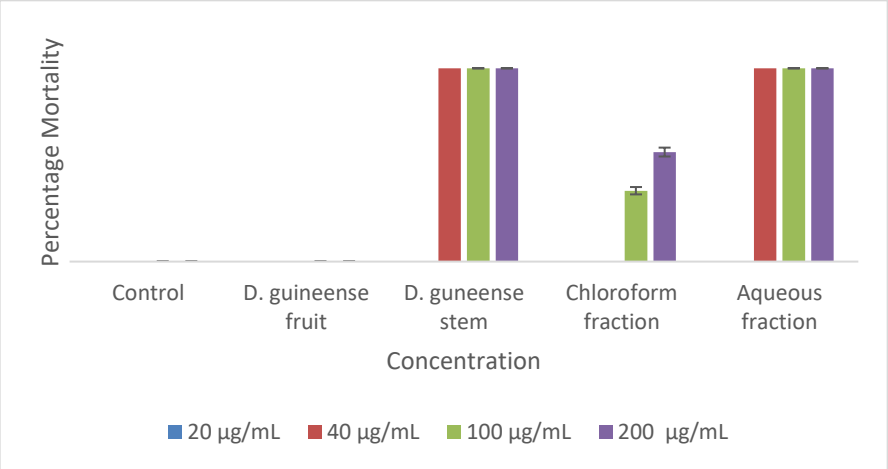

Figure 1: Effect of the extracts and fractions of *D. guineense* on tadpoles of *R. ranninus*

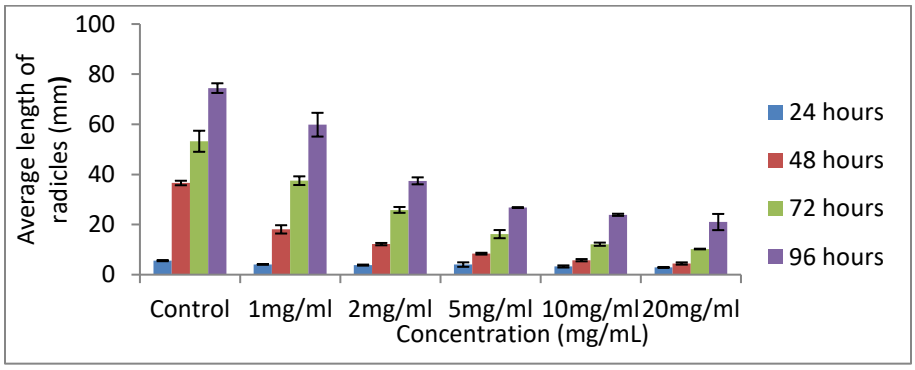

Figure 2: Inhibitory effects of the methanol extract of *D. guineense* stem bark on length of *S. bicolor* radicles

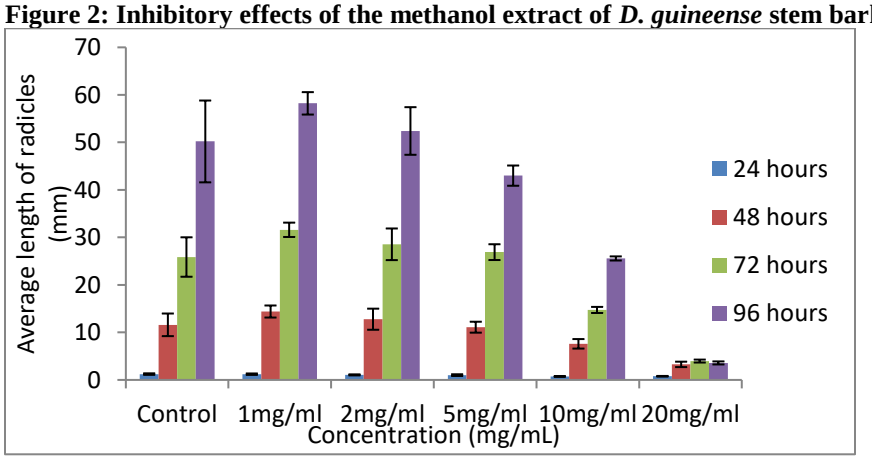

Figure 3: Inhibitory effects of the methanol extract of *D. guineense* fruit on length of *S. bicolor* radicles

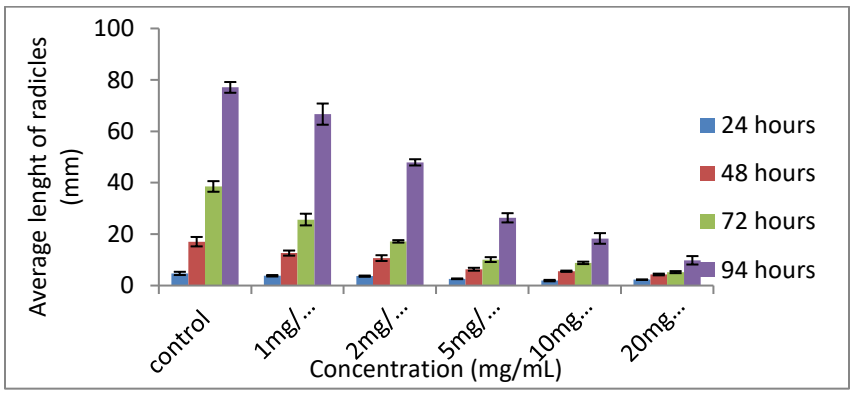

Figure 4: Inhibitory effects of the aqueous fraction of *D. guineense* stem bark on length of *S. bicolor* radicles

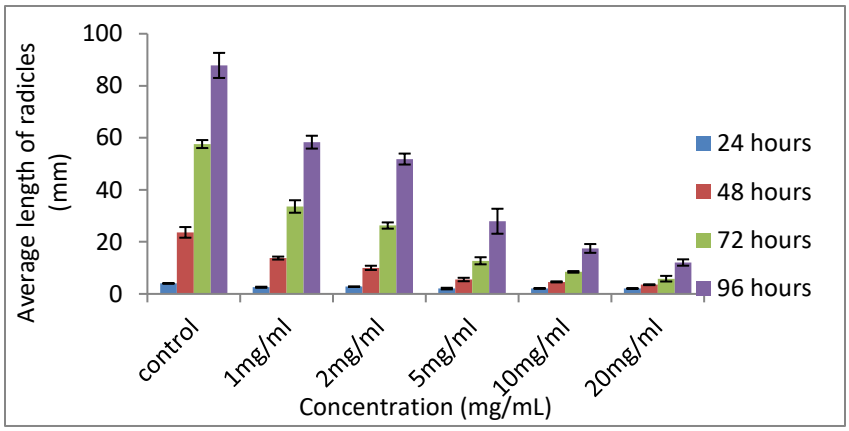

Figure 5: Inhibitory effects of the chloroform fraction of *D. guineense* stem bark on length of *S. bicolor* radicles

Table 1: Cytotoxic activity of *D. guineense* extracts and fractions

Extract/Fraction (50 µg/mL)	Percentage Inhibition (%)
Fruit extract	13.35
Stem bark extract	38.00
Chloroform fraction	46.55
Aqueous fraction	7.37
Doxorubicin	98.46

DISCUSSION

There is a growing interest globally in the pharmacological assessment of various plants used traditionally as medicine. One of such plants is *D. guineense* which has been indicated in the treatment of various diseases in traditional system of medicine including cancer and tumor-related ailments. Discovering plants with potential antitumor activities requires a range of procedures which may not be cost effective. Using simple bench top assays which are reproducible, fast, cheap and reliable have the advantage of eliminating the less promising antitumor plants (Ayinde and Agbakuru, 2010). Two of such assays employed here were the cytotoxic effects of the extracts on tadpoles of *R. ranninus* and their growth inhibitory activities on *S. bicolor* seed radicles.

The stem bark extract was found to have more cytotoxic effect against *R. ranninus* tadpoles than the fruit extract with 100 % inhibition obtained at 40 µg/mL while no effect was observed with the fruit extract at this concentration. The growth inhibitory test using *S. bicolor* seeds produced similar effects with the stem bark extract demonstrating higher percentage reduction in growth of the seed radicles at 96 h (67.92%) compared to the fruit extract (49.06 %). The cytotoxic assay using cell line confirmed the potency of the plant parts with the stem bark extract

producing 38 % inhibition while the fruit extract gave only 13.35 % inhibition against AU 565 breast cancer. Biological activities of medicinal plants are a direct reflection of the nature and effect of the phytochemicals they contain. Analysis of *D. guineense* stem bark showed the presence of flavonoids, alkaloids, tannins, and saponins but the fruits were devoid of glycosides and phenols (Gideon and Raphael 2012). Phenolic compounds are reported to exhibit a wide range of physiological properties, such as anti-allergenic, anti-artherogenic, anti-inflammatory, anti-microbial, antioxidant, anti-thrombotic, cardioprotective and vasodilatory effects (Haq *et al*., 2011). Their presence could have been responsible for the cytotoxic effects against the tadpoles observed with the stem bark extract which was absent in the fruit extract. They could also have been responsible for the activity against AU 565 cell line as saponins and tannins especially are known to exhibit anticancer properties (Isil and Turkan, 2015).

Plants are known to contain many constituents of varying polarities and molecular mass. Using two appropriate immiscible solvents ensures the separation of the constituents based on their relative solubilities in the solvents. Partitioning of methanol extract of *D. guineense* stem bark improved the cytotoxic activity with the chloroform fraction giving higher inhibition of 46.55 %, showing that the active phytochemicals are less polar in nature.

Plants in the family Fabaceae have been reported to possess anticancer activity. They include *Astragalus membranaceus* whose root extract exhibited anti proliferative activity and induced the apoptosis of MDA-MB-231, MCF-7, and SK-BR-3 breast cancer cell lines (Zhou *et al*., 2018). *Cajanus cajan* ethanolic leaf extract also reportedly repressed CaCo-2, MCF-7 and HeLa cancer cell lines (Schuster *et al*., 2016). Furthermore *Derris scandens* extract repressed human colon cancer HT29 cells through apoptosis and mitotic inhibition when augmented with γ-irradiation (Arunee *et al*., 2014).

This study showed the *in vitro* cytotoxic potential of *D. guineense* stem bark extract against AU 565 breast cancer. Further studies using other cell lines as well as determination of the mechanism of action are currently ongoing to further validate this ethno medicinal claimed potency.

CONCLUSION

In conclusion, the present study suggests that *D. guineense* possesses cytotoxic effects which is more resident in the chloroform fraction of the stem bark extract. These results lend credence to its folkloric use for the treatment of cancer.

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