Original Research

Apoptosis-inducing potential of *Myrothamnus flabellifolius*, an edible medicinal plant, on human myeloid leukemia HL-60 cells

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Summary. Conventional therapies for treating acute myeloid leukemia involve chemotherapy and radiation. This approach causes damage to both normal and cancerous cells resulting in several side effects. There is a dire need to discover novel drugs that selectively target only the cancer cells with minimal effects on normal cells. Our research is an effort to identify a novel plant based drug which is edible and selectively targets only the leukemic cells with negligible effects on the normal cells. In this study, extracts from *Myrothamnus flabellifolius*, a South African resurrection plant, were used against human leukemic cells (HL-60). *M. flabellifolius* is known for its anti-viral, anti-microbial, and anti-inflammatory properties. Extracts from this plant also contain derivatives of galloyl and quinic acid. In literature, galloyl and quinic acid have been demonstrated to show anti-cancerous effects. Here, we investigated the anti-cancerous effects of the methanolic and petroleum ether extract of this plant on human leukemic cells (HL-60) compared to normal lymphocytic cells (TK6). The methanolic extract depicted reduced HL-60 cell viability while the petroleum ether extract did not. The loss in HL-60 viability in response to the methanolic extract was accompanied by the induction of caspase-dependent apoptosis by way of caspase-7 and Poly (ADP-ribose) polymerase cleavage. This study establishes an IC₅₀ of 62.5 µg/ml of dry *Myrothamnus* extract on HL-60 leukemic cells.

Industrial Relevance. The outcome of our study depicts the potential of *M. flabellifolius* as a cancer drug due to its selective biological activity against cancer cells. The anti-cancer effects of this plant extract did not manifest toxic side effects as it did not harm the normal lymphocytic cells. The edible nature of *M. flabellifolius* marks it as having a potential role in cancer treatment as a complementary medicine to the existing treatment options.

Keywords. *Myrothamnus flabellifolius*; Anticancer; HL-60; TK6; Leukemia

INTRODUCTION

Acute myeloid leukemia (AML) treatments include chemotherapy, radiation and stem cell transplantation. These treatments have been associated with incomplete remission and have severe side effects as they harm normal cells (Abdel-Wahab & Levine 2010). Hence, there is a need to look for novel drugs that are effective in cancer treatment but only have minimal side effects. Many plant-based formulations are known to have anti-cancer properties and have also been explored as alternative or complementary medicine in cancer treatment (Cragg et al., 2009). In this study, we have investigated the anti-cancer effects of *Myrothamnus flabellifolius*. This plant is generally grown in South Africa and is used commonly in African traditional medicine (Moore et al., 2007).

*M. flabellifolius* is woody, desiccation-tolerant and is commonly known as the resurrection plant. Natives of Africa utilize this plant in tea and decoctions to treat influenza, kidney disorders, hemorrhoids, and gingivitis (Moore et al., 2007; Henley-Smith et al., 2013). Most commonly, this plant is brewed and used as treatment for the common cold and chest complaints (Maroyi 2013). *M. flabellifolius* is rich in polyphenols and particularly galloylquinic acids. 3, 4, 5 tri-O-galloylquinic acid makes up over 90% of the low molecular weight polyphenols found in the plant (Russo et al., 2010; Moore et al., 2005). The compound, 3, 4, 5 tri-O-galloylquinic acid, is also a strong scavenger of free radicals (Baratto et al., 2003). Galloylquinic acids are medicinally known for their wound healing properties. These compounds possess high activity against bronchial hyper-reactivity, allergic reactions, HIV reverse transcriptase, and DNA polymerase (Moore et al., 2007). Additionally, plant polyphenols are known to protect cells from oxidative stress and thus are known to aid as anti-tumor, anti-viral, anti-bacterial, and anti-mutagenic agents. Flavonoids, one of the major polyphenols, have shown to cause a concentration dependent decrease in cancerous cell viability (Russo et al., 2010; Sergiediené et al., 1999). The high polyphenolic content of *M. flabellifolius*, particularly flavonoid content (Molefe-Khamanga 2012), led us to investigate if these extracts would induce cell death or inhibit cell proliferation in human myeloid leukemic cells.

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In this study, we measured the dose-dependent effects of *Myrothamnus flabellifolius* extract on leukemic cells after 24 hours of treatment. To ensure that the anti-cancer effects are specific to HL-60 cells, a non-leukemic cell line (TK6) was included in the study. Two different solvents were used for extraction which included petroleum ether and methanol. Petroleum ether solubilizes sterols, triterpenes, omega 3 fatty acids, omega 6 fatty acids, and other lipid derivatives (Seida et al., 2013). Methanol solubilizes phytochemicals such as flavonoids, alkaloids, saponins, tannins, terpenoids, and glycosides (Chinsembu & Hedimbi M. 2013; Tedesse et al., 2012). Both the extracts were tested for selectivity for HL-60 cells.

**MATERIALS AND METHODS**

**Cell Culture.** HL-60 (ATCC® CCL-240™) and TK6 (ATCC® CRL8015™) cell lines were purchased from ATCC (Manassas, VA). Cells were cultured in RPMI-1640 Medium (ATCC® 30-2001™) with 10% heat inactivated Fetal Bovine Serum (ATCC® 30-2020™). Incubator was kept at 37°C in a humid atmosphere containing 5% CO₂. Cells utilized for assays possessed viability above 94%. To evaluate the effects of *M. flabellifolius* against leukemic and non-leukemic cell lines, the cells were seeded and identically treated with equal doses of the two types of plant extract for 24 hours.

**M. flabellifolius Extract Preparation.** Separate extractions of *M. flabellifolius* were conducted using methanol as one solvent and petroleum ether as another solvent. Stems and leaves of the plant were ground to a fine powder using a mortar and pestle. One milliliter of methanol or petroleum ether was added to 0.1 gram of the plant powder. The dissolved powder was incubated at 4°C overnight, and the supernatant was then evaporated using rotary evaporator. The extract kept -80°C for storage and dissolved in DMSO prior to usage. DMSO dissolved extract was directly added to cell culture media (RPMI-1640 + 10% FBS) prior to treatment with cells. The final amount of extract to enter cell suspension contained 0.6% DMSO.

**Cell Viability Assay.** A series of 6 serial half dilutions of *M. flabellifolius* extract in RPMI + 10% FBS were prepared in 96 well plates. The dilutions from the first well to the sixth well contained the following concentrations of *M. flabellifolius* respectively: 500µg/ml, 250µg/ml, 125µg/ml, 62.5µg/ml, 31.25µg/ml, and 15.625µg/ml. Experimental wells contained 100 µL of plant extract and 100µl of cell suspensions containing 10⁵ cells. Controls included a cell suspension in standard cell culture media and a DMSO control containing 0.6% DMSO. Each well contained a final volume of 200µl. Cells were incubated for 24 hours in standard incubator conditions. After 24 hours, the extract was removed from the cells and viability was determined through a propidium iodide assay. First, the plates were spun in a refrigerated centrifuge at 1800 RPM for 5 minutes to remove the extract. Half of the treated and control wells were killed with 100µL of 70% ethanol in order to determine the total fluorescence of all the cells in those wells. Next, all cells were stained with 0.01mg/mL of propidium iodide (Molecular Probes®) and incubated for 15 minutes at 4°C. Finally, the propidium iodide was centrifuged off and cells were resuspended in complete media. A fluorometer was then used at an excitation of 485/20 and an emission of 590/35 to measure fluorescence. The fluorescence values were utilized to determine cell viability using the following equation: \([\text{extract treated cells – background control)} / (\text{70% ethanol killed cells – background control}) * 100]\). Note that the background is the media (RPMI + 10% FBS) in which the cells were suspended.

**IC₅₀ values calculations.** The concentration of extract inducing 50% inhibition of cell growth was calculated using the ed50v10 excel add-in (downloaded from sciencegateway.org). This add-in is specifically for the calculation of IC50/EC50 values. Data gathered from the cell viability assay was used to determine the concentration of *M. flabellifolius* needed to for 50% growth inhibition of HL-60 and TK6 cells.

**Western Blot.** After treatment with extract, cells were harvested, washed once in 1X phosphate-buffered saline, and lysed in laemmli sample buffer (62.5 mM Tris-HCl pH 6.8, 25% glycerol, 2% SDS) containing 1% protease (Sigma) and 1% phosphatase (Calbiochem) inhibitor cocktails. Protein concentrations were determined using the 660 nm protein assay supplemented with an ionic detergent compatibility reagent (Pierce). Samples were boiled for 5 minutes and 25µg protein was loaded onto 6-5% (PARP) or 11% (caspase-7) SDS-PAGE gels. Protein was transferred to nitrocellulose and membranes were blocked for 1h at RT in 5% non-fat dry milk. Membranes were probed overnight at 4°C with anti-human caspase-7 (1:1000, Cell Signaling), anti-PARP (1:1000, Cell Signaling), or anti-β actin (1:2000, Santa Cruz) antibodies, washed in tris buffered saline containing 0.1% tween-20, and incubated for 1h at RT with horseradish-peroxidase linked anti-Ms or anti-Rb (1:1000-1:3000, Cell Signaling) IgGs. Bands were detected by enhanced chemiluminescence.

**Statistical analysis.** All the results are expressed as average ± SD of three independent experiments. Statistical comparisons were performed using a two-sided Student’s t-test. P values less than .05 were considered to be significant.

**RESULTS AND DISCUSSION**

**Cell viability assay and determining IC₅₀.** Incubation of HL-60 and TK6 cells in methanolic extract for 24 hours resulted in a significant reduction in HL-60 counts. However, a similar reduction in cell counts was not observed in TK6 cells. This decrease in cell counts was observable in the results of the cell viability assay as well as through phase contrast microscopy (Fig. 1). Thus, the methanolic extract was not as detrimental to the growth of TK6 cells as it was to HL-60 cells.
To determine cell viability post 24 hour treatment with extract, cells were stained with propidium iodide. HL-60 cells suspended in the methanolic extract of *M. flabellifolius* depicted a decrease in cell viability as the extract concentration was increased (Fig. 1). The IC$_{50}$ of the methanolic extract was determined to be at a concentration of 62.5µg/ml for HL-60 cells and 248µg/ml for TK6 cells (Fig. 2A, Table 1).

In contrast, the IC$_{50}$ of the petroleum ether extract occurred at 250µg/ml for HL-60 cells and 195.5µg/ml for TK6 cells (Fig. 2B). Based on this, we conclude that the petroleum ether extract of *M. flabellifolius* was not selective against the cancerous cells and was more detrimental to the growth of TK6 cells. Moreover, the methanol extract of *M. flabellifolius* can be concluded to be the more effective cytotoxic agent as compared to the petroleum ether. A previous study has shown presence of alkaloids, flavonoids, gums, glycosides, proteins and amino acids, phenolics and tannins, saponins and steroids in the methanolic extracts of *M. flabellifolius* (Molefe-Khamanga, 2012). The predominant polyphenol in the leaves of this plant is 3, 4, 5 tri-O-galloylquinic acid. This compound is localized in the vacuoles of the leaves and contributes to sustaining hydration in the leaves. However, the effects of 3, 4, 5 tri-O-galloylquinic acid on cancer cells has yet to be studied.
Table 1. Comparisons of IC50 values of *M. flabellifolius* extract inhibiting cell growth 24 hours post treatment.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Methanolic Extract (µg/ml)</th>
<th>Petroleum Ether Extract (µg/ml)</th>
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<tbody>
<tr>
<td>HL-60</td>
<td>62.5±0.40 *</td>
<td>250±0.40</td>
</tr>
<tr>
<td>TK6</td>
<td>248±0.50</td>
<td>195.5±0.40</td>
</tr>
</tbody>
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*p<0.05 for HL-60 vs. TK6 cells

Determining apoptosis induction mechanisms by methanolic extracts. To determine whether the reduction in cell viability caused by *M. flabellifolius* methanolic extract was a consequence of apoptosis induction, two well established markers of apoptosis were measured. Upon cleavage by initiator caspases, the effector caspase-7 cleaves substrates involved in the demolition phase of apoptosis (Shi 2002). Activation of caspase-7, evident by an increase in the active p20 cleaved fragment, was induced after 24h incubation with the HL-60 IC50 concentration (62.5 µg/ml) of *M. flabellifolius* methanolic extract in HL-60, but not TK6 cells (Fig 3). Poly (ADP-ribose) polymerase (PARP), a DNA repair protein that responds to environmental stress, is cleaved by caspases during apoptosis to facilitate cellular disassembly (Oliver et al., 1998). In agreement with caspase activation, cleavage of PARP was observed in HL-60, but not TK6 cells after incubation with the HL-60 IC50 concentration (62.5 µg/ml) of methanolic extract (Fig 3). The presence of some cleaved caspase-7 and PARP in controls and TK-6 samples reflects a small number of cells undergoing apoptosis in normal cultures, a common occurrence variable with cell type. Nevertheless, no increase in cleavage was observed in TK-6 cells upon treatment and thus, *M. flabellifolius* extract selectively induced caspase-dependent apoptosis in leukemic HL-60 cells.

Figure 3. Methanolic extract from *M. flabellifolius* selectively induced apoptosis in HL-60 cells. HL-60 and TK-6 cells were treated with a low (31.25µg/ml) and the HL-60 IC50 (62.5 µg/ml) concentration of methanolic extract for 24h. No treatment and DMSO only controls were included. Cells were lysed and protein was harvested for SDS-PAGE and western blot analysis of caspase-7 and PARP cleavage. Full length (procaspase-7, PARP), cleaved caspase-7 (active p20 fragment) and cleaved PARP are shown. β-actin was used as a loading control. Three replicates of the above experiment was performed to ensure reproducibility.
We show here that the methanolic extract of this plant is selectively inducing apoptosis in leukemic cells with minimal effects on their non-leukemic counterparts. Although the mechanisms of the extract are unclear at the moment, it is noteworthy that the extract shows selective apoptosis induction in leukemic cells. This observation suggests this compound is targeting a key cellular or molecular difference that exists between normal and leukemic cells. Many current therapies for leukemia are treated with drugs that affect both cancer and normal cells, yielding unwanted side effects. Thus, finding novel drugs with increased cancer cell specificity is highly important. Our results suggest that there is a potential for *M. flabellifolius* in anticancer drug development. Furthermore, identification of the anticancer properties of *M. flabellifolius* offers an edible, plant-based treatment option for leukemia. This work lays ground work for a potential alternative complementary medicine to the already existing therapeutic approaches.

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**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

**REFERENCES**


