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Achillea fragrantissima extract exerts its anticancer effect via induction of differentiation, cell cycle arrest and apoptosis in chronic myeloid leukemia (CML) cell line K562

Asma M. Alenad[1,2], Nabila A. Al-Jaber[2], Soundararajan Krishnaswamy[3,4], Sobhy M. Yakout[3,4], Nasser M. Al-Daghri[3,4]* and Majed S. Alokail[3,4]

[1]Chemistry Department, Faculty of Sciences, Al Jouf University, Sakaka, Al Jouf, Saudi Arabia.
[2]Department of Chemistry, College of Science, King Saud University, Riyadh, Saudi Arabia.
[3]Biomarkers Research Program, Department of Biochemistry, College of Science, King Saud University, Riyadh, Saudi Arabia.
[4]Center of Excellence in Biotechnology Research, King Saud University, Riyadh, Saudi Arabia.

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The advantages of herbs in cancer therapy/prevention are attributed to their broad inhibitory activity against multiple aberrant cancer specific pathways and non-toxic nature. *Achillea fragrantissima* (Af) is a traditional herb used to treat viral fever and chronic diseases such as arthritis and diabetes. However, the anticancer potential of Af has not been studied yet. Anticancer properties of extract from Af leaves were studied using human chronic myeloid leukemia (CML) (K562), T cell lymphoma (Jurkat) and hepatocellular carcinoma (HepG2) cell lines. Af extract induced morphological changes were studied using inverted light microscope and its effect on viability of cells was measured by trypan blue assay. Af extract altered the morphology of K562 and Jurkat cells from spherical to spindle shape. This shape change was considered indicative of initiation of differentiation and occurred at a low concentration of the extract (0.5 mg/ml) in K562 cells and at this concentration viability of K562 cells was not significantly affected. At higher (≥1 mg/ml) concentrations, Af extract induced elongation of K562 cells, which was considered indicative of cell cycle arrest. Increasing concentration of Af extract also caused plasma membrane rupture in a higher proportion of K562 cells resulting in reduced viability. Af extract reduced the viability of K562 cells in a time and dose dependent manner. Results from our study indicated Af extract as a potent inducer of differentiation, cell cycle arrest and apoptosis in K562 and Jurkat cells. Af extract activated these anticancer mechanisms in a concentration dependent manner. Anticancer activity of Af extract on K562 and Jurkat cells, mediated by multiple independent mechanisms, suggests its potential use in the treatment of drug resistant leukemia and other cancers.

Key words: Chronic myeloid leukemia, chronic myeloid leukemia (CML), differentiation therapy, K562, *Achillea fragrantissima*, Jurkat, hepatocellular carcinoma (HepG2), cell cycle arrest, apoptosis.

INTRODUCTION

Plant constituents and their derivatives hold great promise in the treatment and prevention of various cancers (Abdel-Hamid et al., 2011; Pezzuto, 1997; Balunas and and Kinghorn, 2005). Plant components exhibit superior anticancer properties by overcoming limitations of chemo- and targeted-therapies. Several plant compounds

*Corresponding author. E-mail: aldaghri2011@gmail.com. Tel: 00966-4675-939. Fax: 00966-4675-931.

used in traditional medicine are common dietary ingredients, and hence, are also considered safe (Haque et al., 2010).

Plant-derived anticancer agents are broad specific and affect multiple pathways simultaneously. Plant-derived antioxidants have been effective at reducing inflammation and level of reactive oxygen species (ROS), the two common hallmarks of the genesis and progression of all cancers (Gupta et al., 2010; Sung et al., 2012). Certain plant components capable of killing cancer cells are also known to suppress the tumor promoting actions of immune and other tumor stromal cells (Jagetia and Aggarwal, 2007). In addition, plant products are less expensive and readily available. Intense research involving plant derivatives has lead to multiple ongoing clinical trials, while a few have already been approved for human use (Mehta et al., (2010); Cragg and Newman, 2005; Srivastava et al., 2005; Asher and Spelman, 2013).

The genus *Achillea*, consisting of about 140 perennial herbs, has traditionally been used in Middle Eastern countries, to treat digestive problems, liver and gall-bladder conditions, menstrual irregularities, cramps, fever and wound healing, among others (Nemeth and Bernath, 2008). Recent research on Af has confirmed its analgesic, anti-ulcer, hepatoprotective and wound healing activities (Nemeth and Bernath, 2008). Af extract exhibited antiviral activity against herpes simplex-1 virus (HSV), poliomyelitis-1 virus (POLIO) and vesicular stomatitis virus (VSV) (Soltan and Zaki, 2009).

Recent reports on the anti-inflammatory and antioxidant functions of Af extract imply potential anticancer activity. Af inhibited lipopolysaccharide (LPS) induced synthesis of nitric oxide (NO) and various proinflammatory mediators, like IL-1β, TNF-α, MMP-9, COX-2 and iNOS (Elmann et al. 2011). Out of 65 plant extracts tested for various anti-inflammatory functions, Af was the most potent in inhibiting NO synthesis (Elmann et al., 2011). Af has also been shown to possess strong antioxidant potential (Khaled et al., 2010).

In this study, the anticancer properties of Af extract was evaluated using CML cell line K562 as an *in vitro* model. Results indicate that this extract can induce differentiation, cell cycle arrest and apoptosis leading to reduced viability of K562 cells.

MATERIALS AND METHODS

The cell culture media [Roswell Park Memorial Institute medium (RPMI)-1640 and Dulbecco's modified eagle's medium (DMEM)], fetal bovine serum (FBS) and penicillin-streptomycin were purchased from Gibco BRL (Life technology). Cell lines K562, Jurkat and HepG2 were obtained from American Type Culture Collection (ATCC), USA.

Af extract preparation

Af leaves collected around Riyadh in Saudi Arabia were washed to remove dirt, allowed to dry at room temperature for seven days and finely powdered. One kilogram of powder was extracted thrice with methanol under reflux. The extract was concentrated to dryness in rotary evaporator under reduced pressure at <35°C and dried over anhydrous sodium sulphate. 100 mg of dried Af extract was redissolved in 200 µl of water to yield a solution with a final concentration of 0.5 mg/ml, centrifuged to remove any undissolved material and the supernatant used for treating cells.

Cell culture

K562 and Jurkat cells were grown in RPMI-1640 medium supplemented with FBS (10%, v/v), streptomycin (100 µg/ml) and penicillin (100 U/ml). 5×10^4 cells/ml of each of K562 and Jurkat cells were suspended in 24 well plates (1 ml/well) and incubated under 5% CO_2 in a humidified atmosphere at 37°C. HepG2 cells were grown as an adherent culture in DMEM medium supplemented with 10% FBS and streptomycin and pencillin antibiotics. 24 h later, appropriate amount of Af extract was added from the aqueous stock solution and incubations continued for various durations.

Morphological evaluation of differentiated cells

Control and Af treated cells were incubated for 24 h and photographed using inverted light microscope, at high (400x) magnification. Percentage of morphologically altered (spherical to spindle shape) cells was determined from the number of cells having spindle shapes.

Statistical analysis

Chi-square analysis was applied to determine the significance of difference in cell viability at various time points and concentrations of Af extract. P values <0.05 were considered significant.

Cell viability assay

Cell numbers and viabilities were assessed using a hemocytometer based on the ability of the viable cells to exclude trypan blue (Strober, 2001). Briefly, at the end of Af extract treatment period, K562 cells in the well were mixed well and an aliquot of cells was mixed with an equal volume of 0.4% trypan blue and after 2 to 3 min, were counted by hemocytometer and viable cells were expressed as a percentage. Number of viable cells in the untreated well at the end of the incubation period was considered 100%. Cell numbers with standard deviation were averaged from 3 independent experiments.

Gas chromatography-mass spectrometry (GC-MS) analysis

GC-MS analysis of 50% ethanolic extract of fenugreek was performed using an Agilent 7890 GC System Gas Chromatograph interfaced to a Mass Spectrometer (GC/MS) equipped with a VF-5ms capillary column (30 m × 0.25 mm ID × 0.25 µm). GC/MS detection was achieved using an electron ionization system with 70 eV ionization energy. Helium (99.999%) was used as carrier gas at a constant flow rate of 1 ml/min. An injection volume of 2 µl was employed in splitless mode. Injector and ion-source were maintained at 250 and 280°C, respectively. The oven temperature was raised from 100 (isothermal for 2 min) to 200°C at a rate of 5°C min^{-1} and further up to 240°C by 10°C min^{-1} and maintained at 240°C for 11 min. Total GC running time was 40 min. The relative amount of each constituent was calculated by measuring

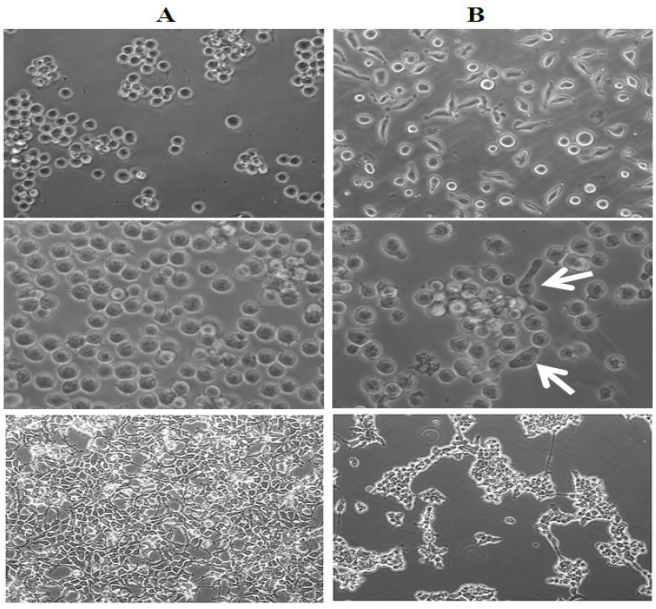

Figure 1. *A. Fragrantissima* extract induced morphological alterations in cancer cell lines. Representative inverted light microscope (400× magnification) pictures of different cell types treated with *A. fragrantissima* extract. K562 cells in RPMI medium were incubated overnight (16 h) without (column A) or with (column B) *A. fragrantissima* extract at 0.5 mg/ml. Treatment with *A. fragrantissima* extract altered the morphology of some of the K562 (top panel) and Jurkat (middle panel) cells from spherical to spindle or elongated shapes as indicated by arrows. Unlike K562 and Jurkat, which are suspended cells, adherent HEPG2 (bottom panel) cells did not show similar morphological changes, even though their numbers were reduced.

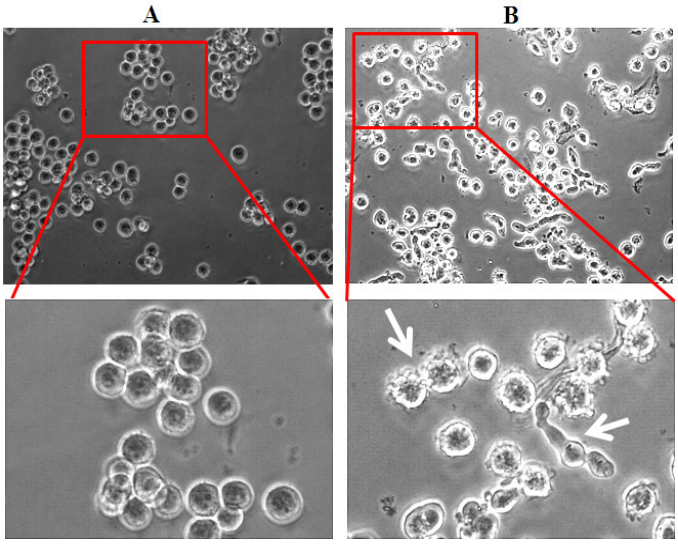

Figure 2. *A. fragrantissima* extract induced cell membrane disintegration in K562 cells. Close-up images of K562 cells undergoing cell elongation and membrane rupture and following incubation with *A. fragrantissima* extract. K562 cells in RPMI medium. (A) K562 cells in RPMI + *A. fragrantissima* extract (1 mg/ml). (B) Arrows point to elongated cells or cells undergoing membrane rupture.

corresponding peak area and represented as a percentage of the sum of areas of all peaks. MS Workstation 7.0 software was used to analyze mass spectra and chromatograms.

RESULTS

Effect of Af extract on morphology of K562, Jurkat and HepG2 cells

K562, Jurkat and HepG2 cells incubated for 16 h with Af extract at 0.5 mg/m showed substantial morphological changes. Morphological changes involved alteration from the original spherical to elliptical shape in K562 and Jurkat cells (Figure 1). In addition, some of the cells also appeared elongated. The shape change was observed only in K562 and Jurkat cells, the two suspension cell types, whereas, HepG2 cells were released from the bottom of the flasks upon treatment with Af extract. At 1 mg/ml of extract, cells were more elongated (cells indicated by arrows in Figure 2). Here, the cells appeared to continue to grow even after the division had stopped resulting in long cells. Approximately 30% of K562 and 10% of Jurkat cells had elliptical shape at 16 h following treatment with 0.5 mg/ml Af extract. At concentrations ≥1 mg/ml of Af extract, number of cells with ruptured plasma membrane and cell death seemed to predominate over those with shape change. Based on the substantially larger morphological changes seen upon treatment with Af extract, viability studies were pursued only with K562 cells.

Effect of Af extract on cell viability

At a low concentration of 0.5 mg/ml, Af extract inhibited the growth of K562 cells, while the untreated cells grew exponentially for up to 3 days (Figure 3). The difference in the percentage of viable cells between the control and Af treated cells was significant at 24 ($P=0.001$), 48 ($P<0.001$) and 72 ($P<0.001$) h. To check the effect of concentration, K562 cells were incubated with different concentrations of Af extract and viable cells determined after different time durations. A range of Af extract concentrations starting from 0.1 mg/ml, reduced survival of K562 cells and level of growth inhibition increased with increasing concentration (Figure 4). At concentrations above 1 mg/ml, death of some cells occurred at 24 h. At concentrations of 2 and 4 mg/ml, most of the cells were dead by 48 h. Statistically, significant ($P \leq 0.05$) difference in the viability of cells was seen at or above Af concentration of 0.05 mg/ml.

GC-MS analysis of Af extract

GC-MS analysis of Af extract revealed the presence of 16 unique compounds whose identities are given in Table 1.

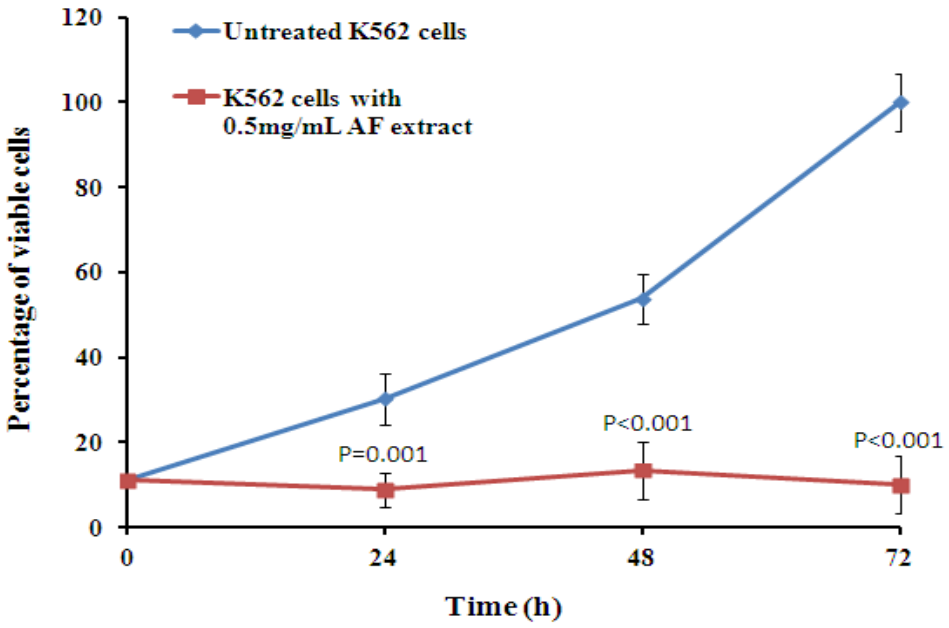

Figure 3. *A. fragrantissima* extract inhibits K562 cell proliferation. K562 cells incubated with 0.5 mg/ml of *A. fragrantissima* extract were followed for 72 h for cell growth. *A. fragrantissima* extract inhibited proliferation completely while the control cells grew exponentially during this period. Viable cells were determined by trypan blue assay. Cell numbers with standard deviation were averaged from three independent experiments. Number of cells in the untreated well at the end of the incubation period was considered 100%. Chi-square analysis of data was performed between control and treated cells at different time points.

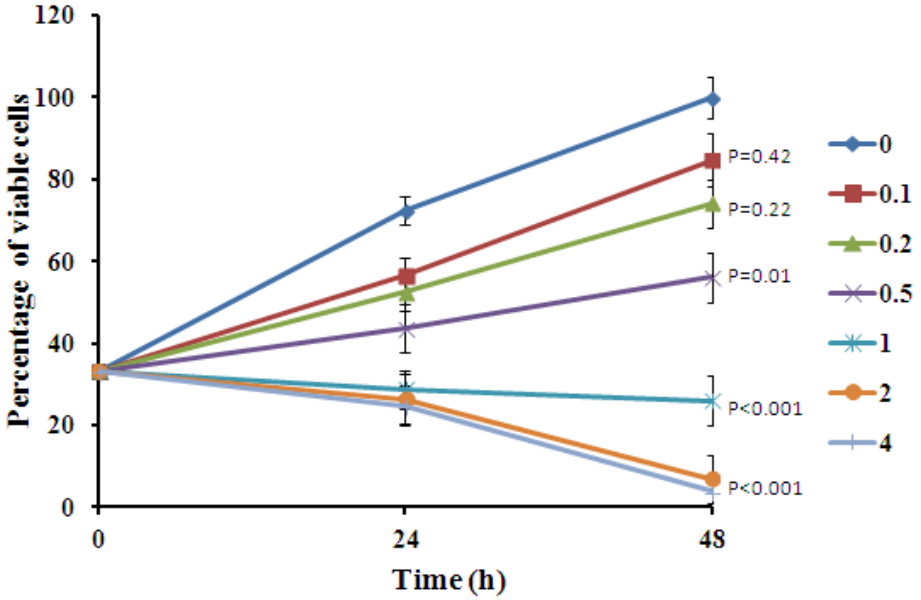

Figure 4. Viability of K562 cells following incubation with *A. fragrantissima* extract. K562 cells incubated with increasing concentrations of *A. fragrantissima* extract (mg/ml) were followed for 48 h for cell growth. Viability of K562 cells was reduced by *A. fragrantissima* extract in a time- and dose-dependent manner. Viable cells were determined by trypan blue assay. Cell numbers with standard deviation were averaged from 3 independent experiments. Number of cells in the untreated wells at the end of the incubation period was considered 100%. Chi-square analysis of data was performed between control cells and cells treated with different concentrations of *A. fragrantissima* extract.

Table 1. Chemical composition of *A. fragrantissima* extract.

Retention time (min)	Compound name	Formula	Total%
6.88	6-Methyl-3-cyclohexen-1-carboxaldehyde	$C_8H_{12}O$	3.12
8.56	2-Methoxy-4-vinylphenol	$C_9H_{10}O_2$	4.76
9.34	Syringol	$C_8H_{10}O_3$	0.56
11.31	Landrin B	$C_{11}H_{15}NO_2$	5.86
12.65	1,4-dihydroxy-p-menth-2-ene	$C_{10}H_{18}O_2$	0.66
13.35	5,5-Dimethyl-1,5-oxasilonan-9-one	$C_9H_{18}O_2Si$	1.16
15.57	[3-(Dimethylamino)propyl]ethylcarbodiimide	$C_8H_{17}N_3$	2.29
18.69	12-Oxatricyclo[4.4.3.0(1,6)]tridecane-3,11-dione	$C_{12}H_{16}O_3$	2.01
18.91	5-Hexyl-2,4-dimethyl-1,3-oxazole	$C_{11}H_{19}NO$	3.60
19.49	9,10-Dimethyltricyclo[4.2.1.1(2,5)]decane-9,10-diol	$C_{12}H_{20}O_2$	0.66
23.18	Pyrethrone	$C_{11}H_{14}O$	1.48
28.75	Flavone, 5,7-dihydroxy-3',4',5'-trimethoxy	$C_{18}H_{16}O_7$	0.78
28.91	Anobin	$C_{15}H_{20}O_5$	17.36
30.95	γ-Himachalene	$C_{15}H_{24}$	2.16
33.15	Tetraneurin D	$C_{17}H_{24}O_6$	9.25
33.69	Bis(2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$	44.27

DISCUSSION

Previously, Af was shown to exhibit antiviral and anti-inflammatory properties. In this study, Af extract induced morphological changes involving spherical to spindle and elongated shapes in K562 and Jurkat cells representing differentiation and cell cycle arrest, respectively. Af extract reduced the proliferation and caused death of K562 cells in a dose and time dependent manner. At low concentration of Af extract, differentiation of K562 was more prominent while at higher Af concentrations cell cycle arrest and plasma membrane rupture were predominant. Results from this study suggest that Af extract may serve as a novel potential therapeutic capable of inducing differentiation, cell cycle arrest and apoptosis in chronic myelogenous leukemia (CML) cells.

Proliferation, differentiation and apoptosis of normal hematopoietic cells are closely intertwined, and under normal circumstances are carefully balanced. Under normal conditions, induction of differentiation is accompanied by a loss of proliferative capacity, and cell maturation leads to eventual cell death. Disruption of the differentiation process of hematopoietic progenitors leading to unrestricted proliferation is a crucial factor in the pathogenesis of leukemias (Tsiftsoglou et al., 2003).

Cancer treatments using small molecule inhibitors, usually targeting specific tyrosine kinases, eventually lead to development of resistance due to the inherent heterogeneity of cancer cells in tumors (Lee and Swanton, 2012). To overcome drug resistance and relapse of disease therapy with minimal side effects were highly desired in the clinical field, and differentiation induction therapy appeared to be an ideal solution (Koeffler, 1983; Spira and Carducci, 2003). Differentiation therapy promised to be an alternative approach to treatment due to its higher specificity towards cancer cells compared to the chemo and targeted therapies (Sell, 2006).

Differentiation therapy is based on the principle that employing agents capable of inducing differentiation and terminal cell division will eventually lead to apoptosis of cancer cells (Leszczyniecka et al., 2001; Pettersson et al., 2011). Differentiation of cancer cells induced by safe plant components, derived from traditional medicinal plants, was considered a less toxic approach to treating cancers. Induction of differentiation in cancer cells by Af extract, therefore, offers a promising approach to treat leukemias and other cancers. Also, combining differentiation inducing agents with cytotoxic drugs for cancer therapy was suggested to increase the chances of cure through accelerated apoptosis, prevention of re-growth of malignant cells and drug resistance (Waxman et al., 1990; Hozumi, 1998; Huang and Waxman, 1998; Niitsu and Honma, 1999; Waxman, 2000).

The mechanism underlying the death of K562 cells caused by Af extract could be explained by the various morphological changes. The morphological change in K562 cells from spherical to spindle shape was shown to be a reliable indicator of differentiation and typically induced by 12-O-tetradecanoylphorbol-13-acetate (TPA) or phorbol 12-myristate 13-acetate (PMA) (Rosson and O'Brien, 1995; Jin et al., 2008; Ghanbarvand and Dehnad, 2011). Similarly, TPA was also shown to induce terminal differentiation and hence block proliferation of Jurkat cells (Makover et al., 1991).

In our study, higher proportion of spindle shaped cells found at lower concentration of Af extract, indicate differentiation as an intermediate stage before final death of cells. At higher concentrations of extract, apoptosis was more prominent as seen from the higher proportion of K562 cells with ruptured plasma membrane. It has been

been shown previously that many agents at low concentrations induce differentiation and terminal cell division with minimal apoptosis, while at higher concentration, usually by an alternative pathway, induced apoptosis (Finnin et al., 1999; Maurer et al., 1999; Suh et al., 1999; Richon et al., 2001; Wu et al., 2001).

Af extract treatment also resulted in elongation of K562 and Jurkat cells. Elongated cells have been shown to be the phenotypic result of cell cycle arrest at G1 (Carreira et al., 2006) or G2 (Huard et al., 2008) phase. G2 phase cell cycle arrest was shown to result in elongation of Hela cells (Shenker et al., 1999). Gastric adenocarcinoma (AGS, a gastric cancer cell line) cells appeared elongated after G2/M phase cell cycle arrest caused by a natural product GKBM, a combination of extracts from several medicinal plants (Luk et al., 2005).

Several reports showed apoptosis to follow differentiation/and cell cycle arrest. K562 cell death by apoptosis following differentiation has been reported recently for several plant components (Subhashini et al., 2004; Miao et al., 2011). Resveratrol was shown to eliminate imatinib resistant CML cells by causing differentiation, apoptosis and caspase-independent death (Puissant et al., 2008).

Incubating K562 cells with ethanolic extract of Af plant dramatically reduced the viability in a time and dose dependent manner. It was inferred from morphological changes that the underlying cell death mechanism may involve induction of differentiation, cell cycle arrest and apoptosis. Confirmation of each of these anticancer mechanisms, by analyzing the expression of corresponding molecular markers, may lead to eventual application of Af for treating drug resistant leukemias and other cancers. Gas chromatography-mass spectrometry (GC-MS) analysis showed the presence of 16 unique compounds in Af extract, and more focused studies (planned for the future) may lead to identification of specific constituents responsible for each of these anticancer actions.

Conclusion

Extract from Af, a herbal plant with known anti-inflammatory and antiviral properties, induced differenttiation, cell cycle arrest and apoptosis, and these processes collectively reduced the viability of K652 cells. Due to its non-toxic nature, Af extract may serve as an effective therapeutic in treating patients with drug resistant CML and warrants further research on other cancers.

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