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In vitro evaluation of the antiproliferative activity of *Carpobrotus edulis* on human neuroblastoma cells

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ABSTRACT

Neuroblastoma is a solid neuroendocrine tumour located outside the cranial cavity and contributes about 15% of all cancer-associated deaths in children. Treatment of neuroblastoma is quite challenging and involves the use of chemotherapy, surgery and radiotherapy. Despite treatment strategies, systemic toxicity are setbacks to patient well-being, hence the need for a new and affordable approach. Medicinal plants are of importance in the field of drug discovery for cancer as some notable anti-cancer agents have been isolated from them. In the present study, the anti-cancer activity of aqueous extract of *Carpobrotus edulis* (*C. edulis*), a ground-creeping edible medicinal plant was investigated in SK-N-BE(2) and SH-SY5Y neuroblastoma cells. The effect of *C. edulis* on cell viability and survival was determined using MTT (3-[4,5-dimethylthiazol-2-yl]−2,5 diphenyltetrazolium bromide) and clonogenic assays respectively. Apoptosis was determined using a Caspase-9 assay kit and flow cytometry was used to measure intracellular reactive oxygen species (ROS) and depolarization of mitochondrial membrane potential. The results show that *C. edulis* inhibits cell viability (IC_{50} of 0.86 mg/ml and 1.45 mg/ml for SK-N-BE (2) and SHSY5Y cells respectively) and colony formation in the neuroblastoma cells as well as induce apoptosis, which is evidenced by an increase in caspase-9 activity in the cells. *C. edulis* also led to a loss of mitochondrial membrane potential and increased production of ROS. Collectively, these results suggest that *C. edulis* induces cell death via induction of mitochondrial-mediated apoptosis and accumulation of intracellular ROS, thus providing a rationale for further investigations.

1. Introduction

Neuroblastoma is a solid neuroendocrine tumour located outside the cranial cavity in children contributes to about 15% of all cancer-associated deaths in children (Louis and Shohet, 2015, Fusco et al., 2018). Neuroblastoma originates from the tissues of the parasympathetic nervous system and frequently develops from the adrenal glands above the kidneys (Brisse et al., 2011). They may also arise from other areas including, the pelvis, abdomen, chest and neck region (Brodeur, 2003). Neuroblastoma patients are categorized as high-risk category when the patients are older than one year of age, and the 5-year overall survival rate in this category of patients is about 40%. However, the low-risk category of patients usually presents about 75% 5-year overall survival (Haupt et al., 2010). The high-risk category makes up about half of the new cases per year and treatment involves the use of chemotherapy, surgery and radiotherapy. Despite the improvement of patient survival rates using this multifaceted therapeutic approach, tumour relapse is still evident in a significant number of patients (Gilman et al., 2009). Furthermore, systemic toxicity to other organs of the body and high cost of chemotherapeutic agents are also challenging to the treatment of cancer. Hence, researchers have continuously explored medicinal plants and plant-derived products as potential sources of effective chemotherapeutic agents as they are readily available, affordable and may show less toxicity to normal cells (Lichota and Gwozdzinski, 2018, Tilaoui et al., 2018).

The use of medicinal plants as a form of complementary and/or alternative medicine is almost as old as humanity and the World Health Organisation (WHO) reported that over 4 billion of the world population (80%) make use of plant or plant-derived products as a treatment for diseases and infections (De Pasquale, 1984, Fabricant and Farnsworth, 2001). Several medicinal plants have been reported to possess pharmacological activities due to the presence of metabolites (active ingredients) which share similar molecular targets as pharmaceutical drugs (Ahn, 2017). These metabolites include saponins, flavonoids, tannins, alkaloids, terpenoids, glycosides, steroids amongst others and are responsible for the biological activities possessed by medicinal

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plants (Shakya, 2016). Some notable anti-cancer agents including, camptothecin, taxol and vinblastine have been identified and characterized from *Camptotheca acuminate*, *Taxus brevifolia* and *Catharanthus roseus* medicinal plants respectively (Cragg and Newman, 2005). More so, herbal medicines play critical roles in slowing down cancer progression as they have been reported to induce apoptosis, regulate DNA repair process as well as inhibit the enzymes and hormones needed for tumour progression (Lee et al., 2002, Ghagane et al., 2017, Russo et al., 2019). It has also been reported that herbal medicines can help to curb the effects of drug-induced systemic toxicity faced by some cancer patients (Rao et al., 2008).

Carpobrotus edulis (*C. edulis*) [family, Aizoaceae] is a ground-creeping edible medicinal plant indigenous to South Africa. Its common names include sour fig, cape fig, hottentots fig (English); *kaapsevy, perdevy, vyerank* (Afrikaans.); *ikhambi-lamabulawo, umgongozi* (Zulu); *igcukuma* (Xhosa). Traditionally, the juice from the leaves is used as a remedy for diarrhoea stomach upset, tuberculosis and skin infections (eczema, dermatitis, and sunburns) (Mathabe et al., 2006, Thring and Weitz, 2006, Scott and Hewett, 2008). Scientifically, previous studies have validated its antimicrobial, antioxidant, anticholinesterase, and immune modulating activities (Ordway et al., 2003, Martins et al., 2011, Ibtissem et al., 2012, Custódio et al., 2012). Its anti-cancer activity in mouse lymphoma cells has been reported with inhibition of p-glycoprotein as a mechanism of action (Martins et al., 2010). Its aqueous extract, and ethanol-water (1:1 v/v) extract was shown to inhibit glycation and induce cytotoxicity in HCT-116 human colon cancer cells (Hafsa et al., 2016). Due to the reported effects of this plant, this study was designed to evaluate the antiproliferative activity of *C. edulis* aqueous extract on human neuroblastoma cells, SK-N-BE(2) and SH-SY5Y.

2. Materials and methods

2.1. Preparation of C. edulis aqueous extract

Fresh leaves of *C. edulis* plant were obtained from the environs of the University of the Western Cape (33.9335° S, 18.6280° E), Cape Town, South Africa. The leaves were identified and authenticated by an expert botanist, Professor Christopher Cupido using standard plant identifications methods. Voucher specimen (UFH 2021–9–01) was deposited in the Giffen Herbarium, Botany Department, University of Fort Hare. Leaves were air-dried to reduce moisture content and reduced to powder using a blender and 1 kg of the powdered leaves were soaked in boiled water and left overnight (12 h) for extraction. The resultant extract was filtered, freeze-dried and stored in airtight containers at − 20 °C until needed. On each day of an experiment, the needed amount of extract was weighed and dissolved in phosphate buffered saline (PBS, Lonza Group Ltd., Verviers, Belgium) to give a stock solution for further dilutions in media.

2.2. Cell lines and culture conditions

The human malignant neuroblastoma cell lines SK-N-BE(2) and SH-SY5Y were a donation by Dr Antonio Serafin, Division of Radiology, Faculty of Medicine and Health Sciences, Stellenbosch University, while, the non-cancerous KMST6 fibroblast was a donation by Prof Mervin Meyer, Department of Biotechnology, University of the Western Cape, South Africa. Cells were cultured in monolayer using Dulbecco Modified Eagles Medium (DMEM, Lonza Group Ltd., Verviers, Belgium), supplemented with 10% foetal bovine serum (FBS, Gibco, Life Technologies Corporation, Paisley, UK) and 1% 100 U/ml penicillin and 100 µg/ml streptomycin (Lonza Group Ltd. Verviers, Belgium). Cells were grown at 37 °C, in a humidified atmosphere of 5% CO2 and 95% air with routine media change. Cells were subcultured at 80% confluency using a solution of 0.25% trypsin EDTA (Lonza Group Ltd., Verviers, Belgium).

2.3. Cell viability assay

The 3-[4,5-dimethylthiazol-2-yl]− 2,5 diphenyltetrazolium bromide (MTT) assay kit (Roche, USA) was used to determine cell viability. Briefly, SK-N-BE(2), SH-SY5Y and KMST6 cells (control non-cancerous cells) were seeded in 96-well cell culture plates at a cell density of 2500, 5000, and 5000 respectively in each well and were allowed to attach for a period of 24 h. After attachment, fresh media containing increasing concentrations of *C. edulis* (0.5, 1, 1.5, 2–2.5 mg/ml) were introduced in each well and incubated for 48 h with the untreated wells as control. Thereafter, 10 µl of 5 mg/ml MTT solution was next added according to manufacturer's instruction and absorbance was read with a BMG Labtech Omega® POLARStar multiwell microplate reader. Percentage cell viability was calculated relative to control, and the half maximal inhibitory concentration (IC_{50}) determined from sigmoidal plots using GraphPad Prism6 software (GraphPad Software, San Diego, CA, USA). Experiments were performed in quadruplicate wells of three technical repeats.

2.4. Clonogenic assay

The impact of *C. edu*lis extract on colony formation was determined using the clonogenic assay. Neuroblastoma cells were seeded in 60 mm dishes and allowed to attach for 24 h before the introduction of medium containing half the IC_{50} and IC_{50} of *C. edulis* extract for another 24 h and untreated cells served as control. After treatments, cells were harvested and re-plated at a density of 500 cells per dish in 35 mm dishes and were left for fourteen days with routine media change to allow for colony formation. At the end of the experiment, formed colonies were fixed with a solution of methanol and glacial acetic acid (3:1) and stained with 0.5% crystal violet stain. Finally, images of dishes were taken to obtain the colony area using ImageJ software.

2.5. Detection of apoptosis

Detection of apoptosis was with the Caspase-9 ApoTarget® apoptotic assay kit (Invitrogen, Life Technologies, USA). SK-N-BE(2) and SH-SY5Y cells were seeded in 60 mm dishes at a density of 1.7×10^5 and left overnight before treatment with the IC_{50} of *C. edulis* for 48 h and untreated cells were used as control. Cells were harvested and lysed for 10 min in cold lysis buffer, centrifuged at 10000 x g for 1 min and prepared according to the manufacturer's procedure for absorbance reading in a microplate reader as previously reported (Omoruyi et al., 2018). Caspase-9 activity was expressed as a percentage of the control treated cells.

2.6. Investigating intracellular reactive oxygen species

The fluorescent probe 2′,7′-Dichlorofluorescin diacetate (DCFH-DA, Sigma St Louis, USA) was utilized to determine intracellular ROS activity in SK-N-BE(2) and SH-SY5Y cells. Briefly, cells were plated in 60 mm at a density of 1.7×10^5 dishes and allowed to attach overnight before exposure to IC_{50} of *C. edulis* for 48 h with untreated cells serving as control. Following treatment, cells were harvested, washed with 1X PBS, and centrifuged at 3000 revolutions per minute (rpm) for 3 min. The resultant pellets were stained with DCFH-DA for 1 h. After staining, cells were pelleted, resuspended in 500 µl of PBS and fluorescence was acquired with the BD Accuri CSampler Flow Cytometer (BD Biosciences Pharmingen, San Diego, CA) and mean fluorescence intensity was expressed as fold of the control cells.

2.7. Determination of changes in mitochondria membrane potential

The changes in the mitochondrial membrane potential (MMP) of SK-N-BE(2) and SH-SY5Y cells was determined using the rhodamine 123 fluorescent dye (Sigma St Louis, USA). Briefly, cells were seeded in 60

mm at a density of 1.7 × 10^5 dishes and allowed to attach overnight before exposure to IC_{50} of *C. edulis* for 48 h and the untreated cells were used as control. After treatment, cells were harvested, washed with PBS Cells and centrifuged for 3 min at 3000 rpm. The resultant pellets were stained with 10 μm of rhodamine 123 in supplemented DMEM for 30 min. After staining, cells were pelleted at 3000 rpm for 3 min, resuspended in 500 μl PBS and fluorescence intensity was measured using the BD Accuri CSampler Flow Cytometer and mean fluorescence intensity was expressed as fold of the control cells.

2.8. Statistical analysis

Data generated from this study was expressed as means ± standard error of means (SEM) of three independent experiments and analyzed using the GraphPad Prism 6 software. Groups were compared using either *t*-test or ANOVA, and the level of significance set at $P \leq 0.05$.

3. Results

3.1. C. edulis induces cytotoxicity in SK-N-BE(2) and SH-SY5Y neuroblastoma cells

Cytotoxicity of *C. edulis* on SK-N-BE(2) and SH-SY5Y neuroblastoma cells was investigated using MTT assays following exposure of cells to increasing concentrations of the extract. Results show that *C. edulis* induced dose-dependent cytotoxicity in SK-N-BE(2) and SH-SY5Y cells when compared to control cells (Fig. 1a and b). Calculated IC_{50} values from sigmoidal plots were, 0.86 mg/ml and 1.45 mg/ml for SK-N-BE(2) and SHSY5Y cells respectively indicating that the SK-N-BE(2) cells were more sensitive to treatment. Contrary to the results obtained from the neuroblastoma cells, *C. edulis* showed selectivity as they were less cytotoxic to the non-cancerous KMST6 fibroblast (Fig. 1c). Indeed, at the highest concentration of 2.5 mg/ml, cell viability obtained from the KMST6 was 52.22%. Altogether, these results show that *C. edulis* induced cytotoxicity in neuroblastoma cells with less effect on the KMST6 non-cancerous cells.

3.2. C. edulis inhibits proliferation and survival in neuroblastoma cells

The clonogenic assay was conducted to ascertain the impact of *C. edulis* treatment on proliferation and the long-term survival of cells. Fig. 2 shows that *C. edulis* treatment dose-dependently inhibited colony formation in both SK-N-BE(2) and SH-SY5Y cells when compared to control. Furthermore, colonies were quantified, and the results show a significant reduction in colony area. Together, these results indicate that *C. edulis* interferes with the reproductive capacity of both SK-N-BE(2) and SH-SY5Y neuroblastoma cells.

3.3. C. edulis induces apoptosis in neuroblastoma cells

To understand the molecular mechanism involved in the cytotoxic activity of *C. edulis*, the caspase-9 activity in the cells following treatment was investigated. Caspases are widely known as drivers of apoptosis, and the activation of caspase-9 is associated with the intrinsic mitochondrial pathway of apoptosis (Shalini et al., 2015, Kiraz et al., 2016). Results obtained show that *C. edulis* treatment increased

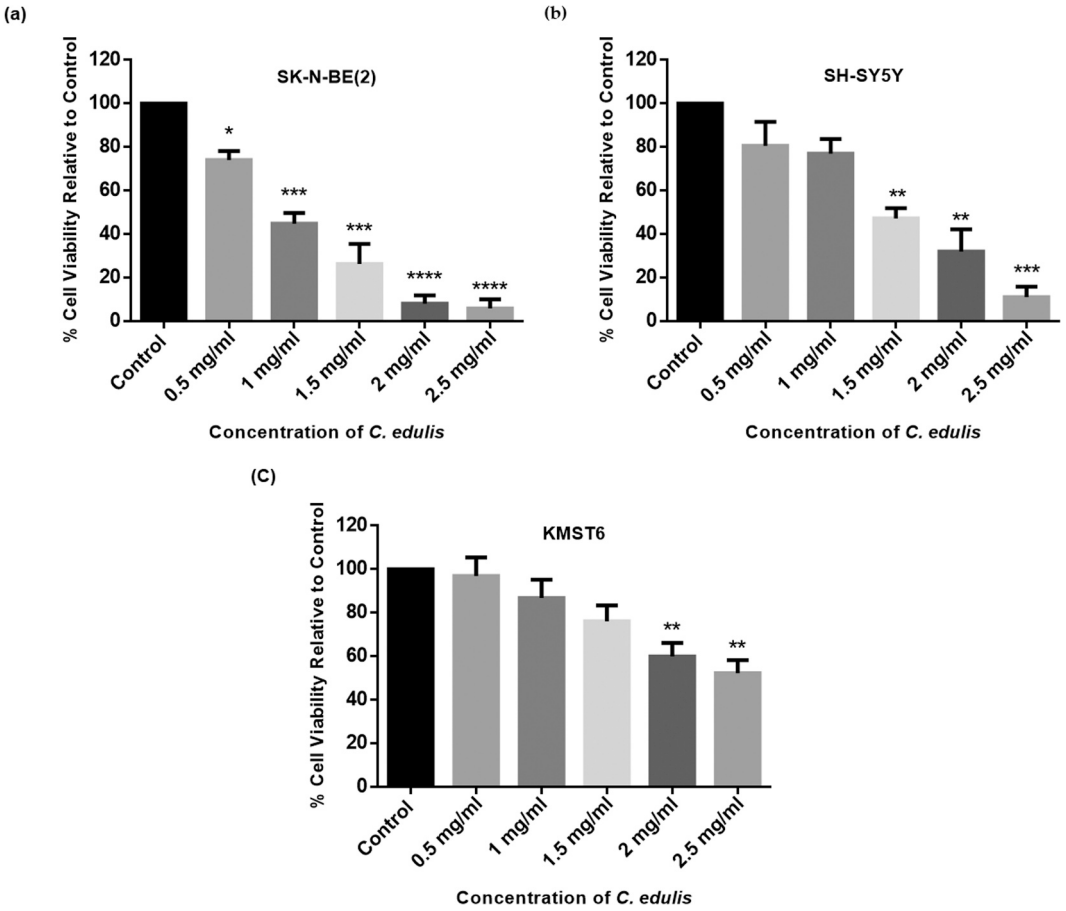

Fig. 1. *C. edulis* inhibits cell viability in neuroblastoma cells. *C. edulis* induced a significant dose-dependent response in (a) SK-N-BE(2) with *p* values 0.0395, 0.0010, 0.0002, < 0.0001, < 0.0001 for 0.5, 1, 1.5, 2 and 2.5 mg/ml respectively (b) SH-SY5Y with *p* values 0.0075, 0.0021, 0.0005 for 1.5, 2 and 2.5 mg/ml respectively and (c) KMST6 with *p* values 0.0048, 0.0012 for 2 and 2.5 mg/ml respectively. Bars represent means ± SEM of three independent experiments performed in quadruplicate wells.

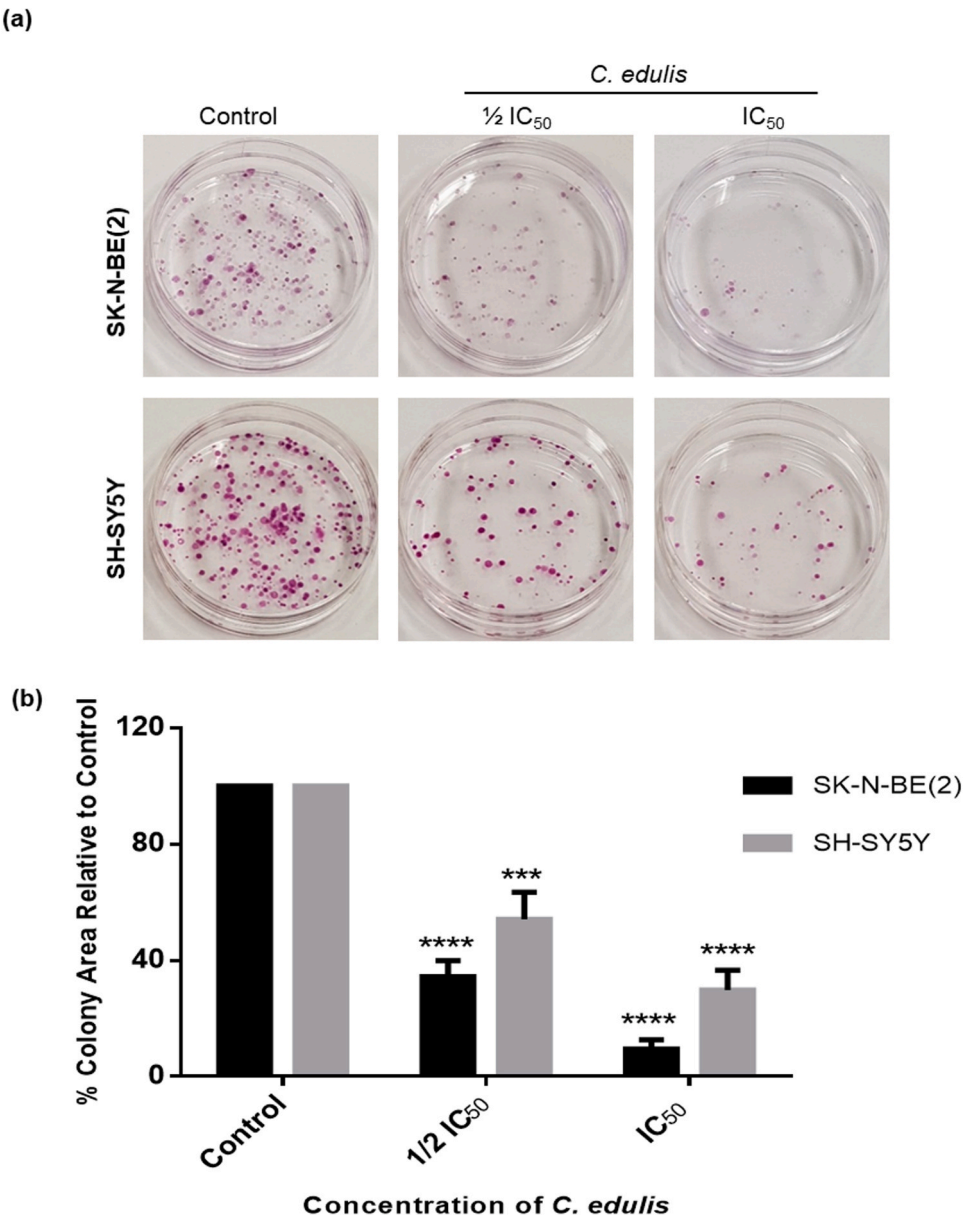

Fig. 2. *C. edulis* inhibits cell survival in Neuroblastoma cells. (a) SK-N-BE(2) and SH-SY5Y cells treated with *C. edulis* and colonies stained with crystal violet. (b) Quantification of colonies show a reduction of colony area with each bar representing means ± SEM and significance of difference p value for SK-N-BE(2) was < 0.0001 for both ½IC_{50} and IC_{50} while SH-SY5Y cells had a $p = 0.006$ and < 0.0001 for ½IC_{50} and IC_{50} respectively.

caspase-9 activity in SK-N-BE(2) and SH-SY5Y neuroblastoma cells (Fig. 3). These results indicate that induction of apoptosis may be involved in *C. edulis* induced cytotoxicity in neuroblastoma cells.

3.4. *C. edulis increases ROS generation in cells*

Excess production of ROS in association with reduced clearance by antioxidants lead to oxidative stress in cells, and this is linked to cytotoxicity in cancer cells (Lau et al., 2008, Tong et al., 2015). Medicinal plants have also been reported to induce ROS-mediated death/toxicity in cancer cells (Dong et al., 2017, Ryu et al., 2017). To this end, flow cytometry was performed to investigate ROS activity in cells using the DCFH-DA fluorescent dye. Fig. 4a and b show that when compared to control, *C. edulis* treatment led to a shift in fluorescence from left to right for both cell lines tested. Furthermore, the fluorescence intensity of treated cells was expressed as fold of control and *C. edulis* significantly increased ROS levels in cells (Fig. 4c and d). Altogether ROS generation may in part contribute to the cytotoxicity of *C. edulis* in neuroblastoma cells.

3.5. *C. edulis alters mitochondrial membrane potential in neuroblastoma cells*

Mitochondrial dysfunction has been reported to contribute to the induction of apoptosis and sustained loss of MMP will result in cellular energy depletion and eventually cell death (Ly et al., 2003, Vakifahmetoglu-Norberg et al., 2017). Considering how critical mitochondrial function is to cell survival, the impact of *C. edulis* on MMP was investigated next using Rhodamine 123 dye. Results show that *C. edulis* treatment led to the depolarization of MMP evidenced by a shift in fluorescence to the left in both cell lines tested (Fig. 5a and b). Furthermore, comparing the fluorescence intensity of treated cells as fold of control, *C. edulis* significantly induced loss of MMP (Fig. 5c and d). Together, these results may suggest that depolarization of MMP is

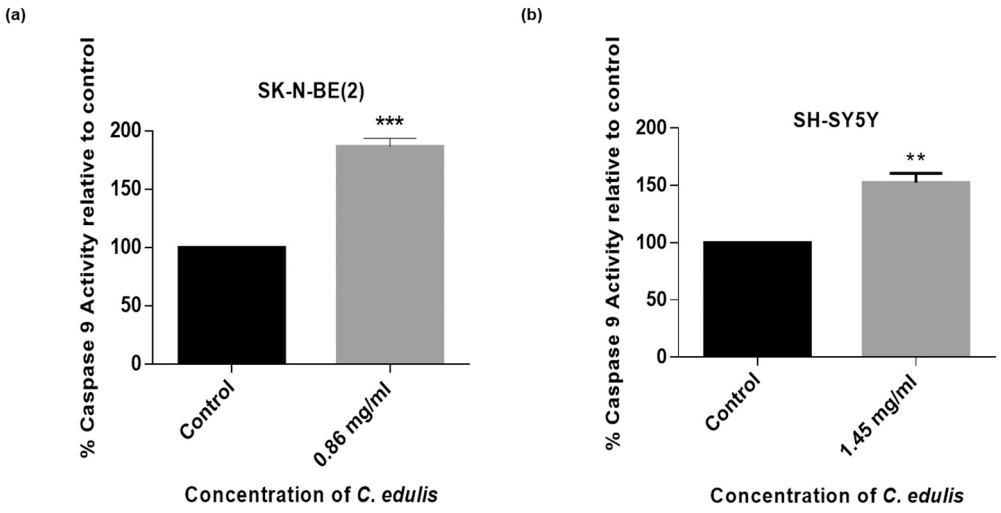

Fig. 3. *C. edulis* activates caspase-9 activity. (a) SK-N-BE(2) and (b) SH-SY5Y treated with 0.86 and 1.45 mg/ml of *C. edulis* respectively for 48 h showed significant ($p = 0.0001$ for SK-N-BE(2) and $p = 0.0014$ for SH-SY5Y) activation of Caspase-9 when expressed as percentage of control. Each bar represents means ± SEM.

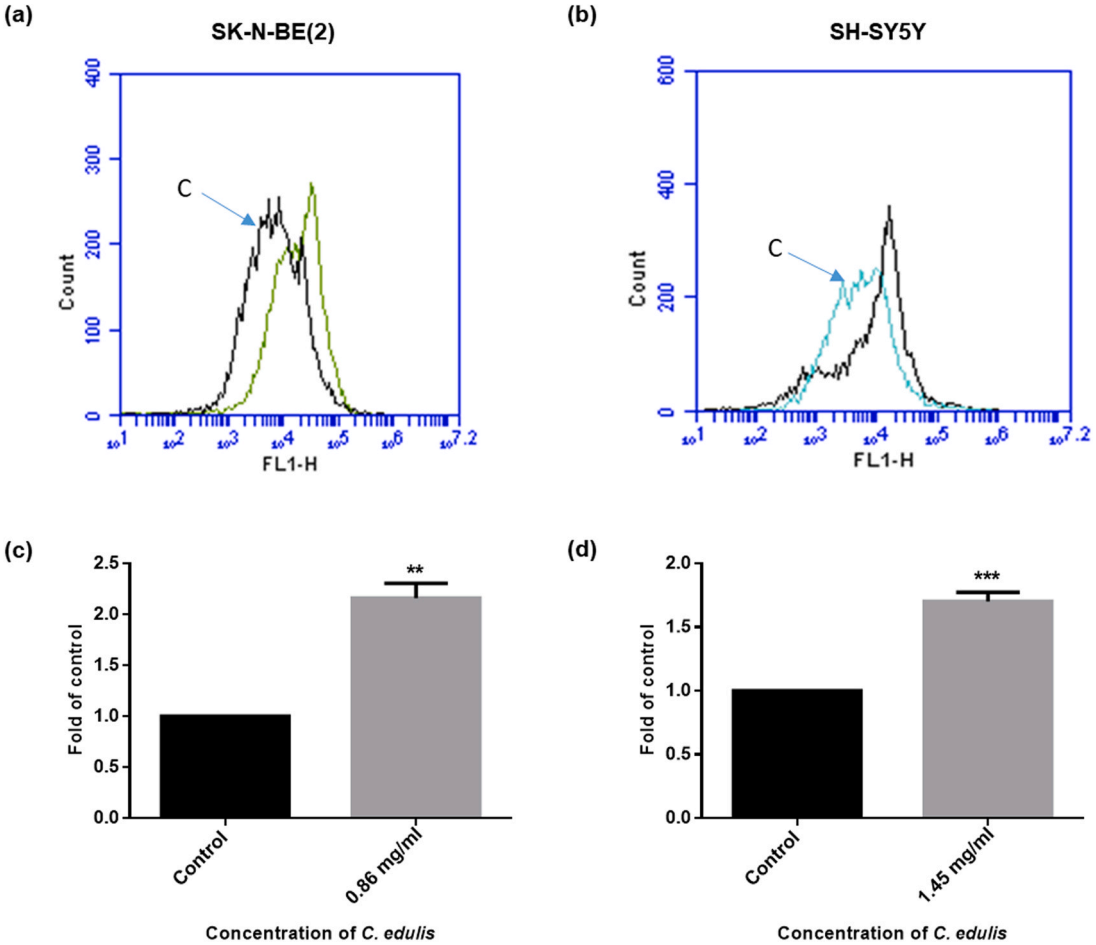

Fig. 4. *C. edulis* induces Intracellular ROS accumulation. Representative flow cytometry profile of (a) SK-N-BE(2) and (b) SH-SY5Y treated with 0.86 and 1.45 mg/ml of *C. edulis* respectively for 48 h when compared to control [C]. The fluorescence intensity of treated cells was expressed as fold of control (c) SK-N-BE(2) with $p = 0.0013$ and (d) SH-SY5Y with $p = 0.0006$. Each bar represents means ± SEM.

involved in the cytotoxicity of *C. edulis* in neuroblastoma cells.

4. Discussion

Although there has been enormous progress in the development of novel therapeutic agents for the treatment of cancer, deaths arising from

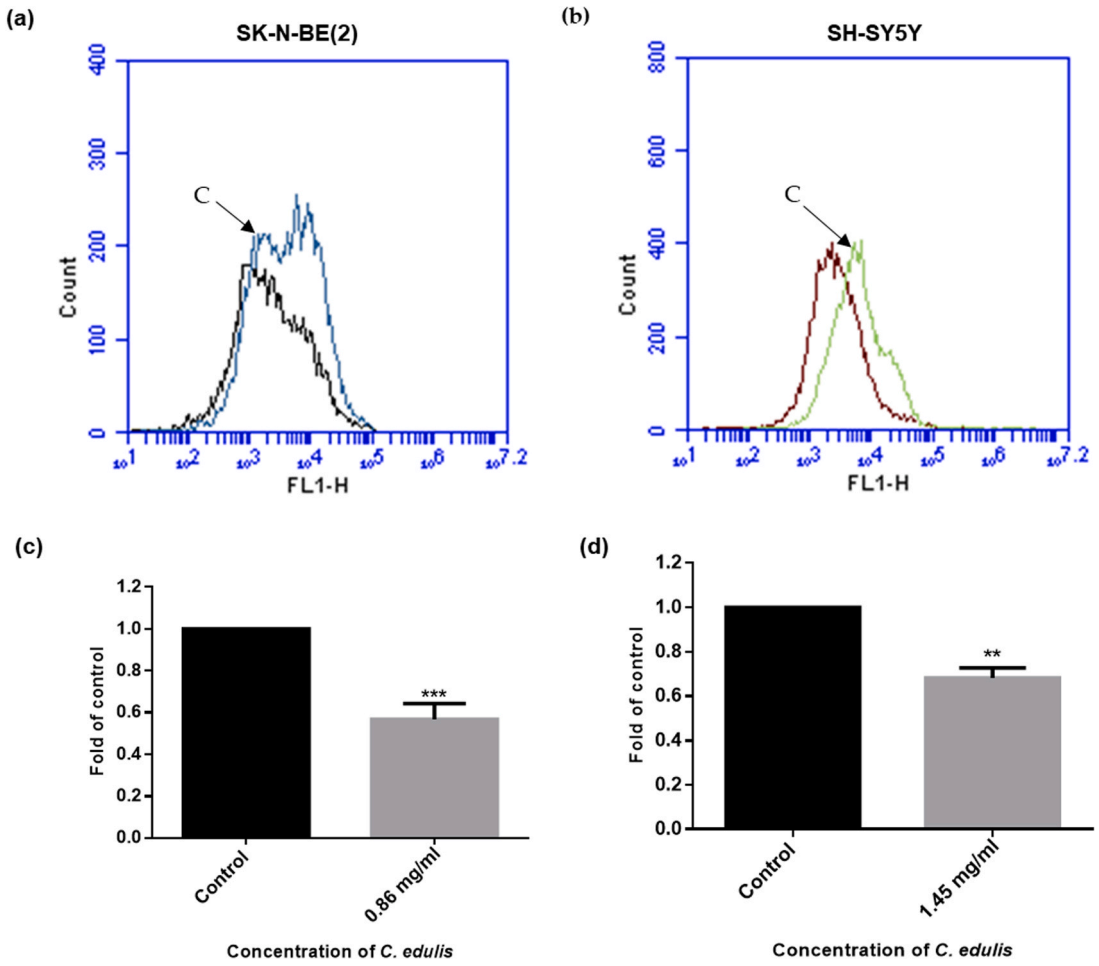

Fig. 5. *C. edulis* alters MMP in neuroblastoma cells. Representative flow cytometry profile of (a) SK-N-BE(2) and (b) SH-SY5Y treated with 0.86 and 1.45 mg/ml of *C. edulis* respectively for 48 h when compared to control [C]. The fluorescence intensity of treated cells was expressed as fold of control (c) SK-N-BE(2) with $p = 0.0006$ and (d) SH-SY5Y with $p = 0.0023$ and each bar represents means ± SEM.

cancer still rank second in the world next to cardiovascular diseases (Siegel et al., 2015). For several decades, medicinal plants and plant-derived products have played a pivotal role in the development of notable anti-cancer drugs. These medicinal plants are instrumental in the prevention or slowing down of oncogenesis as they are readily available and present with less systemic toxicity compared to conventional chemotherapy (Wong et al., 2013, Akindele et al., 2015, Al-Rimawi et al., 2016). This present study investigates the anti-proliferative activity of *C. edulis*, a groundcover medicinal plant indigenous to the people of South Africa in SK-N-BE(2) and SH-SY5Y cells. Data generated from this study shows that *C. edulis* induced cytotoxicity in both neuroblastoma cell lines. Furthermore, it is necessary for an anti-cancer agent to show selectivity with cancer cells while sparing healthy cells (Blagosklonny, 2004). In line with this, *C. edulis* displayed selectivity to cancer cells as it was less cytotoxic to the KMST6 fibroblasts. While it seems the extract also induced some levels of toxicity in the KMST6 cells, the authors cannot speculate what impact this might have on the cells at long-term exposure as this experiment only lasted for 48 h. However, at the highest concentration of 2.5 mg/ml, the percentage cell viability for the KMST6 normal control was 52.2% whereas the viability of SKNBE(2) and SH-S5Y was 5.94% and 11.19% respectively.

Rapid division and proliferation of cells is a hallmark of cancer and it will be of importance for an anticancer agent aside from being cytotoxic in the short-term to also inhibit the long-term survival of cells. In the present study, findings show that *C. edulis* inhibited colony formation in the SK-N-BE(2) and SH-SY5Y cells indicating a reduction in cell division and proliferation. Results obtained are consistent with previous reports for some medicinal plants (Gu and Leonard, 2006, Kurapati et al., 2012, Purushotham et al., 2016, Chowdhury et al., 2017).

Apoptosis, a form of programmed cell death, plays an integral role in the development and maintenance of cellular homeostasis. However, disruption of apoptosis has its implication in several diseases including neurodegenerative diseases, cancer and auto-immune diseases. In short, evasion of apoptosis is a hallmark of cancer as cancer cells no longer respond to death signals due to altered genetic profile (Hanahan and Weinberg, 2000). Thus, researchers have identified apoptotic signalling as a therapeutic target for cancer treatment (Pore et al., 2013, Signore et al., 2013). A molecular marker of apoptosis is a group of enzymes known as caspases, and a reduction of their cellular level is known to promote evasion of cell death (Wong, 2011). In the present study, results obtained show that *C. edulis* induced apoptosis in SK-N-BE(2) and SH-SY5Y neuroblastoma cells via the increase in activity of caspase-9 which is associated with the intrinsic or mitochondrial apoptotic pathway (Wurstle et al., 2012). Apoptosis observed in this study may in part be induced via the accumulation of ROS and loss of MMP on both cell lines tested.

ROS are quite critical for normal cellular physiological processes including proliferation, cell cycle and migration at a moderate level, but overproduction of ROS could lead to stress in cells and eventually cell death (Covarrubias et al., 2008). Existing data shows that some cancerous cells are known to have a marked increase in ROS levels and in-turn depend on endogenous antioxidants to maintain redox balance to continue proliferation (Wang et al., 2017). Invariably, depleting

endogenous antioxidant status in cancer cells could be a therapeutic approach (Raj et al., 2011). Consistent with this, findings from this current study show that exposure of SK-N-BE(2) and SH-SY5Y cells to *C. edulis* led to an accumulation of ROS in the cells and may in part be responsible for the inhibition of cell proliferation. In support of this claim, medicinal plants and plant-derived compounds are widely known to induce cytotoxicity by increased generation of intracellular ROS (Yang et al., 2006, Madi et al., 2016, Liang et al., 2016).

Reports show that increased ROS generation is accompanied by depolarization of MMP which triggers the activation of the intrinsic apoptotic pathway (mitochondrial-mediated apoptosis) (Chen et al., 2009, Wang et al., 2017). The loss of MMP initiates a cascade of events leading to increased caspase-9 activity; this is in line with the results from our study which demonstrate a loss of MMP and subsequent activation of caspase-9 in the neuroblastoma cells upon exposure to *C. edulis*. These are consistent with previous reports for other medicinal plants and natural products (Madi et al., 2016, Ryu et al., 2017).

The activity of *C. edulis* observed can be attributed to the bioactive composition of the plant. A previous study analysed its phenolic components and identified seven bioactive compounds including sinapic acid, ferulic acid, luteolin7-o-glucoside, hyperoside, isoquercitrin, ellagic acid and isorhamnetin 3-O-rutinoside (Hafsa et al., 2016). Sinapic and ferulic acid have been reported to decrease cell proliferation in T47D breast cancer cells and the Caco2 human colon carcinoma cells (Kampa et al., 2003, Janicke et al., 2005, Eroğlu et al., 2018). Similarly, luteolin7-o-glucoside was reported to induce apoptosis by scavenging free radicals and inhibition of β-catenin expression in COLO 320 DM human colon cancer cells (Baskar et al., 2011). In addition, a study which characterised the biochemical composition of the aqueous extract of *C. edulis* reported the plant to be rich in phenolics, proanthocyanidins and tanins among others (Omoruyi et al., 2012). In light of this, the cytotoxic activity observed in the present study using the aqueous extract of *C. edulis* can be attributed to the phenolic components as the presence of phenols in medicinal plants is strongly associated with their cytotoxicity (Pandey and Rizvi, 2009, Boutennoun et al., 2017, Padmapriya et al., 2017, Nizioł-Łukaszewska et al., 2018).

5. Conclusions

Conclusively, this study highlights the cytotoxic, antiproliferative and ROS-inducing activity of C. *edulis* aqueous extract in human neuroblastoma cells. Also, it shows that *C. edulis*-induced apoptosis may be via the mitochondrial apoptotic pathway due to an increase in caspase-9 activity. Altogether, this study suggests that *C. edulis* may be useful in the treatment of neuroblastoma and thus provides a template for further investigations into its mechanism of action and isolation of bioactive components responsible for its anti-cancer activity.

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CRediT authorship contribution statement

Sylvester I. Omoruyi: Conceptualization, Methodology, Investigation, Writing – original draft, Software. **Adaze B. Enogieru**: Data curation, Methodology, Reviewing. **Okobi E Ekpo**: Supervision, Reviewing and Editing.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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