Inhibition of phosphatidylinositol 3-kinase (PI3K) enzyme and human skin carcinoma cell growth by *Combretum apiculatum* Sond.

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Abstract

The aim of this study was to determine the antiproliferative effect of an ethanolic leaf extract of *Combretum apiculatum* (CA) against human epidermoid carcinoma (A431) and human malignant melanoma (UCT-MEL1) cells, the toxic potential of CA on non-cancerous human keratinocytes (HaCat) and to evaluate the inhibitory activity against the phosphatidylinositol-3-kinase (PI3K) enzyme, which is involved in tumor survival and proliferation. The protective effect of CA on hepatocellular carcinoma (HepG2) cells, in which toxicity was induced using acetaminophen, was further determined. The CA extract was found to exhibit a 50% inhibitory concentration (IC_{50}) of 56.40 ± 6.11 and 90.53 ± 4.94 µg/mL on A431 and UCT-MEL-1 cells respectively. Against non-cancerous HaCat cells an IC_{50} value of 62.20 ± 3.52 µg/mL was obtained, resulting in a selectivity index of 1.10 and 0.69 on A431 and UCT-MEL-1 cells respectively. Therefore, CA showed a higher selective antiproliferative effect towards squamous cell carcinoma cells. *Combretum apiculatum* was also found to have a protective effect against acetaminophen induced toxicity in HepG2 cells, with an 11% protection at 5 µg/mL. Lastly, the results from the PI3K assay revealed that CA was able to significantly inhibit the PI3K enzyme at each of the tested concentrations, with the highest inhibition noted at a concentration of 0.4 µg/mL with a relative percentage of 106.38 ± 26.36 %, which was comparable to the positive control, wortmannin. This study provides the first report of the activity of *Combretum apiculatum* against skin cancer cell lines and the potential inhibitory activity of this species on the PI3K enzyme.

Keywords: Antiproliferative activity, Human epidermoid carcinoma, Human keratinocytes, PI3K inhibition, Apoptosis, Hepatoprotection, *Combretum apiculatum*

Abbreviations: 143B: Human osteosarcoma cells, A2058, A375, Colo 800, SK-MEL5, SK-MEL28, UACC-903, UCT-MEL-1: Human malignant melanoma; A431: Cutaneous squamous cell carcinoma; AKT: Protein kinase B; B16F10: Murine malignant melanoma; Bax: B-cell associated protein X; BCC: Basal cell carcinoma; Bcl-2: B-cell lymphoma 2 family; B-PIP3:

Biotinylated-Phosphatidylinositol (3,4,5) triphosphate; BxPC-3: human pancreatic cells; CaCo2: Colorectal adenocarcinoma; CPD: Cyclobutane pyrimidine dimer; DMEM: Dulbecco's Modified Eagles Medium; DPPH: 2,2-diphenyl-1-picryhydrazyl; EGFR: Epidermal growth factor receptor; GRP-1: General receptor of phosphoinositides 1; HaCat: Human keratinocytes; HeLa: Cervical cancer; Hep2: Human epithelial 2; HepG2: Human hepatocellular carcinoma; HL 60: Promyelocytic leukemia; IC_{50}: 50% inhibitory concentration; LPS: Lipopolysaccharide; MM: Malignant melanoma; MCF-7: Human breast adenocarcinoma cells; mTOR: Mechanistic target of rapamycin; PARP: Poly (ADP ribose) polymerase; PI3K: Phosphotidylinositol-3-kinase; PIP2: Phosphatidylinositol (4,5)-bisphosphate; PIP3: Phosphatidylinositol (3,4,5)-triphosphate; PTEN: Phosphatase tensin homolog; Raw 264.7: Murine macrophages; SA-HRP: Streptavidin-horse radish peroxidase; Saos-2: Human osteosarcoma cells; SCC: Squamous cell carcinoma; SI: Selectivity index; T24: Human bladder cancer cells; TMB: 3,3',5,5'-Tetramethylbenzidine; UV: Ultraviolet; XIAP: X-linked inhibitor of apoptosis

1. Introduction

According to the World Health Organization, 2-3 million non-melanoma skin cancers and approximately 132,000 malignant melanoma cases occur globally every year (World Health Organization, 2020). There are three major types of skin cancer, which can be classified into two groups; non-melanoma skin cancer; consisting of squamous cell carcinoma (SCC) and basal cell carcinoma (BCC), and malignant melanoma (MM) (Sing et al., 2015). Basal cell carcinoma, which originates from pluripotent cells in the epithelium, most often occurs due to frequent sun exposure, which typically gives rise to slow growing tumours that rarely spread to other sites in the body. The severity of BCC is often noted when lesions go undetected or untreated (Lear et al., 2007). Squamous cell carcinoma, is a malignant tumour arising from squamous cells in the epidermis of the skin. The potential of SCCs to metastasize is often low, however it increases significantly when lesions are not treated (Motley et al., 2002). Malignant melanoma, which is the most

lethal type of skin cancer, arises from melanocytes and has a high metastatic potential which often leads to a poor prognosis (Liu et al., 2018).

South Africans have a high risk of developing skin cancer due to year-round exposure to high solar ultraviolet radiation (Norval et al., 2014; Wright et al., 2012). South Africa has the second highest incidence of skin cancer in the world following Australia, with approximately 20,000 skin cancer cases reported from the ~115,000 cancer cases diagnosed each year (CANSA, 2014; 2010). Every person is at risk of developing skin cancer, however individuals with a fair skin colour are at a higher risk due to the reduced amount of melanin present within their skin. Norval et al (2014), reported that Caucasians have the highest incidence rate of SCC, BCC and MM followed by mixed-race individuals, Africans and Asians (Norval et al., 2014).

Melanin, which is not only responsible for skin pigmentation, also plays a role in photoprotection. Exposure to solar ultraviolet radiation is one of the major contributing factors which causes DNA damage in both the epidermal and dermal layer of the skin resulting in the development of skin cancer. The mutagenic photoproduct, cyclobutane pyrimidine dimer (CPD) is one of the main contributing factors to developing skin cancer. A study by Fajuyigbe et al (2018), found that melanin was able to protect against the CPD mutagen by 59-fold in the basal layer of African skin, followed by 16.5, 5, 8-fold protection in the middle-, upper epidermis and overall epidermis layer, which correlates with the distribution of melanin, which is higher in the basal layer (Fajuyigbe et al., 2018; Leiter and Garbe, 2008; Pfeifer et al., 2005). Although the risk for Africans to develop skin cancer is less, the development of melanoma is often diagnosed at a late stage where the cancer has metastasized to other organs within the body, leading to a poor prognosis and a higher mortality rate (Stein et al., 2008).

Phosphatidylinositol-3-kinases (PI3Ks) and its isoforms (p110α, p110β, p110γ and p110 δ) are lipid kinases involved in physiological and cellular processes such as cell proliferation, growth and survival (Engelman et al., 2006). Alterations in the activation of PI3K are common in several cancers, including

skin cancer, therefore providing a valuable therapeutic target for anticancer drug development (Liu et al., 2009). In clinical trials, drugs targeting PI3K, such as non-isoform selective pan PI3K inhibitors, have not produced significant results, however recent pre-clinical studies have shown that PI3K isoforms possess different roles in cellular signaling and cancer proliferation. This suggests that inhibitors which target specific isoforms may achieve greater therapeutic efficacy (Martini et al., 2013). In skin cancer cells, the PI3K/Akt/mTOR pathway is typically activated by mutations in the tumour suppressor gene, phosphatase tensin homologue (PTEN), which enables the phosphorylation of phosphatidylinositol 4,5 bisphosphate (PIP2) by PI3K, thus converting PIP2 to phosphatidylinositol (3,4,5)-triphosphate (PIP3). Subsequently, PIP3 activates pathways containing anti-apoptotic genes resulting in uncontrolled proliferation (Noorolyai et al., 2019). In a previous study, enhanced levels of PI3K were detected in human cutaneous malignant melanoma (UACC-903) cells due to loss of PTEN, which resulted in increased tumorigenicity. Furthermore, hybrids of UACC-903 where PTEN was introduced, displayed normal physiological functions such as growth, cell survival and apoptosis. After several days, these cells subsequently became tumorigenic due to either epigenetic, deletion or mutation alterations in PTEN resulting in the loss of normal physiological functioning (Stahl et al., 2003). Similarly, loss of PTEN in cutaneous squamous cell carcinoma cells (A431) was reported in a study which found that A431 cells were resistant to the epidermal growth factor receptor (EGFR) inhibitor, EKB-569. High levels of EGFR are detected under conditions where PTEN loss is evident (Vivanco et al., 2010). The loss of PTEN, an antagonist of the PI3K/AKT/mTOR pathway, supported the enhanced levels of PI3K observed in skin cancer (Ming and He, 2009).

Several medicinal plants have been reported for their antiproliferative activity against skin cancer cells. The methanolic extract of *Sutherlandia frutescens* (L.) R.Br., a well-researched South African medicinal plant, tested at 0.625 mg/mL inhibited the proliferation of human malignant melanoma (A375 and Colo 800) cells, after a 72h period by 38 and 57% respectively (van der Walt et al., 2016). Furthermore, *Aniba*

rosaeodora Ducke essential oil at 400 nl/mL inhibited proliferation in human cutaneous squamous carcinoma (A431) cells, by 80% (Sœur et al., 2011).

Combretum caffrum (Eckl.& Zehy.) Kuntze, a species within the Combretaceae family, is traditionally used by the Xhosa tribe in the Eastern Cape, where the bark is combined with other herbs and used as a treatment for cancer (Schmelzer, 2008). This species is well known for the combretastatin compounds such as stilbenes (Combretastatin A), dihydrostilbenes (Combretastatin B), phenanthrenes (Combretastatin C) and macrocyclic lactones (Combretastatin D), however the Combretastatin A family has garnered the most interest due to their anti-angiogenic potential resulting in loss of blood supply to tumours and ultimately tumour necrosis (Cragg et al., 2010, Karatoprak et al., 2020). Combretastatin A4, which was first isolated from *Combretum caffrum*, was shown to inhibit tumor cell growth and tubulin polymerization. However, due to its limited water solubility, the water-soluble pro-drug, combretastatin A4 phosphate was synthesized, a tubulin depolymerizing agent which disrupts the cytoskeleton and has anti-angiogenic properties which causes cell death by inhibiting the flow of blood through the vascular system, thereby starving tumor cells of oxygen and nutrients (West and Price, 2004). In 2003, the US Food and Drug Administration (FDA) designated combretastatin A4 phosphate an orphan drug for the treatment of thyroid cancer and in 2006 it was designated an orphan drug for the treatment of ovarian cancer (Cragg and Pezzuto, 2016).

For this study, a plant belonging to the Combretaceae family, *Combretum apiculatum*, was investigated. The Combretaceae family consists of 18 genera, the largest of which are *Combretum* with approximately 370 species and Terminalia, with about 200 species (McGaw et al., 2001). *Combretum apiculatum*, native to Southern Africa, is a semi-evergreen tree growing up to 10 m tall, which is commonly referred to as red bushwillow (English), rooiboswilg (Afrikaans), umbondwe (Zulu), imbondvo (Swazi), Mohwidiri (Tswana), Mohwelere (Sepedi), Muvuvha (Tshivenda) (Masupa and Rampho, 2011; Schmidt et al., 2002). *Combretum apiculatum* possesses several ethnobotanical uses, the sap from the roots has been used for the treatment of snakebites and decoctions, prepared from the leaves, have been used for the

treatment of scorpion stings. The leaves have been used for abdominal disorders and the stem bark has also been used for conjunctivitis, whereas a decoction prepared using the leaves has been used for stomach ailments (Kokwaro, 1976; Von Koenen, 1996; Watt and Breyer-Brandwijk, 1962).

The present study was conducted to determine the effect of an ethanolic leaf extract of *C. apiculatum* (CA), against skin cancer cells; human epidermoid carcinoma (A431) and human malignant melanoma (UCT-MEL1), and on non-cancerous human keratinocytes (HaCat). The mechanism of action was investigated by testing the inhibitory potential of CA on the PI3K enzyme. *Combretum apiculatum* was further evaluated for its hepatoprotective activity.

2. Materials and Methods

2.1 Materials, chemicals and reagents

Human malignant melanoma (UCT-MEL1) and human keratinocytes (HaCat) were donated by Dr Lester Davids from the University of Cape Town (South Africa). The human hepatocellular carcinoma (HepG2) cells were donated by Prof Lyn-Marie Birkholtz from the University of Pretoria (South Africa) and human cutaneous squamous carcinoma (A431) cells were purchased from the European collection of authenticated cell cultures (ECACC). The Dulbecco's modified Eagle's medium (DMEM), phenol-red trypsin-EDTA (0.25%), phosphate buffered saline, foetal bovine serum, antibiotics, antifungal agents and PrestoBlue were purchased from Thermofisher Scientific (Johannesburg, South Africa). Cell culture plates and flasks were sourced from Lasec SA Pty Ltd. (Midrand, South Africa). The PI3 Kinase Activity/Inhibitor ELISA kit (Cat # 17-493) and other chemicals and reagents, including dimethyl sulfoxide (DMSO), actinomycin D, acetaminophen and silymarin were purchased from Sigma-Aldrich (Johannesburg, South Africa).

2.2 Methods

2.2.1 Plant collection

Combretum apiculatum leaves were collected from the University of Pretoria Experimental farm (Pretoria, South Africa) during February 2018. The plant material was identified by Ms Magda Nel at the University of Pretoria HGWJ Schweickerdt Herbarium (PRU) based on its morphological features where a voucher specimen (PRU 124380) was deposited. The plant name was confirmed using The Plant List database available at http://www.theplantlist.org.

2.2.2 Plant extract preparation

The ethanolic extract of *C. apiculatum* (CA) was prepared with fresh leaf material (425 g) using ethanol in a 1:4 ratio, in which one part of the fresh plant material was blended in four parts of ethanol (1700 mL). The resulting solution was vacuum filtered through Whatman No.1 filter paper using a Büchner funnel. Thereafter the extract was rotary evaporated using a Buchi R-200 rotavapor system. The extract was placed in an air-tight container and refrigerated at -20˚C until use.

2.2.3 Cell culture

The human epidermoid carcinoma (A431), human malignant melanoma (UCT-MEL-1), human hepatocellular carcinoma (HepG2) and human keratinocyte (HaCat) cell lines were grown in tissue culture flasks, which contained Dulbecco's Eagles medium (DMEM), at 37ºC and 5 % CO_2. The DMEM media was supplemented with 10 % heat inactivated foetal bovine serum, 1 % antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin) and 1 % antifungal agent (250 µg/mL amphotericin B). Cells were passaged once an 80 % monolayer formed. Trypsin-EDTA (0.25 % trypsin containing 0.01 % EDTA) was used to detach the cells followed by the addition of complete medium (10 % DMEM) to inhibit the reaction.

2.2.4 In vitro antiproliferative activity

The antiproliferative activity of the CA extract on A431, UCT-MEL-1, HepG2 and HaCat cells was determined using the PrestoBlue cell viability assay as described by (Lall et al., 2013). The cells were

seeded in a 96-well microtitre plate at the following concentrations; 1×10^5 cells/mL (A431), 5×10^4 cells/mL (UCT-MEL-1 and HaCat) and 1×10^3 cells/mL (HepG2), and incubated for 24 h at 37˚C and 5 % CO_2 to allow for cell attachment. A stock solution of the extract (20 mg/mL) and the positive control, actinomycin D (1 mg/mL) was prepared, followed by serial dilutions to obtain final concentrations ranging from 400 - 3.125 µg/mL for the extract and 0.05 - 3.9×10^{-4} µg/mL for actinomycin D, in the 96-well plates. Controls included, the negative control (cells grown in media), the vehicle control (2 % DMSO) and a control which contained no cells, only media (PrestoBlue control). Plates were incubated for an additional 72 h except for the HepG2 cells which were incubated for 24 h, thereafter 20 µl PrestoBlue cell viability reagent was added to each of the wells. After an additional 2 h incubation, the fluorescence was measured at an excitation wavelength of 560 and an emission of 590 nm (Victor Nivo Multimode plate reader). The samples were tested in triplicate to calculate percentage cell viability (using the below equation) and the 50 % inhibitory concentration (IC_{50}). The data was analysed using GraphPad prism 7 software.

$$(\%\, Viability) = \frac{Fluor.sample - Flour.PB\ control}{Flour.vehicle\ control - Flour.PB\ control} \times 100$$

Where $Flour_{sample}$ is the fluorescence of (sample including PrestoBlue), $Flour_{PB}$ is the fluorescence of (PrestoBlue control, without any cells, 0 % viability), and $Flour_{vehicle\ control}$ is the fluorescence of (vehicle control including PrestoBlue, 100 % viability).

2.2.5 In vitro hepatoprotective assay

The method as described by Oosthuizen et al. (2017) was used to determine the hepatoprotective effect of CA. The HepG2 cells were seeded in a 96 well microtitre plate at a concentration of 1×10^3 cells/mL and incubated for 24 h at 37°C and 5 % CO_2 to allow for cell attachment. Thereafter, the CA extract was added at final concentrations of 50, 10 and 5 µg/mL, followed by the addition of the toxic inducer, acetaminophen, at a final concentration of 25 mM. Controls included, a vehicle control (2 % DMSO), the

positive control Silymarin (50 µg/mL) with the addition of acetaminophen, the toxic inducer (25 mM), cells grown in media only and a PrestoBlue control without the addition of cells. The plates were incubated for an additional 18 h at 37ºC, 5 % CO_2. Thereafter, 20 µL of PrestoBlue was added to all the wells and incubated for another hour. The fluorescence was measured at an excitation wavelength of 560 and an emission of 590 nm (Victor Nivo Multimode plate reader). The samples were tested in triplicate to calculate the hepatoprotective effect (using the below equation). The data was analysed using GraphPad prism 7 software.

$$\%\ Hepatoprotection = \frac{Flour.sample - Flour.toxic\ inducer}{Flour.vehicle\ control - Flour.toxic\ inducer} \times 100$$

Where $Flour_{sample}$ is the fluorescence of (sample including PrestoBlue), $Flour_{toxic\ inducer}$ is the fluorescence of (acetaminophen control, 0% viability), and $Flour_{vehicle\ control}$ is the fluorescence of (vehicle control including PrestoBlue, 100% viability).

2.2.6 Inhibition of PI3K activity

The inhibitory potential of CA against the PI3K isoform (p110β) was determined using the PI3 kinase activity/ inhibitor ELISA assay (Merck Millipore, Cat # 17-493) according to the manufacturer's instructions (Sigma Aldrich, 2020). The assay is based on the principle that PI3 kinase binds to and phosphorylates the substrate, phosphatidylinositol (4,5)-bisphosphate (PIP2) converting it to phosphatidylinositol (3,4,5)-triphosphate (PIP3). The capture antibody, general receptor of phosphoinositides-1 (GRP-1), has a high binding affinity for PIP3, generated as a product of the reaction between the PI3K enzyme and PIP2 substrate. The GRP-1 capture antibody is also able to capture the biotinylated-PIP3 (B-PIP3), which can be detected by streptavidin-horse radish peroxidase (SA-HRP) conjugate, resulting in a colour reaction upon addition of 3,3',5,5'-tetramethylbenzidine (TMB). A stop solution is added resulting in yellow colour development, where a low colour intensity correlates with high PI3 kinase activity. The CA extract was tested at final concentrations of 50, 10, 2 and 0.4 µg/mL, and controls included, a vehicle control (2 % DMSO) and the positive control, wortmannin (100 nM). The

plate was read at 450 nm using the BIO-TEK power-wave XS multi-well plate reader (A.D.P, Weltevreden Park, South Africa). An additional control, 100 % B-PIP3 was used to calculate the relative percentage to B-PIP3, using the below equation. The relative percentage to B-PIP3, reflects the inhibitory effect against the PI3K enzyme. The samples were tested in triplicate and the data was analysed using GraphPad Prism 7.

$$Relative\ \%\ to\ B-PIP3 = \frac{Absorbance\ of\ sample}{Absorbance\ of\ Biotinylated-PIP3}\ x\ 100$$

2.2.7 Statistical analysis

The experimental results are presented as means ± standard error of the mean (SEM)/ standard deviation (SD) of three independent experiments (n =3). The IC_{50} values were calculated using nonlinear regression analysis of the sigmoidal dose response curves using GraphPad Prism 7. The significance of the data was analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test using GraphPad Prism 7, where $^{*}P$ indicates significance when compared to the control (+) as described in the results section.

3. Results and discussion

3.1 In vitro antiproliferative activity

This study evaluated the antiproliferative activity of the CA extract against two malignant skin cancer cell lines using the PrestoBlue cell viability reagent. Actinomycin D, an antibiotic peptide produced by *Streptomyces* species, was used as the positive control as it is able to cause DNA strand breaks which results in a cytotoxic effect towards cells (Kleeff et al., 2000). The assay is based on the ability of viable cells to reduce resazurin to resorufin (Lall et al., 2013). The extract was able to inhibit the proliferation of the cancerous cell lines in a dose-dependent manner with higher antiproliferative activity observed against the A431 cells when compared to the activity against the UCT-MEL-1 cells (Table 1). The antiproliferative potential against normal keratinocytes (HaCat) was determined, which showed an IC_{50} value of 62.20 ± 3.82 µg/mL. The selectivity index (SI) was calculated to determine whether the extract

was more selective towards the cancerous cells than the non-cancerous cells, using the below equation (Suffness and Pezzuto, 1990).

$$Selectivity\ index\ (SI) = \frac{IC_{50}\ against\ non-cancerous\ cells}{IC_{50}\ against\ cancerous\ cells}$$

Both the positive control, actinomycin D and the extract showed a higher selective antiproliferative activity towards the non-cancerous cells when compared to UCT-MEL-1, with SI values of 0.8 and 0.69 respectively. However, when compared to A431, the extract and actinomycin D were more selective towards the cancerous cells with SI values of 1.10 and 1.33 respectively (Table 1). In pre-clinical studies (*in vitro*), it has been reported that a drug candidate should be considered for *in vivo* studies if the selectivity index is greater than that of the standard anticancer drug. However, isolation and identification of novel compounds could result in a significantly higher selectivity index when compared to the standard anticancer agent (Calderón-Montaño et al., 2014, López-Lázaro, 2015), therefore this should be considered for future studies.

Table 1

Antiproliferative activity of *Combretum apiculatum* against skin cancer cells and human keratinocytes

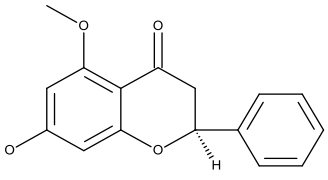
Sample	A431	UCT-MEL-1	HaCat	SI[c]: HaCat vs A431	SI: HaCat vs UCT-MEL-1
	IC^a_{50} (µg/mL ± SD[b])				
Combretum apiculatum Sond.	56.40 ± 6.11	90.53 ± 4.94	62.20 ± 3.82	1.10	0.69
Actinomycin D[d]	0.03 ± 0.03	0.05 ± 0.05	0.04 ± 0.002	1.33	0.8

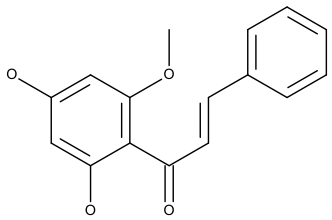
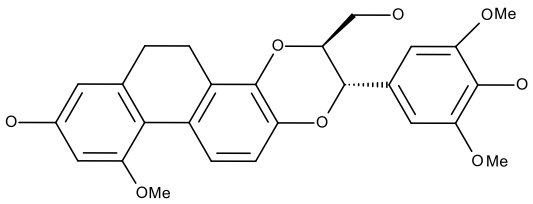
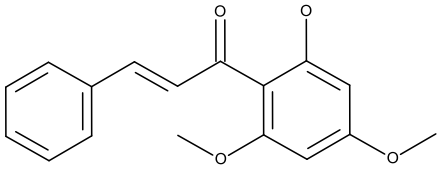
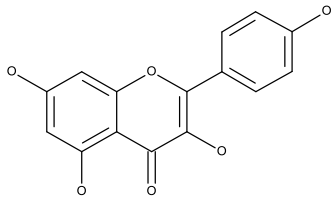
[a]Fifty percent inhibitory concentration; [b]Standard deviation; [c]Selectivity index (calculated using equation 3); [d]Positive control for antiproliferative activity

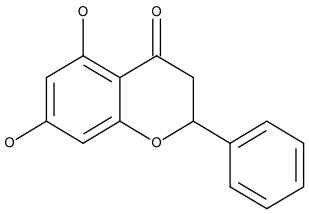
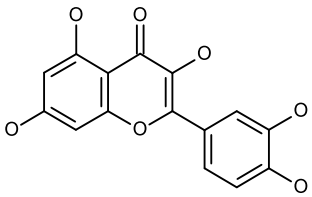
This species has been extensively studied for its various biological activities such as its anti-fungal, anti-inflammatory and cytotoxicity (Fyhrquist et al., 2006; Masoko et al., 2007; McGaw et al., 2001). The cytotoxic potential of *Combretum* species against cancer cell lines has been previously reported. Fyhrquist et al. (2006) investigated the cytotoxic effects of seven *Combretum* species against bladder cancer (T24), cervical cancer (HeLa) and breast cancer (MCF-7). The methanolic extract prepared using the leaves of *Combretum fragrans* F. Hoffm , tested at 25 µg/mL, inhibited the growth of T24, HeLa and MCF-7 cells by 7.0 ± 0.6, 6.8 ± 0.08 and 13.3 ± 1.3% respectively, whereas the methanolic extract prepared using the leaves of *C. apiculatum,* tested at 25 µg/mL, showed significantly higher activity. This extract inhibited the growth of bladder cancer cells (T24) and breast adenocarcinoma cells (MCF-7) by 65.0 ± 17 and 40.1 ± 6.8% respectively (Fyhrquist et al., 2006). Numerous compounds have been isolated from *C. apiculatum,* which have been tested for their antiproliferative activity against cancerous cells (Table 2). Furthermore, Combretastatins (Combretastatins A1-6) isolated from *Combretum caffrum* have been reported to possess significant anticancer properties, where Combretastatin A4 and Combretastatin A1 (cis-stilbenes) displayed the most significant activity, however, the Combretastatin A family of compounds is yet to be identified in *Combretum apiculatum.* (Karatoprak et al., 2020).

Table 2

Compounds isolated from *Combretum apiculatum* which have been tested for their antiproliferative activity

Compound name and structure	Biological activity against cancer	References
Alpinetin 	Alpinetin induced apoptosis in human pancreatic cells (BxPC-3), possibly through the regulation of the B-cell lymphoma 2 family (Bcl-2), X-linked inhibitor of apoptosis protein (XIAP) expression and the release of cytochrome c.	Du et al., 2012

Cardamonin 	Cardamonin significantly lowered the proliferation of malignant melanoma, A375, cells at concentrations ≥ 5 µM and induced apoptosis through increased procaspase 3 activity and Poly (ADP-ribose) polymerase (PARP) cleavage.	Berning et al., 2019
Erathrin A (9,10-dihydrophenanthrene derivative) 	Cytotoxic activity was observed against promyelocytic leukemia (HL-60) cells with an IC_{50} of 14.50 µM.	Rui et al., 2016
Flavokawain B 	Flavokawain B significantly inhibited nitric oxide secreted by lipopolysaccharide induced murine macrophages (Raw 264.7) cells with an IC_{50} of 9.8 µM. In another study, Flavokawain B exhibited significant cytotoxicity against osteosarcoma cells (143B and Saos-2) and induced apoptosis through the activation of procaspase 3 and 9; and the downregulation of Bcl-2.	Ji et al., (2013); Lin et al., (2009)
Kaempferol 	Kaempferol significantly lowered the proliferation of malignant melanoma, cells (A375) with an IC_{50} of 20 µM. Furthermore, key proteins of the PI3K/Akt/mTOR pathway (p-PI3K, p-Akt and p-mTOR) were downregulated by Kaempferol.	Yang et al., 2018

Pinocembrin 	Pinocembrin induced apoptosis in the human malignant melanoma (A375) cells and in the murine malignant melanoma (B16-F10) cells. The proportion of apoptotic cells in B16F10 and A375 increased from 9.46% ± 1.46% and 10.53% ± 0.32% to 26.49 ± 1.19% and 32.57% ± 2.29% respectively at a concentration of 200μM after 48h.	Zheng et al., 2018
Quercetin 	Quercetin inhibited the proliferation of two human malignant melanoma cell lines, A375 and A2058, with IC_{50} values of 99.59 ± 10 and 118.1 ± 14.2 μM and induced apoptosis in both cell lines.	Cao et al., 2013

3.2 In vitro hepatoprotective assay

The hepatoprotective activity of CA was conducted at non-toxic concentrations (50, 10 and 5 μg/mL) as determined by the antiproliferative assay which showed an IC_{50} value of 66.92 ± 4.49 μg/mL (Fig.1a). At a concentration of 50 μg/mL, a cell viability of 82.20 ± 3.16 % was observed after 72h of exposure to CA , which was statistically similar ($P>0.05$; unpaired t-test) to that of the vehicle control (2% DMSO). A significant decrease in cell viability was observed on HepG2 cells treated with acetaminophen (25 mM). *Combretum apiculatum* exhibited 11% protection at 5 μg/mL, whereas silymarin, displayed a protective effect of 60% (Fig. 1b). In a study conducted by Banskota et al (2000), the methanol extract prepared using the leaves of *Combretum quadrangulare* Kurz showed significant *in vivo* hepatoprotective effects on D-galactosamine and lipopolysacharride treated mice with a

76.5% protective effect (Banskota et al., 2000).

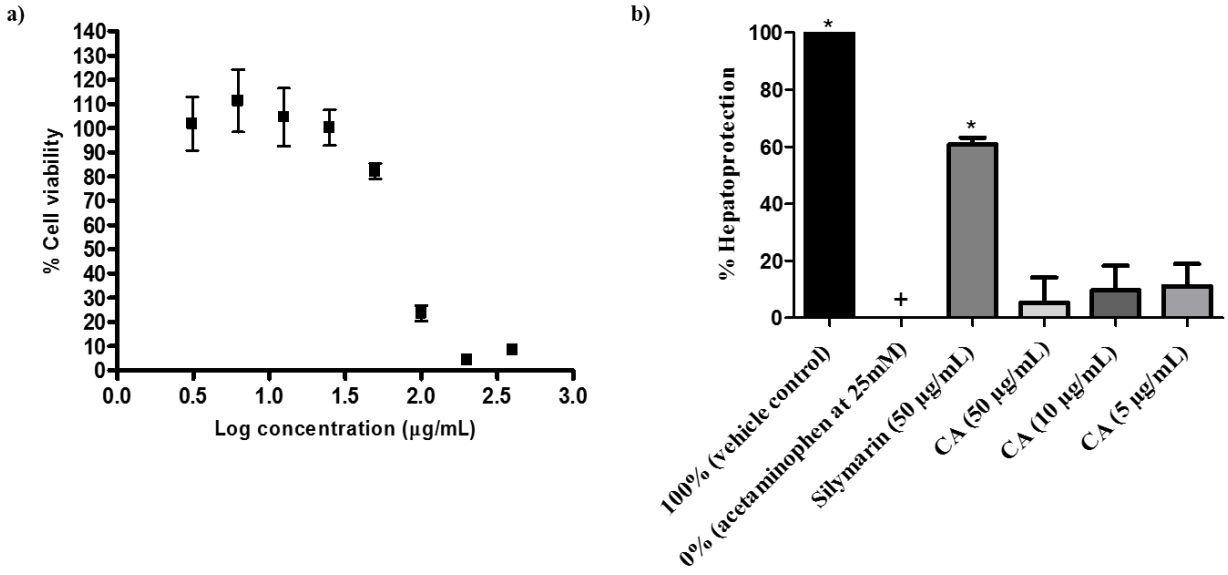


Fig. 1a) Percentage cell viability of HepG2 cells after 72h exposure to *Combretum apiculatum* ethanolic leaf extract at concentrations ranging from 400 – 3.125 µg/mL. Data is represented as log concentrations (in µg/mL) versus cell viability as mean ± SD (n=3); b) Hepatoprotective effect of *Combretum apiculatum* ethanolic leaf extract (50, 10 and 5 µg/mL) on HepG2 cells treated with acetaminophen (25 mM). Controls included silymarin (50 µg/mL), vehicle control (2 % DMSO) and acetaminophen (25 mM). Data is represented as mean ± SEM (n=3). Statistical significance was determined using one-way ANOVA followed by Dunnett's multiple comparison test, where *P <0.001 indicates significance when compared with acetaminophen (25 mM) (+).

3.3 Inhibition of PI3K activity

The PI3K enzyme is a heterodimer comprising of a p85 and p110 subunit. Different isoforms of the PI3K enzyme exist such as p110α, p110β, p110γ and p110δ (Engelman et al., 2006). In this study, p110β was targeted as tumours with PTEN loss are reliant on p110β for PI3K signaling. Furthermore, it was found that tumorigenesis occurred in 30-60 % melanomas due to loss of PTEN function (deletions, mutations and epigenetic silencing). Loss of PTEN function was also detected in cutaneous squamous cell

carcinoma where the level of EGFR was upregulated and the EGFR inhibitor was not effective due to PTEN loss (Davies, 2012; Stahl et al., 2003; Vivanco et al., 2010).

The CA extract was able to significantly inhibit the PI3K enzyme, with relative percentage values similar to that of the B-PIP3 control. At concentrations of 50, 10 and 2 µg/mL of the extract, relative percentage to B-PIP3 values of 82.64 ± 21.64, 98.12 ± 7.74 % and 104.36 ± 7.58 % were obtained respectively. The relative percentage to B-PIP3 correlates to the inhibitory effect against the PI3K enzyme. At the lowest concentration of 0.4 µg/mL, the relative percentage to B-PIP3 was 106.38 ± 26.36 %, where the extract showed inhibition that was comparable to the positive control, wortmannin, a class I general PI3K inhibitor, which inhibited the PI3K enzyme with relative percentage statistically similar to the B-PIP3 control, of 114.80 ± 22.66 % (Fig.2).

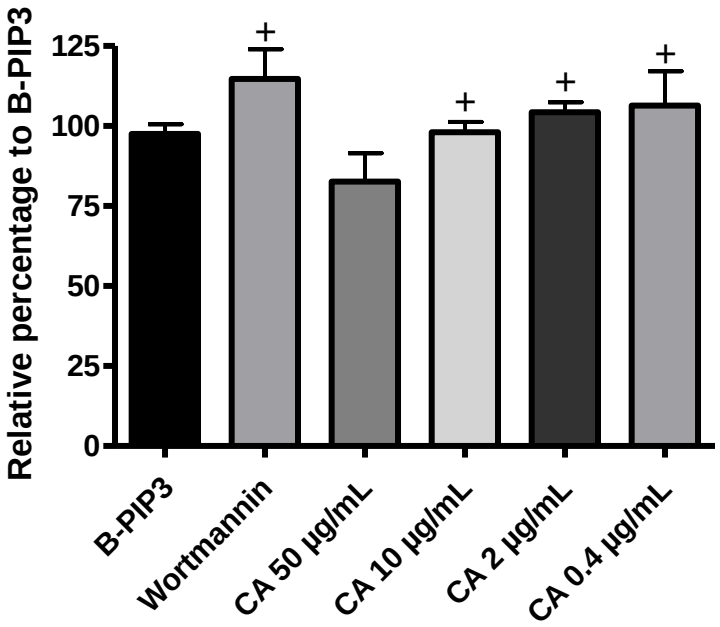


Fig 2. Inhibitory potential of *Combretum apiculatum* ethanolic leaf extract (50, 10, 2 and 0.4 µg/mL) against the PI3K enzyme (p110β isoform). Controls included wortmannin (100 nM) and the biotinylated-PIP3 (100%). Data is represented as mean ± SEM (n=3). Statistical significance was determined using one-way ANOVA followed by

Dunnett's multiple comparison test. Samples which showed statistically similar activity to the wortmannin control were identified (+).

The inhibition of the PI3K enzyme at the lowest concentration (0.4 µg/mL) could be due to competitive inhibition whereby the PI3K enzyme binds a substrate, PIP2, to form an enzyme-substrate complex or it binds an inhibitor (CA plant extract or wortmannin) forming an enzyme-inhibitor complex. The enzyme cannot bind both the inhibitor and substrate thus, if the inhibitor is present in high concentrations then an enzyme-inhibitor complex is able to form. The formation of an enzyme-inhibitor complex indicates that small concentrations of the enzyme are free to bind to the substrate to form PIP3, which activates the Akt and mTOR pathways leading to an influx of anti-apoptotic proteins (Berg et al., 2002).

Conclusion

The ethanolic leaf extract of *Combretum apiculatum* showed antiproliferative activity against the A431 cell line with an IC_{50} of 56.40 ± 6.11 µg/mL. The antiproliferative effect of the CA extract could be due to the numerous compounds within the CA extract, of which several have been identified and evaluated for their activity against cancer cells. However, there is a potential to isolate novel compounds from this species, which could possibly show increased selectivity against cancer cell lines and activity against therapeutic targets associated with tumorigenesis. Furthermore, the CA extract was able to significantly inhibit the PI3K enzyme at the lowest concentration (0.4 µg/mL) with a relative percentage to B-PIP3 of (106.68 ± 26.36 %), however the type on inhibition (competitive, non-competitive or uncompetitive) needs to be confirmed. The PI3K enzyme, which is involved in the activation of anti-apoptotic genes, provides a valuable target for the potential activation of apoptosis in cancer cells. However, future studies such as apoptosis detection, cell cycle analysis and the effect the extract has on anti-apoptotic proteins such as B-cell lymphoma-2 (Bcl-2) and the pro-apoptotic protein, B-cell associated protein X, as well as determining the potential to activate procaspase-3 and 9, should be considered to confirm apoptosis in the cancer cells. Additionally, western blotting should also be conducted to determine the phosphorylation status of PI3K and AKT (downstream of PI3K).

Furthermore, the CA extract showed a protective effect of 11% at 5 µg/mL on HepG2 cells exposed to acetaminophen. The hepatoprotective effect of compounds isolated from the CA extract should be considered for testing. This was the first report on the activity of *Combretum apiculatum* against skin cancer cell lines and the inhibitory potential against the PI3K enzyme.

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Conflict of interest

The authors declare that there is no conflict of interest

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