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Original Article

Apoptotic effect of *Bulbine Natalensis* and *Chlorophytum Comosum* in myelogenous Leukemia K562 cell line

Efeito apoptótico da natalensia bulbina e do clorofito comoso na linhagem celular mielogênea da leucemia K562

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Abstract

Bulbine natalensis and *Chorophytum comosum* are potential medicinal source for the treatment of cancers. Chronic myeloid leukaemia is a hematopoietic stem cells disorder treated by tyrosine kinase inhibitors but often cause recurrence of the leukaemia after cessation of therapy, hence require alternative treatment. This study determines the anti-cancer effect of leaf, root and bulb methanolic and aqueous extracts of *B. natalensis and C. comosum* in chronic human myelogenous leukaemia (K562) cell line by MTT, Hoechst bis-benzimide nuclear and annexin V stain assays. The root methanolic extract of *B. natalensis and C. comosum* showed a high cytotoxicity of 8.6% and 16.7% respectively on the K562 cell line at 1,000 µg/ml concentration. Morphological loss of cell membrane integrity causing degradation of the cell and fragmentation were observed in the root methanolic extract of both plants. A high apoptosis ($p < 0.0001$) was induced in the K562 cells by both leaf and root extracts of the *C. comosum* compared to the *B. natalensis*. This study shows both plants possess apoptotic effect against *in vitro* myelogenous leukaemia which contributes to the overall anti-cancer properties of *B. natalensis* and *C. comosum* to justify future therapeutic applications against chronic myelogenous leukaemia blood cancer.

Keyword: Bulbine natalensis, *Chlorophytum comosum,* leukemia K562, apoptosis, cytotoxicity.

Resumo

Bulbine natalensis Baker e *Chorophytum comosum* (Thunb.) Jacques são potenciais fontes medicinais para o tratamento de cânceres. A Leucemia Mieloide Crônica (LMC) é um distúrbio das células-tronco hematopoiéticas que é tratado com inibidores da tirosina quinase, mas frequentemente, causa recorrência da leucemia após a interrupção da terapia, portanto, requer um tratamento alternativo. Este estudo determinou o efeito anticancerígeno de extratos metanólicos e aquosos de folha, raiz e bulbo de *B. natalensis* e *C. comosum* na linhagem celular de leucemia mieloide humana crônica (K562) por ensaios de MTT, Hoechst bis-benzimida nuclear e anexina V. O extrato metanólico da raiz de *B. natalensis* e *C. comosum* apresentou alta citotoxidade de 8,6% e 16,7% respectivamente, na linhagem celular K562 com a concentração de 1,000 µg / ml. Perda morfológica da integridade da membrana celular causando degradação dos núcleos, citoplasma e encolhimento celular foi observada no extrato metanólico da raiz de ambas as plantas. Uma alta apoptose (p <0,0001) foi induzida nas células K562 por extratos de folhas e raízes de *C. comosum* em comparação com *B. natalensis*. Este estudo mostrou que ambas as plantas possuem efeito apoptótico contra leucemia mieloide *in vitro* que contribui para as propriedades anticâncer gerais de *B. natalensis* e *C. comosum* para justificar futuras aplicações terapêuticas contra câncer de sangue de LMC.

Palavras-chave: *Bulbine natalensis, Chlorophytum comosum,* leucemia K562, apoptose, citotoxidade.

1. Introduction

Cancer is a major health burden and the second leading cause of global death accounting for an estimated 8.2 million deaths annually (Stewart and Wild, 2014). The mortality rates of cancer are expected to increase in developing countries (Made et al., 2017). Chronic myeloid leukaemia (CML) is a malignant blood cancer disease that affects the bone marrow cells characterized by the presence of an active tyrosine kinase (Apperley, 2015). Although, data on incidence and prevalence of CML in South Africa is lacking and limited as disease is often combined with the general category of leukaemia or other cancers (Stefan, 2015; Torre et al., 2015), the tendency of

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increased cases on a yearly basis is very high (Statistics South Africa, 2014). It has been projected there will be an estimate of 181,000 CML patients in the USA from 2050 (Huang et al., 2012). Certain anti-cancer drugs that are tyrosine kinase inhibitors are often the first line of treatment employed. However, they often cause adverse reactions, drug resistance and active recurrence of the leukaemia upon cessation of drug (Huang et al., 2012; O'Hare and Deininger, 2008). Hence, the development of an effective natural anti-cancer drug as an alternative medicine to treat this disease is required as a way to combat the increasing prevalence of the cancer.

Bulbine natalensis is a member of the family Asphodelaceae and a predominant species in the genus (Van Wyk and Gericke, 2000). This bulb-like indigenous soft plant is widely distributed in the northern and eastern province of South Africa. It is commonly known as Natal Bulbinella with a local name Ibhucu in Zulu and rooiwortel in afrikaans (Hutchings, 1996; Pather et al., 2011). *B. natalensis* has found considerable attention for traditional medicinal applications for the treatment of skin diseases and wounds (Pather, 2009; Pather et al., 2011), sexual dysfunction (Ajao et al., 2019; Yakubu and Afolayan, 2009) urinary infections and human immunodeficiency virus infection (Plessis-Stoman et al., 2009). *Chorophytum comosum* is a grass-like evergreen perennial of the family Liliaceae and native to South Africa. *C. comosum* is widely distributed ranging from Sierra Leone to Ethiopia. However, it is a native plant from South Africa and has been found to be used in folk medicine to treat a range of ailments such as bronchitis, fractures and burn (Matsushita et al., 2005) Previous reports have shown the protective effect of *C. comosum* against liver damage in rodent model (Areshidze et al., 2016) and potential to stimulate intestinal microbiome (Bondareva et al., 2017).

Few studies have reported *B. natalensis* and *C. comosum* to be a potential major source for the treatment of cancers through its apoptotic roles and cytotoxic effect against some cancer cell lines including laryngeal carcinoma (HEp-2), human breast cancer (MDA-MB-231 and T47D), lung carcinoma (A549), human embryonic kidney 293 (HEK293), cervical carcinoma (HeLa), human promyelocytic leukaemia (HL-60) and pro-monocytic leukaemia (U937) (Kasumbwe et al., 2017; Kushwaha et al., 2019; Matsushita et al., 2005; Singh and Reddy, 2012). However, no study has been reported, to the best of our knowledge, on the anti-cancer effect of *B. natalensis* and *C. comosum* on chronic human myelogenous leukaemia (K562) cell line. Hence, we investigated the apoptotic effect of *B. natalensis and C. comosum* organic and aqueous extracts through cytotoxicity, Hoechst bis-benzimide nuclear and flow cytometry annexin V staining of the K562 cells.

2. Materials and Methods

2.1. Plant material and extraction

Bulbine natalensis and *Chorophytum comosum* were obtained from the Reservoir Hills, Durban. The leaves, roots and bulbs of the plants were collected, washed with distilled water and air-dried for 48 h. The plant materials were blended to a fine powder and then stored. 10g of the powdered samples were added to 200 ml of sterile distilled water for the aqueous extracts and 100 ml methanol for the organic extracts. The plant material solvents were shaken at 156 rpm in an incubator of 37°C for 48 h. The leaf, root and bulb methanolic extracts for *B. natalensis* (BLM, BRM and BBM respectively) and leaf and root methanolic extracts for *C. comosum* (CLM and CRM respectively) were filtered through a 40µm filter disk (Whatman No 1, UK). The methanolic extracts were concentrated in a rotary evaporator at 65°C and subsequently stored in the biofreezer at -70°C. The root aqueous extract for *B. natalensis* (BRA) and leaf and root aqueous extracts for *C. comosum* (CLA and CRA respectively) were freeze dried for 24 h and then stored.

2.2. Cell lines

A human myelogenous (erythromyeloid) leukaemia K562 cell line was purchased from Highveld Biological, South Africa. These cells were non-adherent, rounded and resemble undifferentiated granulocytes. The cells were maintained with growth medium containing 10% foetal bovine serum (FBS) supplemented with 1% antibiotic (penicillin: 10,000 µ/ml, streptomycin sulphate: 10,000 µ/ml) in a humidified 5% CO_2 air incubator at 37°C. Cultures were examined daily by the morphological observation, colour of medium, cells density and growth pattern using Zeiss inverted light microscope. Cells were washed and preserved in medium containing 90% FBS and 10% (100 µl) dimethyl sulfoxide (DMSO) at -70°C.

2.3. Determination of cytotoxicity effect of B. natalensis and C. comosum on K562 cells using MTT Assay

The cytotoxicity effect of *B. natalensis and C. comosum* methanolic and aqueous extracts were quantified using the MTT assay following the method by (Jun et al., 2003). The percentage of cytotoxicity (%) were calculated using Equation 1 as described below (Singh and Reddy, 2012). Briefly, the K562 cells (8×10^4 cells/ml) were seeded in a 96-well microtiter plate (100 µl/well) and subsequently treated with the *B. natalensis and C. comosum* methanolic and aqueous extracts at 100 and 1,000 µg/ml concentrations for 24 h. Untreated cultured wells containing the K562 cells with complete culture medium and 2% DMSO were used as the control. All samples were assayed in triplicate. The plate was centrifuged at 400 g for 10 min and the cell supernatant was removed. MTT solution (10 µl) was added to each well and incubated for 3 h at 37°C humid chamber. The purple formazan crystals were solubilized with 100 µl DMSO and incubated further for 30 min. The absorbance was read at 570 nm with a reference wavelength of 650 nm using a microplate reader (BioTek Instruments, Inc. USA). The cytotoxicity (%) was calculated:

$$Cytotoxicity(\%) = 100 - \frac{Average\ absorbance\ of\ treated\ cells}{Average\ absorbance\ of\ control\ cells} \times 100 \quad (1)$$

(Singh and Reddy, 2012)

2.4. Determination of DNA fragmentation and condensation in K562 cells

The Hoechst bis-benzimide (H33342), a blue-fluorescent dye that binds to the minor groves of DNA determines the condensed pycnotic nuclei in apoptotic cells. Briefly, 300 µl of 5 x 10^4 cells/ml in 24-well microtiter plate treated with 6 µl of the *B. natalensis* and *C. comosum* methanolic and aqueous extracts at 37°C were incubated for 24 - 48 h. Positive control cells were treated with 10 µg/ml camptothecin while untreated cells remains as negative control. Following incubation, after cells were centrifuged at 1500 rpm for 5 min, the cell pellets were washed with 500 µl of 0.1M PBS and centrifuged again as previously described. H33342 solution (100 µl) was added and incubated for 15 min at 37°C. The cell pellets were retrieved and washed as previously described. The cells were fixed in 10% paraformaldehyde solution for 5 min, centrifuged and washed. Finally, cells were re-suspended in 100 µl of PBS, wet mounted on coverslips and observed immediately under the Zeiss AxioPlan fluorescence (Zeiss, Germany) microscope with excitation filter of 350 nm and barrier filter of 450 nm.

2.5. Flow cytometry apoptotic cell death analysis by annexin V staining

The induced apoptotic cell death population by the methanolic and aqueous extracts of *B. natalensis* and *C. comosum* in K562 cells was determined using flow cytometry technique as previously described (Wlodkowic et al., 2009). A 400 µl of 2 x 10^5 cells/ml were incubated in T25^2 flasks for 24 h at 37°C. Cells were treated with 8 µl of the methanolic and aqueous extracts of *B. natalensis* and *C. comosum* and incubated further for 24 h at 37°C. Cells were washed in cold PBS and centrifuged at 1,500 rpm for 10 min. Following another washing, cells were re-suspended in a prepared 1X annexin binding buffer which contains 1 ml of 5X annexin binding buffer added to 4 ml of de-ionized water. Alexa fluor 488 annexin V (5 µl) and SYTOX green working solution (1 µl) were added to the cells and incubated at room temperature for 15 min. Furthermore, 400 µl of 1X annexin binding buffer was added to the cells, mixed gently while kept on ice and stained cells were immediately analysed by flow cytometry measuring the fluorescence emission at 530 nm.

2.6. Statistical analysis

All data were presented as the mean ± standard deviation at triplicate independent experiments. All statistical data were analysed using GraphPad Prism version 5.01 software. Statistical comparisons between the treatments extracts were performed using t-test and one-way analysis of variance. $P < 0.0001$ was considered to indicate a statistically significant difference.

3. Result and Discussion

This present *in vitro* study utilised the leaf, root, and bulb methanolic and aqueous extracts of both *B. natalensis* and *C. comosum* to assess the apoptotic effect of the two plants in myelogenous leukaemia K562 cell line. Few studies have reported anti-cancer properties of *B. natalensis in vitro* and chemoprotective properties of *Cholophytum comosum* in a liver damaged in vivo model (Areshidze et al., 2016; Singh and Reddy, 2012). Potential metabolites in *B. natalensis* and *C. comosum* has been shown to inhibit the growth of some cancer cells lines. However, there is little or no report known till date about the effects of these two medicinal plants on K562 cells, a type of cell line originated from a CML patient with hematopoietic stem cells disorder.

In this present study, the dose-dependent cytotoxicity effect of the methanolic and aqueous extracts of *B. natalensis* and *C. comosum* on the viability of K562 cells was observed (See Figure 1A). A high cytotoxicity activity was exhibited by the methanolic and aqueous extracts of *C. comosum*. The CRM extract showed a high cytotoxicity (16.7%) against the K562 cell line at 1,000 µg/ml concentration (See Figure 1B). An increase in cytotoxicity was exhibited from 4.9% at 100 µg/ml to 8.6% at 1,000 µg/ml concentration in the K562 cell by the BRM extract. The root methanolic extracts of both plants showed significant cytotoxicity ($p < 0.0001$) against the K562 cell line compared to the leaf, bulb methanolic and root aqueous extracts irrespective of the concentration. This suggests the cytotoxic efficacy of the root methanolic extracts of *B. natalensis* and *C. comosum* against K562 cell proliferation. Conversely, the leaf methanolic (BLM and CLM) extracts of both plants extracts tend to show the lowest cytotoxicity irrespective of the concentration.

The extract treatments of *B. natalensis* and *C. comosum* showed cytotoxic effect to the K562 cells in a concentration-dependent manner. We observed a high cytotoxicity in the *C. comosum* extracts as the root methanolic extract was higher compared to the leaf methanolic and aqueous extracts. Matsushita et al. (2005), showed that root butanol extract of *C. comosum* induces anti-proliferative effect in HL-60 and U937 cell lines However, Adhami et al. (2020) reported the leaf ethanolic extract of *C. comosum* exhibited maximum response of cytotoxicity (IC_{50} 3.08 µg/ml) compared to the root extract. A systematically screening of the cytotoxicity activity of the different phytochemical extracts from the plant extracts may provide a valuable information to this controversy. In addition, we noticed that the root extract *was* more cytotoxic than the other methanolic and aqueous extracts of *B. natalensis* against the K562 cell line at 1,000 µg/ml. Although the cytotoxicity level of the CRM was significantly higher compared to the BRM, it is plausible to assume that the type and sensitivity of the phytochemical compounds such as anthraquinones, chrysophanol and knipholone present in the root has a potent anti-proliferative activity reacting in a different apoptotic pathway (Musara and Aladejana, 2020) and thus may differently affect the proliferation of the K562 cell line.

The morphological and cellular changes in the K562 cell induced by the extracts of *B. natalensis* and *C. comosum* at 1,000 µg/ml concentration after staining with the H33342 fluorescent probe showed indicative apoptotic characteristic such as membrane damage, nuclear fragmentation, vacuolated cytoplasm and possible nuclei disintegration in comparison to the untreated cells (See Figure 2). These were indicative of apoptosis in the treated

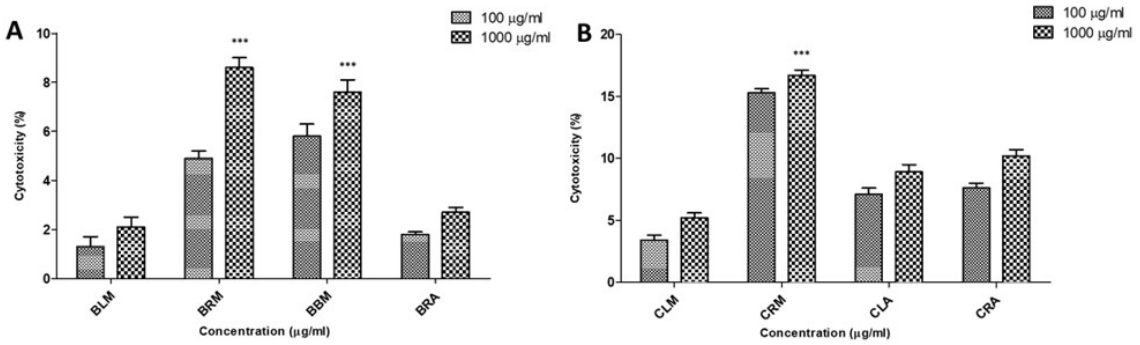

Figure 1. Cytotoxic effect of (A) *Bulbine natalensis* and (B) *Cholophytum comosum* extracts at 100 µg/ml and 1,000 µg/ml concentrations in human myelogenous leukaemia K562 cell line using MTT assay. All *** is $p < 0.0001$ which is considered statistically significant.

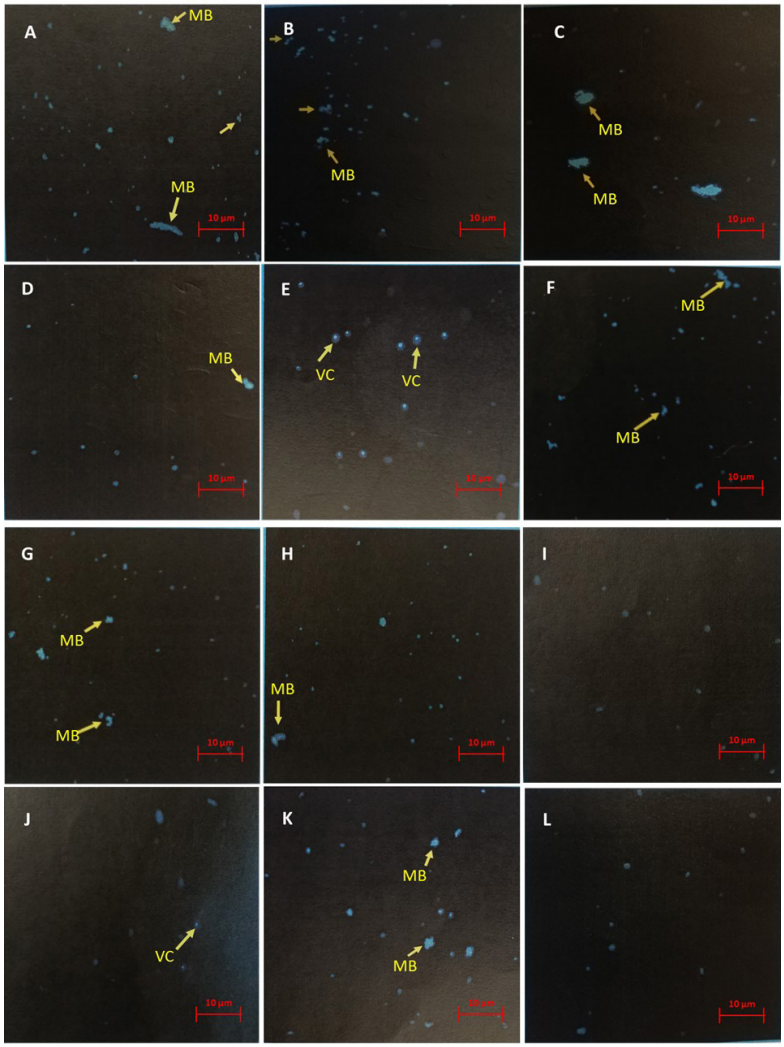

Figure 2. H33342 staining viewed under fluorescent microscope on human myelogenous leukaemia K562 cell morphological changes induced by: 1. 1,000 µg/ml *Bulbine natalensis* (A) leaf methanolic extract, BLM (B) root methanolic extract, BRM (C) bulb methanolic extract, BBM and (D) root aqueous extract, BRA; 2. 1,000 µg/ml Cholophytum comosum (E) leaf methanolic extract, CLM (F) root methanolic extract, CRM (G) leaf aqueous extract, CLA (H) root aqueous extract, CRA; 100 µg/ml *Cholophytum comosum* (I) leaf aqueous extract, CLA (J) root aqueous extract, CRA and 3. Controls (K) camptothecin indicating membrane blebbing (L) untreated cells indicating normal cells. MB- membrane blebbing; VC- vacuolated cytoplasm.

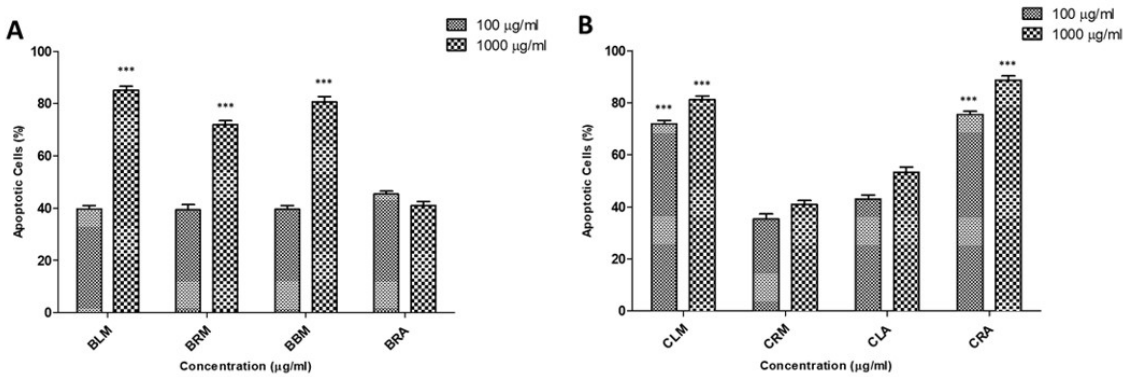

Figure 3. Apoptotic effect of (A) *Bulbine natalensis* and (B) *Cholophytum comosum* extracts at 100 µg/ml and 1,000 µg/ml concentrations in human myelogenous leukaemia K562 cell line using Annexin V. All *** is $p < 0.0001$ which is considered statistically significant.

K562 cells which exhibited different degree of cell and nuclear fragmentation. The BLM, BRM, BBM, CLA and CRA extracts treated K562 cells showed possible morphological loss of cell membrane integrity causing membrane blebbing (MB) and fragmentation (See Figure 2A-2D, 2G, 2H). Degradation of the cell cytoplasm were observed in the BRM and CRM extracts treated K562 cells (see Figure 2F). Vacuolated cytoplasm (VC) was also presented in the CLM and CRA extracts treated K562 cells (see Figure 2E and 2J). This suggests that the root methanolic extracts of both plants is induced more apoptotic activity when compared to the other extracts in their aqueous forms. The K562 cells treated with CLA extracts at 100 µg/ml concentration induced little or no apoptotic activity as cellular disintegration was very minimal (See Figure 2I). The camptothecin treated K562 cells showed vacuolated condensed nuclei with extensive membrane damage (see Figure 2K) compared to the untreated cells exhibiting a normal nuclear morphology (see Figure 2L).

Kushwaha et al. (2019) reported a significant number of apoptotic cells were observed in the MDA-MB-231 breast cancer cells treated with leaf methanolic extracts of *B. frutescens*. The expression of caspase-3 induced by *B. natalensis* ethanol fractions extract in Hep-2 cells as against the aqueous extract was indicative of the apoptosis (Singh and Reddy, 2012). In the present study, the induced K562 cell morphological changes was more evident in the 1,000 µg/ml dose concentration. The different dose treatments applied to K562 cell could possibly induced different morphological alterations of the cellular surface which corroborate with Kushwaha et al. (2019) and Rahman et al. (2013).

Comparatively, the better efficacy of *C. comosum* extracts against K562 cell in the cytotoxicity study motivate for further confirmation by annexin V staining through quantitative flow cytometry analysis to justify the apoptotic potential of both *B. natalensis* and *C. comosum* extracts in K562 cells. *B. natalensis* and *C. comosum* extracts treated K562 cells were stained with a recombinant Annexin V dye conjugated to an Alexa fluor 488 dye to quantify the apoptotic induction ability of the plants extract. Viable cells were stained Annexin V negative and apoptotic cells were Annexin V positive. In this study, *C. comosum* has a significantly higher efficiency of inducing apoptosis most especially the leaf methanolic and root aqueous extracts.

The extracts: BLM (85.1%), BRM (71.9%), BBM (80.6%), CLM (81.1%) and CRA (88.7%) induced a significantly high apoptosis ($p < 0.0001$) in the K562 cells compared to the BRA (40.9%), CRM (41.0%) and CLA (53.2%) at 1,000 µg/ml concentration. All *B. natalensis* extract treated K562 cells showed low apoptotic cell population level below 50% at 100 µg/ml concentration (see Figure 3A). Contrarily, it was observed that CLM (71.9%) and CRA (75.5%) extract treated K562 cells exhibited apoptosis significantly ($p < 0.0001$) high above 50% at 100 µg/ml concentration (see Figure 3B). Furthermore, a significantly high apoptosis ($p < 0.0001$) was induced in the K562 cells by both the leaf and root extracts of the *C. comosum* irrespective of the dose concentration compared to the *B. natalensis* extracts. This further confirms the high cytotoxicity observed in the *C. comosum* extract treated K562 cell. Although, previous studies suggested that a specific hydrolysate, DL – ornithine monohydrochloride, which is present in the aerial part of *C. comosum* may induce significant apoptotic through mitosis, necrotic and anti-proliferation effects (Areshidze et al., 2016; Areshidze et al., 2013). Our study agrees with Areshidze et al. (2016) considering the high cytotoxicity and apoptotic effect of *C. comosum.*

4. Conclusion

In this study, the methanolic and aqueous extracts of *B. natalensis* and *C. comosum* inhibit the proliferation of K562 cells possibly at the early stage. It is plausible that *B. natalensis* and *C. comosum* possess apoptotic effect with considerable cytotoxicity against the *in vitro* myelogenous leukaemia. Previous studies have reported anti-proliferative property of plants extracts against HL-60, U937, HEp-2, MDA-MB-231 and T47D cancer cell lines in a time-dependent fashion within 24, 48 and 72 hr response to treatment (Kushwaha et al., 2019; Matsushita et al., 2005). The limitation to this study is that the 48 – 72 hrs treatment of the *B. natalensis* and *C. comosum* extracts against the K562 cell line which may increase cytotoxicity

against the growth of the cells. However, this warrant further studies to elucidate the time response effect of treatment and ascertain the apoptotic pathway induced by *B. natalensis* and *C. comosum* in both *in vivo* and preclinical models. This might contribute to the overall apoptotic properties of *B. natalensis* and *C. comosum* and to justify future therapeutic applications against chronic myelogenous leukaemia blood cancer.

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