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RANDOMIZED ANTICANCER AND CYTOTOXICITY ACTIVITIES OF *GUIBOURTIA COLEOSPERMA* AND *DIOSPYROS CHAMAETHAMNUS.*

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Abstract

Background: Plants have consistently proven to be a reliable and yet not fully explored source of medicines. In light of this, there is a constant demand for new treatment regimens for cancer. Namibia has a rich diversity of plant species of over 4300 with 17 % of them being endemic to Namibia. Plants growing in Namibia's diverse climatic zones produce many secondary metabolites as part of adaptation to their environment. This article focused on the screening of such phytochemicals and their cytotoxic and anticancer properties *in vitro.* Two Namibian plants *Diospyros chamaethamnus* and *Guibourtia coleosperma* were randomly selected for this purpose.
Materials and Methods: The plants were screened for the presence of coumarins, alkaloids, flavonoids, anthraquinones, steroids and terpenoids using thin layer chromatography. Anticancer screening was performed on a panel of three cancer cell lines, while cytotoxicity was determined using a human fibroblast cell line, both using the SRB method.
Results: Alkaloids, anthraquinones, flavonoids and steroids were detected in both organic and aqueous extracts of the two plants. The organic plant extracts had a greater anti-proliferative effect on the cancer cell lines than the aqueous extracts; the *D. chamaethamnus* organic root extract was the most potent with an IC_{50} of 16.08, 29.12 and 24.67 µg/mL against TK10, UACC62 and MCF7 cells, respectively. Furthermore, cytotoxicity analysis revealed the non-toxic nature of the extracts, except for the organic root extract of *D. chamaethamnus* that showed significant cytotoxicity (IC_{50} 13.03 µg/mL).
Conclusion: *D. chamaethamnus* is a potential candidate for the development of a plant based cancer treatment. The study showed the value of random screening in drug discovery from plants for pharmacological activity that is unrelated to their ethnomedicinal uses.

Key words: medicinal plants, anticancer, cytotoxicity, phytochemicals.

Introduction

Despite the availability of cancer treatments such as surgery (solid tumor), radiotherapy (solid tumor), chemotherapy (malignant cancer), and immunotherapy; cancer remains the second leading cause of death after cardiovascular diseases in developing countries (Akindele *et al*., 2015). An estimated 12.7 million cases and 7.6 million cancer related deaths occurred globally in 2008 (Schwartsmann *et al.*, 2002). Cancer cases and mortalities are expected to rise by 2050 to 24 million and over 16 million, respectively (Jemal *et al*., 2011). The Cancer Association of Namibia reported a 28.6 % upsurge in cancer cases between 2005 and 2009; the predominant cancers being Kaposi sarcoma and prostate cancer in males, and breast and cervical cancer among females (Namibian cancer registry, 2011). Prognosis remains poor at the time of reporting. This is mainly due to a combination of factors such as lack of awareness and long referral processes; thus increasing treatment failure, morbidity and mortality. The current chemotherapy regimens in place produce side effects such as vomiting, loss of hair (alopecia) and lowered bone marrow count; all of which are a result of them being toxic to the localized cells in the gastrointestinal tract, hair and bone marrow. Furthermore, some of these chemotherapeutics are ineffective as a result of developed resistance and because they are insoluble, unstable, have low absorbance (Akindele *et al*., 2015).

Traditional medicines comprise of spiritual rituals, as well as treatments based on plant and or animal preparations. Practitioners perceive the practice of traditional healing to be holistic. This includes social, emotional and physiological facets. Plants have consistently proven to be a reliable and yet not fully explored source of new medicines. The pharmacological properties of plants are due to the presence of biologically active constituents known as secondary metabolites (Afify *et al*., 2011). Usually these phytoconstituents increase the likelihood of survival of the plant in adverse conditions such as heat and drought, and may even act as deterrents to herbivores and or omnivores (Kennedy & Wightmann, 2011). Many plants have been reported as treatments for a number of cancer and cancer related conditions (Nirmala *et al*., 2011; Akindele *et al*., 2015). In addition, current available anticancer drugs such as

camphothecin and vinca alkaloids vincristine and vinblastine were isolated from *Camptotheca acuminate* (Nirmala *et al*., 2011) and from *Catharanthus roseus*, respectively; and are used to treat cancers such as Hodgkin and non-Hodgkin lymphomas, breast cancer and germ cell tumors (Moudi, Go, Yien & Nazre, 2013).

Medicinal plants are useful for treating conditions other than what they are used for in ethnomedicinal setting. An example includes that of *Sutherlandia frutescence* (Cancer bush) which is used to boost immunity in HIV patients but is used for cancer (Mills *et al*., 2005). This study investigated the anticancer and cytotoxic properties of two Namibian plants, *Guibourtia coleosperma* and *Diospyros chamaethamnus* belonging to the Fabaceae and Ebenaceae families, respectively. Within the *Kwanyama* community in North Central Namibia, *G. coleosperma* is known as *Omushii* and *D. chamaethamnus* as *Omukokofi* (Rodin, 1985). The root of *D. chamaethamnus* is used as a treatment for malaria and psychological problems together with *Strychnos pungens*, *Annona stenophylla*, *Diplorhyncus condylocarpon* and *Swartizia madagascariensis* (von Koenen, 2001; Du Preez, 2016). *G. coleosperma* is used for cosmetic purposes while the gum that exudes from the tree is known to be poisonous (Rodin, 1985). The leaves of *G. coleosperma* are also used as a remedy for the common cough (Cheikhyoussef and Embashu, 2013), while the root extract is used for the treatment of wounds (Von Koenen, 2001). In addition, Bushmen of the Kalahari Desert also prepare a decoction from the bark for diarrhea. Other uses include blood clotting, stomach complaints and constipation (Cheikhyoussef and Embashu, 2013). *D. chamaethamnus* has no ethnomedicinal use for cancer treatment or cancer associated symptoms in Namibia or surrounding countries, while *G. coleosperma* is used as an analgesic and for treatment of wounds and other subcutaneous tissue ailments (Royal Botanic Gardens, Kew1999).

Materials and Methods

Plant harvesting

The roots of *G. coleosperma* and *D. chamaethamnus* were harvested in March 2012, from the Zambezi region of Namibia. Voucher specimens were prepared and deposited with herbarium at the National Botanical Research Institute (NBRI) of Namibia, for confirmation of scientific nomenclature. The plants were air dried at room temperature for 4 weeks, ground to a powder and then stored at -20 °C.

Preparation of plant extracts

An aqueous extract was prepared by maceration of plant material using distilled water, while the organic extract was prepared using dichloromethane-methanol (1:1 v/v) at a sample-to-solvent ratio of 1:20 (w/v) (for 2 hours at 60 °C and 48 hours at room temperature, respectively. The plant and solvent mixture was filtered using a Grade 1 Whatman filter paper. The filtrate was concentrated *in vacuo* using a rotary evaporator and was further dried using a freeze dryer.

Thin-layer chromatography

Dry organic and aqueous plant extracts were reconstituted in dichloromethane-methanol (1:1 v/v) and distilled water, respectively at a concentration of 40 mg/mL. An aliquot of 7.5 µL of extract were loaded onto MERCK precoated Silica gel 60 F254 plates. The plates were developed using solvent systems and staining reagents for respective classes of compounds such as alkaloids, anthraquinones, coumarins, flavonoids, steroids and terpenoids (Harborne, 1998).

Maintenance of cell lines

The cancer cell lines, melanoma UACC62, renal TK10 and breast MCF7 were obtained from the American Type Culture Collection (ATCC), while the human fetal lung fibroblast W138 cell line was obtained from the European Collection of Cell Cultures (ECACC). The cancer cells were maintained in RPMI supplemented with 5 % bovine serum, 2 mM L-glutamine and 50 µg/mL gentamycin. The WI38 cells were maintained as a monolayer in EMEM media supplemented with 10 % bovine serum, 2 mM L-glutamine and 50 µg/mL gentamycin. The cells were grown in flasks at 5% carbon dioxide, 100 % relative humidity, and 37 °C.

Anticancer activity

Anticancer activity of the plants was assessed using the SRB assay (Fouche *et al*., 2008). The organic and aqueous extracts were resuspended in dimethyl sulphoxide and double distilled water, respectively, and were further diluted in RPMI media. The three cancer cell lines were seeded in 96 well plates at cell densities ranging between 7-10 000 cells/well. After 24 hours of incubation at 5 % CO_2, 100 % relative humidity, and 37 °C; plant extracts and RPMI media were added to each well to obtain final concentrations of 100, 50, 25, 12.5 6.25 µg/mL and a final DMSO concentration of 0.02 %. This was done in duplicate. Untreated cells were used as the negative control, whilst

Etoposide constituted at 100, 50, 25, 12.5, 6.25 µg/mL in DMSO was used as the positive control. The plates were further incubated at 37 °C, 5% CO_2, 100% relative humidity for another 24 and 48 hours. At end of the incubation period, the viable cells in each well were fixed by adding 50 µL of 50 % aqueous trichloroacetic acid (TCA), followed by incubating the plates at 4 °C for 1 hour. Excess TCA was washed off and 100 µL of 0.4 % Sulphurhodamine B (SRB) protein dye, prepared in 1 % acetic acid solution was used to stain cells for 1 hour. Excess SRB was rinsed off using 1 % acetic acid; 100 µL of 10 mM tris base was then added to solubilize the well content and the absorbance was read at 540 nm using a multi well spectrophotometer. Cell viability was calculated using the formula below.
Cell viability % = (OD_{540} treatment-OD_{540}blank)/(OD_{540}control-OD_{540}blank) x 100 %

Cytotoxicity evaluation

Human fetal lung fibroblast cells WI38 were used to determine the cytotoxicity of the plant extracts. The cells were seeded at a density of 10 000 cells per well in 96 well flat-bottom plates. Plant extracts at concentrations of 100, 50, 25, 12.5 and 6.25 µg/ml were incubated with human fetal lung fibroblast cells. The analysis was conducted as described by Fouche *et al*. (2008).
Data analysis
The percentage cell viability was analyzed using student T tests at $\alpha = 0.05$ for comparison of the effects of the plant extracts in each experimental design. Furthermore, the percentage cell viability was used to calculate the IC_{50} values using non-linear regression analysis in GraphPad Prism Version 6.

Results

Phytochemical analysis

The four plant extracts displayed a similar phytochemical profile with alkaloids, anthraquinones flavonoids and steroids being detected in both the organic and aqueous extracts of *G. coleosperma* and *D. chamaethamnus*, as shown in Table 1. Coumarins were only detected in the extracts (aqueous and organic) of *G. coleosperma*, and terpenoids in the aqueous extracts of both *G. coleosperma* and *D. chamaethamnus*.

Table 1: Phytochemical profile of root extracts of *G. coleosperma* and *D. chamaethamnus* using thin layer chromatography.

	Plant name			
	G. coleosperma		***D. chamaethamnus***	
Phytochemicals	**Organic**	**Aqueous**	**Organic**	**Aqueous**
Alkaloids	+	+	+	+
Anthraquinones	+	+	+	+
Coumarins	+	+	-	-
Flavonoids	+	+	+	+
Terpenoids	+	-	+	-
Steroids	+	+	+	+

Key: Present= +, absent= -

Anticancer activity

The aqueous extract of *G. coleosperma* exhibited anticancer activity against MCF-7 cells (IC_{50} 92.88 µg/mL), as did the organic extract ($IC_{50-=}$ 62.03 µg/mL) (Table 2). However, both these extracts did not inhibit the growth of TK-10 (Renal) and UACC-62 (melanoma) cell lines (IC_{50} >100 µg/mL). The organic extract of *D. chamaethamnus* showed significant anticancer activity on all cell lines; TK10 (IC_{50} = 16.08 µg/mL), UACC-62 (IC_{50} = 29.12 µg/mL) and MCF-7 (IC_{50} = 24.67 µg/mL). The anticancer activity exhibited by the organic extract of *D. chamaethamnus* against the renal cancer cell line, TK10 (IC_{50} = 16.08 µg/mL) was relatively lower than that of the etoposide (21.43 µg/mL). The aqueous extracts of *D. chamaethamnus* exhibited no anticancer activity against any of the cancer cell lines with IC_{50} values >100 µg/mL.

T (root p=0.008) at α=0.05) for the renal cancer cell line, but not for UACC62 melanoma and MCF7 breast cancer cell lines (p=0.915) and (p=0.373), respectively.

Table 2: Anticancer activities (IC_{50}) of *D. chamaethamnus* and *G. coleosperma* root extracts against a panel of three cancer cell lines.

Treatment	Extract	IC_{50} (µg/ml)		
		TK-10	UACC-62	MCF-7
G. coleosperma	Aqueous	>100	>100	92.88
	Organic	>100	>100	62.03
D. chamaethamnus	Aqueous	>100	>100	>100
	Organic	16.08	29.12	24.67
Etoposide		21.43	1.668	1.759

***In vitro* cytotoxicity screen**

The aqueous extract of *G. coleosperma* produced a greater reduction in cell viability (cytotoxicity) in comparison to the organic extract of the same plant (Figure 1). Statistical analysis showed a significant difference in the cell viability across increasing extract concentrations of the two extracts of *G. coleosperma* ($p=0.009$). The organic extract of *D. chamaethamnus*, was more cytotoxic in comparison to its aqueous extract and also in comparison to both the aqueous and organic extracts of *G. coleosperma* (Figure 2, Table 3). Statistical analysis revealed a significant difference between the aqueous and organic extracts of *D. chamaethamnus* ($p<0.001$).

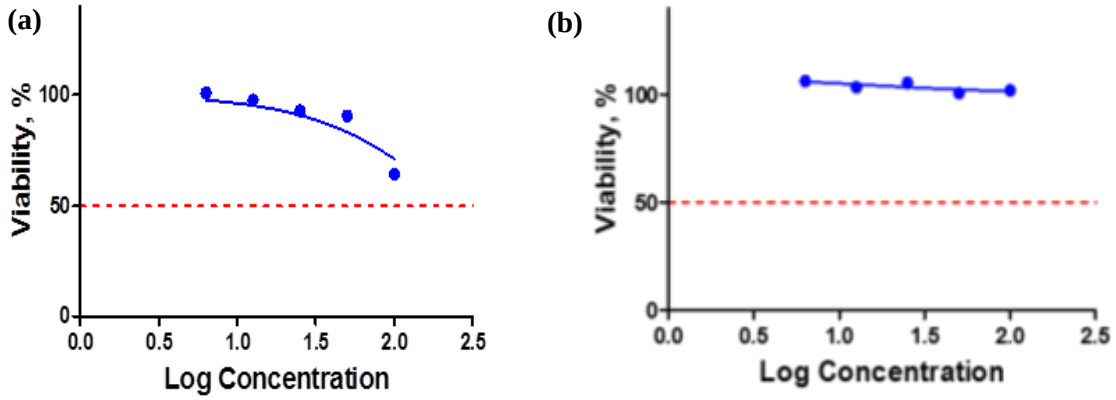

Figure 1: Dose response curves showing the cytotoxic effects of *Guibourtia coleosperma* extracts, both (a) aqueous and (b) organic, on human fetal lung fibroblast W138 cells. Concentrations (µg/mL) were expressed in log form and used to plot the non-linear graphs against percentage cell viability.

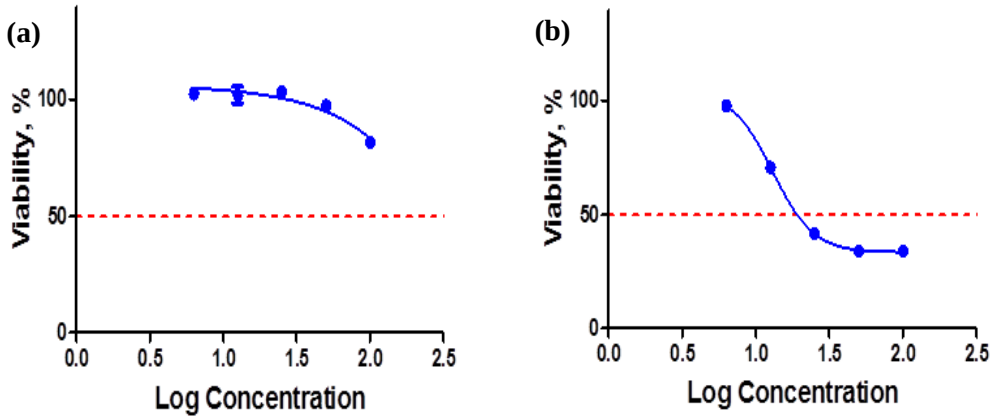

Figure 2: Dose response curves showing the cytotoxic effects of *Diospyros chamaethamnus* extracts, both (a) aqueous and (b) organic, on human fetal lung fibroblast W138 cells. Concentrations (µg/mL) were expressed in log form and used to plot the non-linear graphs against percentage cell viability.

Table 3: Cytotoxic effects (IC_{50}) of *D. chamaethamnus* and *G. coleosperma* root extracts on human fetal lung fibroblast cells, WI38.

Treatment	Solvent	IC_{50} (µg/mL)
G. coleosperma	Aqueous	>100
	Organic	>100
D. chamaethamnus	Aqueous	>100
	Organic	13.03
Etoposide		5.1

Discussion

The presence of phytochemicals such as alkaloids, phenols and terpenoids in plant extracts has been associated with anticancer activity. Alkaloids are a part of many medicinal plants and have been shown to confer various pharmacological activities, including anticancer, with varied modes of action as discussed (Lu *et al*., 2012). Anthraquinones, coumarins and flavonoids are all part of the phenol phytochemical parent group and have been attributed to possess anticancer, antioxidant, anti-inflammatory, antimutagenic properties (Karimi *et al*., 2012). Daphnoretin, a coumarin isolated from *Wikstroemia indica* was found to confer anticancer activity against Ehrlich ascites carcinomas (Nirmala et al., 2011) while anthraquinones have been known to confer *in vivo* pharmacological activities such as antioxidant, antileukemia, analgesic and other functions (Singh and Geetanjali, 2005). Terpenoids are known as anti-inflammatory compounds and have been found to be useful in wound healing and also possessing antitumor properties (Salminen et al., 2008). TLC can confirm the presence or absence of a phytochemical compound class but does not provide any qualitative or quantitative information. Although TLC showed phytochemical profiles of the two plants, more robust analytical techniques such as ultraviolet and visible spectroscopy (Jagessar and Allen, 2012), infrared spectroscopy (Starlin et al., 2012), mass spectroscopy (Soam et al., 2013) and Nuclear Magnetic spectroscopy, together with bioassay guided fractionation (Nyoya et al., 2014) are needed to identify the anticancer phytochemicals.

Table 4: Secondary metabolites reported to have or exhibit anticancer activities.

Reported secondary metabolites with anticancer activities	References
Alkaloids	Mohan *et al*. (2012); Moudi, Go, Yien & Nazre (2013)
Anthraquinones	Yordanova and Koprinarova (2014)
Coumarins	Salem *et al*. (2016)
Flavonoids	Katyal *et al*. (2014)
Terpenoids	Thoppil and Bishayee (2011)
Steroids	Yan *et al*. (2009)

Based on the presence of phytochemicals such as phenols, terpenoids and alkaloids, plants in this study were screened further against a panel of three cancer cells lines with different sensitivity levels to anti-cancer agents to allow more generalization of the interpretation of results. Both the aqueous and organic extracts derived from the root of *G. coleosperma* displayed IC_{50} values below100 µg/ml for MCF7only. It was reported that the slower metabolism of the TK10 cell model (Garner and Eastman, 2010) may contribute to observed differences in the response of the TK10 compared to other cancer cell lines such as MCF7 cells. This study also showed remarkable activity in the organic extract of *D. chamaethamnus* across the three cell lines, which can be indicative of the nonpolar compounds extracted by the organic solvent. However, *D. chamaethamnus* organic extract displayed potent cytotoxicity against the human fetal fibroblast cells, indicating that the extract contains phytochemicals which are non-selective between cancer and non-cancerous cells. The use of a panel of different cell lines for anticancer screen and the cytotoxicity yields an unbiased means solid case to gauge selectivity of extracts against cancer cells. This is indicated by a selectivity index (SI) by the use of IC_{50} obtained against cancerous cell lines (anticancer activity) and non-cancerous cell lines (cytotoxicity) (Mahavorasirikul et al., 2010). The SI of a compound or extract reflects its potential usefulness in the treatment of diseases with minimal side effects on normal body cells (Hafidh et al., 2012). In cancer chemotherapy, the search of new therapeutic medicines stems from the lack of selectivity of many existing clinical chemotherapeutic entities, which often results in adverse side effects such as fatigue, nausea, alopecia and others (Gonzalez-Arrigada et

al., 2013, Ihbe-Heffinger et al., 2013). In this study, SI was not determined owing to the fact that IC_{50} values obtained where higher than 100 µg/ml, which is above range for substantial/notable anticancer activity of a crude plant extract.

According to literature, $IC_{50} \leq 100$ µg/ml for at least two cell lines indicates moderate anticancer activity (29). The NCI standard indicating anticancer activity (Fouche et al., 2008) for a crude plant extract is an IC_{50} value of ≤ 30 µg/ml. The two standards are different because of the size of the panel of cell lines used. The NCI screens a plant extract against a panel consisting of 60 cancer cell lines (Fouche et al., 2008), while the CSIR uses a panel consisting of 3 cell lines (Mashele and Kolesnikova, 2010). In this study, both plants were screened against 3 cancer cell lines, similar to the panel at CSIR, hence the adoption of CSIR's activity criteria. The IC_{50} values in this study are all below 100 µg/ml. The observed anticancer activity coupled with equally cytotoxic activity of the *D. chamaethamnus* extract may be differentiated in future studies through the use of activity guided fractionation (Weller, 2012).

Both *G. coleosperma* and *D. chamaethamnus* have a record for use against febrile ailments and also wound treatment (von Koenen, 2001). The results obtained in this study support that unrelated ethno-medicinal use can be used as a tool for selection of medicinal plant candidates for anticancer screen. Since Namibia has such a rich plant diversity, we propose that plants with unrelated ethnomedicinal history be used in throughput screens in the discovery of effective and safe medicinal candidates for cancer therapy.

This paper reported on the phytochemical profile, anticancer and cytotoxicity activities of *D. chamaethamnus* and *G. coleosperma*. Phytochemicals such as alkaloids, anthraquinones, flavonoids and steroids of both plants were similar. The anticancer screen against a panel consisting of three cancer cell lines showed that the organic extract of *D. chamaethamnus* displayed better activity, even in comparison to etoposide, the positive control. Cytotoxicity screen revealed that the organic extract of *D. chamaethamnus* was toxic. This study also shows the value of random screen or screening of plants for pharmacological activity of plants with unrelated use in a traditional setting. We recommend that future studies focus on fractionation of plant extract to identify active phytochemical/s followed by their characterization, in addition to mechanistic studies to determine the mode of action, which can aid in the identification of novel alternative medicinal entities.

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