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Full Length Research Paper

Antileukemia activity from root cultures of *Vernonia amygdalina*

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***Vernonia amygdalina*, Del (Compositae) is an African medicinal plant well known for producing the anticancer agents' vernodaline and vernolide. It grows wild under severe anthropogenic and environmental pressures. The roots are the principle material for herbal medicine. It is collected from the wild there is great variability in the quality and effectiveness of the root extracts. It is necessary to establish a fast-growing root culture and to test the extracts of cultured roots for activity against leukemia cells in vitro. Leaves were cultured on half-strength MS medium supplemented with different auxin types and concentrations. Basal medium supplemented with indole-3-butyric acid (IBA) at 2.0 mg/l favored induction of the highest number of roots/explant (38.3 ± 1.1). After six weeks well-established roots were separated. About 100 mg of fresh root tissue was cultured in 80 ml full-strength MS liquid medium supplemented with 2.0 mg/l IBA and under continuous agitation (80 rpm). The biomass of root cultures increases by 21 fold after 5 weeks of culture. Cold water, hot water and ethanol extracts from the in vitro cultured roots were prepared and tested for their antioxidant activity and efficacy against leukemia cells. All of the extracts showed significant antioxidant activity. All the extracts could kill the majority (50-75%) of abnormal cells among primary cells harvested from 3 patients with acute lymphoblastic leukemia (ALL) and 3 with acute myeloid leukemia (AML). DNA fragmentation patterns were detected within treated cells and inferred targeted cell death by apoptosis. The metabolites within the extracts may act as tumor inhibitors that promote apoptosis. Therefore in vitro root culture can be an alternative to collection from the wild, cultivation in the field or to chemical synthesis of anticancer agents. In addition the plant extracts may be used to supplement or replace established drugs treatments.**

Key words: Anticancer, natural products, plant extracts

INTRODUCTION

In the early century of mankind, plant derived secondary metabolites have been used by humans to treat acute infections, health disorders and chronic illness. Only during the last 100 years have natural products been largely replaced by synthetic drugs (Wink et al., 2005). However, some important anticancer agents are still extracted from plants because they cannot be synthesized chemically on a commercial scale due to their complex structures that often contain several chiral centers. Further, some patients show resistance to some established treatments based on their genetics or repeated exposures (Mishra et al., 2006). Therefore, new treatments with different modes of action are constantly sought.

Vernonia amygdalina, Del (Compositae), tree between

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1 to 3 m in height (Igile et al., 1995) is a well known African medicinal plant that provides anticancer agents. Earlier investigations by Kupchan et al. (1969) showed that purified compounds from chloroform extracted fractions of *V. amygdalina*, identified as vernodaline, vernolide and vernomygdine, elicited cytotoxic effects in human carcinoma naso-pharynx cells. In addition, the antioxidant activities of compounds (luteolin, luteolin 7-O-β-glucuronoside and luteolin 7-O-β-glucoside flavonoid) isolated from the leaves of *V. amygdalina* have been reported using coupled oxidation of β-carotene linoleic acid (Igile et al., 1994). Subsequently, Jisaka et al. (1992) demonstrated that vernodaline and vernolide elicited antitumor activities in leukemia cells. Recently, Izevbigie (2003) reported that some peptides (edotides) from the aqueous extract of *V. amygdalina* showed cell growth inhibitory effects in prostate cancer cell line (PC-3) but no effect on normal human peripheral blood mononuclear cells (PBMC). Therefore, *V. amygdalina* roots may contain additional bioactive compounds or peptides which are yet to be discovered.

The plants of *V. amygdalina* used in preparing drugs are still being collected from wild plantations in regions where plant genetic resources are considered under severe anthropogenic and environmental pressures. Overgrazing, droughts and firewood harvesting add to the depletion of wild *V. amygdalina* (Robinson, 2005). The quality of roots collected from the wild can vary dramatically. Therefore, in vitro plant tissue culture may provide an alternative to field-grown roots (Dhingra et al., 2000). Shoot and root cultures of plant often produce the same compounds as in the field grown organs, sometimes in useful amounts (Wink et al., 2005). In vitro cultures may provide systems for sustainable bio-production of valuable (>$500/kg) biologically active compound in plants (McCabe et al., 1997).

Several studies have demonstrated that extracts from herbal medicines or mixtures had anticancer potential in vitro or in vivo (Bonham et al., 2002; Hu et al., 2002; El-Shemy et al., 2007). Aqueous extracts from willow (Salix sp.) (Saliceae) leaves prevented proliferation of three cancer cell types acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL) and Ehrlich ascites carcinoma cells (EACC; El-Shemy et al., 2007). Alcohol extracts of Ganoderma lucidum induced apoptosis in MCF-7 human breast cancer cells. In many cases the complex mixtures in crude extracts were more effective than single purified compounds (Hu et al., 2002).

Leukemia is one of the most common cancers (Cervantes et al., 2008). The greater prevalence of leukemia in the modern world may be due to the reduction of incidence of most infectious diseases and the increased life span of humans. Treatments for cancer diseases are expensive with no assurance that even simple leukemia can be cured. For developing countries the use of endogenous medicinal plants as cures against leukemia and other cancers is attractive. This study was therefore designed primarily to induce normal root culture of *V. amygdalina* in vitro and to determine their anti-proliferative effects on two leukemia cell types.

MATERIALS AND METHODS

Plant material

V. amygdalina plants were collected in summer from western part of Sudan and their identity was authenticated by comparisons to reference samples at the Botany Department, in the Medicinal and Aromatic Plant Research Institute, Khartoum, Sudan. Frozen explants, cultures and DNA were available on request.

Surface sterilization and in vitro induced plantlet

Explant surface sterilization and in vitro plantlet induction followed the methods described (Khalafalla et al., 2007). Briefly, leaf explants were collected from in vitro raised plantlet of 3-6-months old. Explants were washed thoroughly under running tap water for 15 min and then were surface-sterilized by submersion in 70% (v/v) ethanol for 60 s, followed by dipping in 25% (v/v) bleach (about 8% NaClO) for 25 min and washed thrice with sterile distilled water.

Culture medium and conditions for root initiation

Adventitious roots were induced on the leaf explants that were placed on half-strength MS (Murashige and Skoog, 1962) medium supplemented with different concentrations (0.5-2.0 mg/l) of different auxins, indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), α-naphthalene acetic acid (NAA). Two explants were cultured in each culture bottle; about 24 explants were cultured per treatment. The cultures were incubated at 25±2°C under 16 h photoperiod provided by cool white fluorescent lambs with 1000 lux intensity. The roots that formed were sub-cultured in fresh media. The extracts were made from root tissue (2-3 g dry weight).

Extract preparation

The extraction used 1 g of freeze-dried, powdered roots from culture suspended in 10 ml of hot water, cold water, or 80% (v/v) ethanol. Extracts were stirred mechanically for 12 h at room temperature (25ºC) except the hot water extract (80ºC) that was made in 30 min. Solids were removed by centrifugation (4,000 g, 10 min) and the supernatant collected. The resulting extracts were completely dried in a rotary evaporator at 40ºC and the lyophilized extracts stored at 4ºC for further process.

Antioxidant activity

The antioxidant activity of the plant extracts was evaluated by using the 2.2'diphenylpicrylhydrazyl (DPPH) assay (Cuendet et al., 1997; Burits and Bucar, 2000). The extracts (5-20 µg in 50 µl) were added to 5 ml of a 0.004% (w/v) of DPHH in methanol (100% v/v). After, a 30 min incubation period at room temperature the absorbance at 517 nm was compared to DPPH in methanol without an extract sample (blank) and quercetin was used as positive control. The percent inhibition of free radical formation (I %) was calculated as

$$I\% = (A\ blank\ \_\ A\ sample / A\ blank) \times 100$$

Where; A blank is the absorbance of the control reaction (containing all reagents except the extract), and A sample is the absorbance of the mixture containing the extract. The experiment

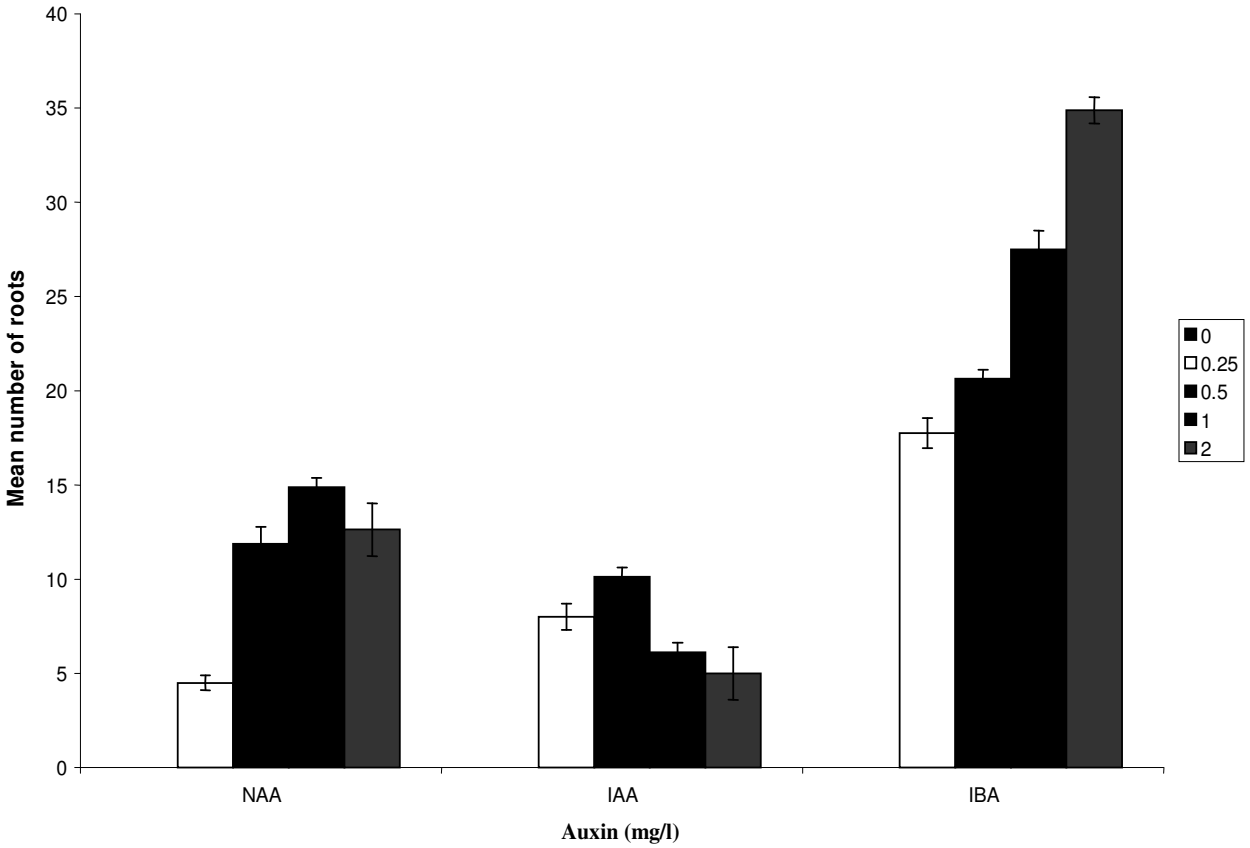

Figure 1. Effect of different auxins in half-strength MS solid medium on root induction of *Vernonia amygdalina.*

was carried out out in triplicate.

Viability of tumor cells

The study was performed on cells harvested from adult leukemia patients or healthy relatives admitted to the National Cancer Institute, Cairo University. International protocols governing the ethical treatment of patient were followed. The experimental samples were taken from healthy volunteer relatives (3 samples) and leukemia patients that included 3 ALL (acute lymphoblastic leukemia) and 3 AML (acute myeloid leukemia, immature monocytes) patients. ALL and AML had been diagnosed by peripheral blood and bone marrow examination and cytochemistry (with immunological markers used in two cases). Mononuclear cells were separated from other blood cells by Ficoll hypaque density gradient (Pharmacia, Uppsala, Sweden). The cells were then washed with three changes of PBS. The cell counts were adjusted so there were 105 cells in 0.1 ml (counting both mature and immature cells). The culture medium was prepared using modified Earle's salt with 1.2 g/l sodium carbonate and L-glutamine (Gibco, Grand, USA), 10% (v/v) inactivated fetal bovine serum (Gibco), 100 µg /ml penicillin and 100 µg/ml streptomycin was added. The medium was filtered through 0.22 µm Millipore filter, one ml of which was transferred into a 1.8 ml screw-capped sterile plastic tube. Next, 0.1 ml of the cell suspension containing 105 cells was added to each of 5 tubes per extract. To three of the tubes, 0.1 ml of the extract was added, while the other two tubes served as negative and positive controls. Culture medium was used instead of the extract for the negative control and the extract was added to the cells from healthy volunteers as a positive control. The tubes were incubated at 37°C in the presence of 5% (v/v) CO2 for 24 h (dark condition, humidified air). The cells were tested for their viability using the trypan blue exclusion test (Bennett et al., 1976). Two hundred cells were counted, and the percentage of viable cells was estimated.

DNA Extraction

DNA was extracted from mature (normal cells) and immature white blood cells (leukemic cells) before and after treatment with each extract. Cells were washed with PBS and then lysed in cold lysis solution (5 mM of Tris, pH 7.4, 20 mM of EDTA, 0.5% (v/v) Triton X-100) for 20 min (Gao et al., 2002). Cell lysates were centrifuged at 27,000 g for 15 min, and DNA was extracted from the aqueous phase with phenol: chloroform: isoamyl alcohol (25:24:1, v:v:v) containing 0.1% (w/v) hydroxyquinoline. DNA was precipitated with 0.3 mM of sodium acetate and 2 cm3 of cold 100% (v/v) ethanol. Agarose gel electrophoresis (1% w/v) at 30 mA for 2 h followed by UV fluorescence was used to determine the degree of DNA fragmentation (Gao et al., 2002; El-Shemy et al., 2007).

RESULTS

Regeneration protocol

In this study rooting responses were observed in leaf explants when cultured on basal medium containing auxins

Table 1. Measurement of antioxidant activity of plant extracts using DPPH.

Extracts (1 µg/ml)	Antioxidant activity (%)
Cold water	49 ± 0.49
Hot water	63 ± 0.96
Ethanol Extract	77 ± 0.51
Quercetin	61± 0.45
LSD	1.072

Each value represents the mean ± S.D (Standard Division) and mean of three replicates ($P \leq 0.05$).

(Figure 1). Explants failed to produce root in media without auxin. Therefore, in vitro root formation from *V. amygdalina* leaf is increased by the presence of auxin in the medium.

Of the various auxins tested, it was observed that IBAwas the best growth regulator for the adventitious root induction of *V. amygdalina* (Figure 1). The highest mean root number per explant (38.3 ± 1.1) was achieved on the basal media supplemented with IBA at 2.0 mg/l. The average rooting number increased from 17.8 to 34.9 when IBA concentration was raised from 0.25 to 2.0 mg/l.

DPPH radical scavenging activity

The plant extracts each showed a concentration-dependent scavenging activity by quenching DPPH radicals (Table 1). As judged by this assay, the ethanol extract showed high antioxidant activity at 77% inhibition of radical formation compared to 49% for the cold water extract at 1 µg/ml (Table 1). In the other hand, the positive control (quercetin) was tested and had the antioxidant activity at 61% inhibition of radical formation. This high antioxidant capacity may be due to the high concentration of phenolics and flavonoids in ethanol extracts.

Antileukemic effect

After 24 h incubation of the mononuclear ALL cells with plant extracts, a remarkable destruction of lymphoblasts (86%, only 14% were viable) occurred (Table 2). Destruction was dose, extract and time dependent ranging from 9 to 86% across the treatments. Responses were not linear, higher doses and longer times could not kill all the leukemia cells. However, cell death was significantly higher than that in the cells treated with media alone (8%, not shown). Similar results were observed, with AML cells, the mean viability of extract treated cells was 30% (70% destroyed) when compared to the cells treated with media alone (93% Table 3). In addition, the extracts were incubated with normal mononuclear cells from healthy volunteer (3 samples). There was no significant difference in killing healthy cells (mean 15.3%) when compared to the 7% caused by the media addition control (negative control; data not shown). Therefore, leukemia cells were more vulnerable to the extract than healthy cells.

DISCUSSION

Generally, the production of adventitious roots in plant was expected to be controlled by growth regulators (Davis and Hassig, 1990), with a key role being played by auxins (Henselova, 2002; Nandagopal and Kumari, 2007). However, the development of efficient and reproducible plant cell and organ culture protocols such as this holds a tremendous potential for the production of high-quality plant based medicines (Murch et al., 2000) and is often not a trivial barrier.

IBA was more effective than NAA in *V. amygdalina* adventitious root induction. The superiority of IBA is not a general rule though. Leonardi et al. (2001), observed that in Grevillea rosmarinifolia, A (Proteaceae), the effects of IBA and NAA on in vitro rooting were similar at a range of different concentrations. Watad et al. (1992) reported that, NAA was more effective than IBA in promoting root formation in some Grevillea species. Similarly, in Panax ginseng, C (Araliaceae) adventitious root cultures, full strength MS medium with either NAA or IBA was found to be suitable for biomass production (Yu, 2000). Therefore, Nandagopal and Kumari (2007) may have correctly inferred that the effect of auxin on adventitious root induction and elongation depended on the plant explants in culture.

After six weeks in culture well-established roots from leaf explants were separated. About 100 mg fresh root tissue was cultured in 80 ml full-strength MS liquid medium supplemented with 0.2 mg/l IBA and under continuous agitation (80 rpm) showed profuse root growth after 2-3 weeks of culture (Figure 2). The maximum fresh weight of roots (2,194.9 mg) was obtained at the 5th week of culture. However, on the 6th week the growth had declined as judged by fresh weight (2,184.6 mg). The decrease of biomass may be due to a reduction in water content related to a reduced proportion of lateral compared

Table 2. The effect of the plant samples on the percentage of viable ALL cells after 24 h of incubation.

ALL (L2)	Extract concentration											
	Cold water				Hot water				Ethanol Extract			
	1 µg	5 µg	10 µg	20 µg	1 µg	5 µg	10 µg	20 µg	1 µg	5 µg	10 µg	20 µg
	Dead %	Dead %	Dead %	Dead %	Dead %	Dead %	Dead %	Dead %	Dead %	Dead %	Dead %	Dead %
6 h	2 ± 0.12	36 ± 2.6	48 ± 4.3	67 ± 5.1	8 ± 0.3	41 ± 4.1	56 ± 2.3	81 ± 1.9	19 ± 1.2	45 ± 1.2	57 ± 3.1	76 ± 5.3
18 h	5 ± 0.36	30 ± 3.1	56 ± 2.8	73 ± 2.3	13 ± 2.1	39 ± 3.1	62 ± 1.3	76 ± 4.2	23 ± 3.6	36 ± 3.2	59 ± 2.8	74 ± 3.8
24 h	4 ± 0.29	20 ± 2.3	59 ± 3.1	77 ± 5.1	16 ± 1.8	47 ± 2.3	76 ± 1.6	84 ± 3.1	36 ± 2.5	52 ± 4.6	71± 4.9	86 ± 3.5
L.S.D. $_{(0.05)}$	1.02	2.1	3.56	1.83	0.87	1.68	3.21	4.24	1.25	2.1	4.23	3.2

Each value represents the mean ± S.D (Standard Division) and mean of three replicates (P ≤ 0.05).

Table 3. The effect of the plant samples on the percentage of viable AML cells after 24 h of incubation.

AML(M3)	Extract concentration											
	Cold water				Hot water				Ethanol Extract			
	1 µg	5 µg	10 µg	20 µg	1 µg	5 µg	10 µg	20 µg	1 µg	5 µg	10 µg	20 µg
	Dead %	Dead %	Dead %	Dead %	Dead %	Dead %	Dead %	Dead %	Dead %	Dead %	Dead %	Dead %
6 h	9 ± 1.2	32 ± 2.023	39 ± 3.25	58 ± 5.3	18 ± 2.2	29 ± 1.45	53 ± 4.1	62 ± 2.1	22 ± 2.2	36 ± 1.25	59 ± 2.12	64 ± 1.23
18 h	26 ± 2.5	35 ± 1.92	47 ± 4.23	66 ± 2.1	15 ± 1.4	36 ± 1.23	63 ± 2.6	71 ± 1.57	31 ± 2.1	39 ± 0.87	68 ± 3.2	77 ± 4.21
24 h	37 ± 1.9	49 ± 3.1	53 ± 1.64	72 ± 1.3	34 ± 3.1	55 ± 3.1	61 ± 3.1	75 ± 1.34	28 ± 3.1	51 ± 3.2	73 ± 3.12	82 ± 5.2
L.S.D. $_{(0.05)}$	1.09	3.55	2.13	1. 8	3.11	2.13	1.89	2.8	3.23	2.21	4.2	3.26

Each value represents the mean ± S.D (Standard Division) and mean of three replicates (P ≤ 0.05).

to primary roots (Nandagopal and Kumari, 2007).

Phenolic and flavonoid compounds are common in medicinal plants, spices, vegetables, fruits, grains, pulses and other seeds. These compounds are an important group of natural antioxidants with possible beneficial effects on human health (Meyer et al., 1997). They canparticipate in protection against the harmful action of reactive oxygen species, mainly oxygen free radicals.

Phytochemicals, especially the phenolics found in medicinal plants, fruits and vegetables, have been proposed as the major bioactive compounds providing the health benefits associated with diets rich in plant-foods (Halliwell and Gutteridge, 1999). In this context, redox and antioxidant systems are among the most promising targets for functional food science. For this reason, many functional foods aim to increase human intake of antioxidants to reduce the risk of chronic diseases linked to oxidative stress. Among the most common dietary sources of natural antioxidants are grapes and berries that are rich in phenolic compounds and particularly flavonoids (Robards et al., 1999; Moure et al., 2001). The results of this study suggest extracts of the herbs like Vernonia can substitute for grapes and berries.

In the earlier reports anti-leukemic plant extracts, the allamandin derivatives that are extracted with water and/or ethanol from Allamanda catharica, L (Apocynaceae) showed significant activity in vivo against the p-388 leukemia in the mouse (Kupchan et al., 1976). In addition, willow leaves showed highly active against ALL and AML cells (El-Shemy et al., 2003, 2007) probably related to salicylic acid derivatives. Gao et al. (2002) investigated the resveratrol-induced DNA fragmentation in 32Dp210 leukemic cells. Resveratrol (a phenolic) induced apoptosis in 32Dp210 cells as shown by the induction of

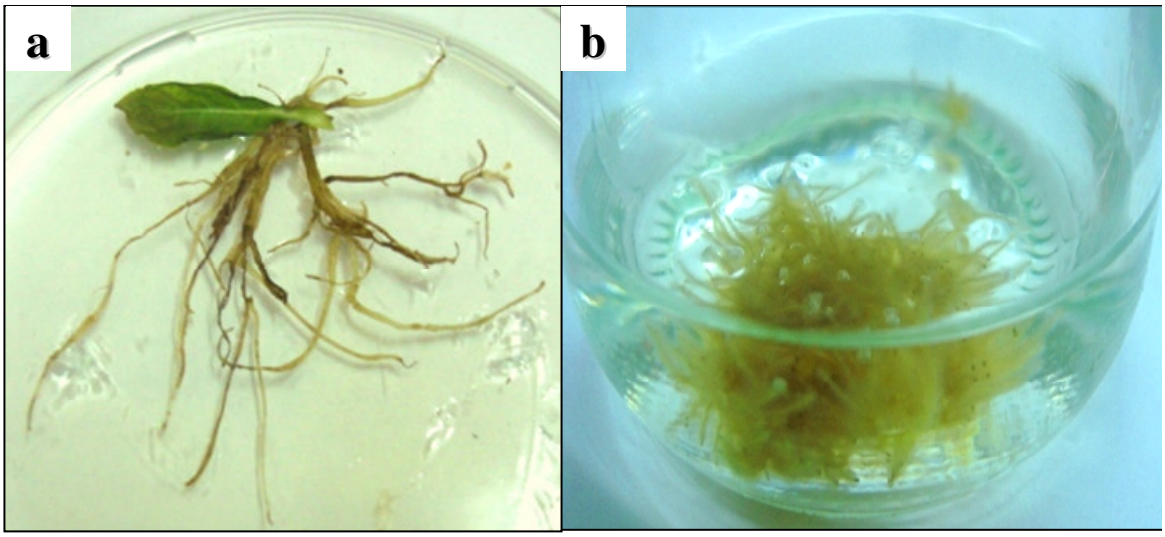

Figure 2. Establishment of root cultures in *Vernonia amygdalina*. Panel a. Root initiation from mature leaf explant cultured in half-strength MS medium supplemented with 2.0 mg/l IBA. Panel b. Root proliferation from subcultured roots in MS liquid medium supplemented with 2.0 mg/l IBA.

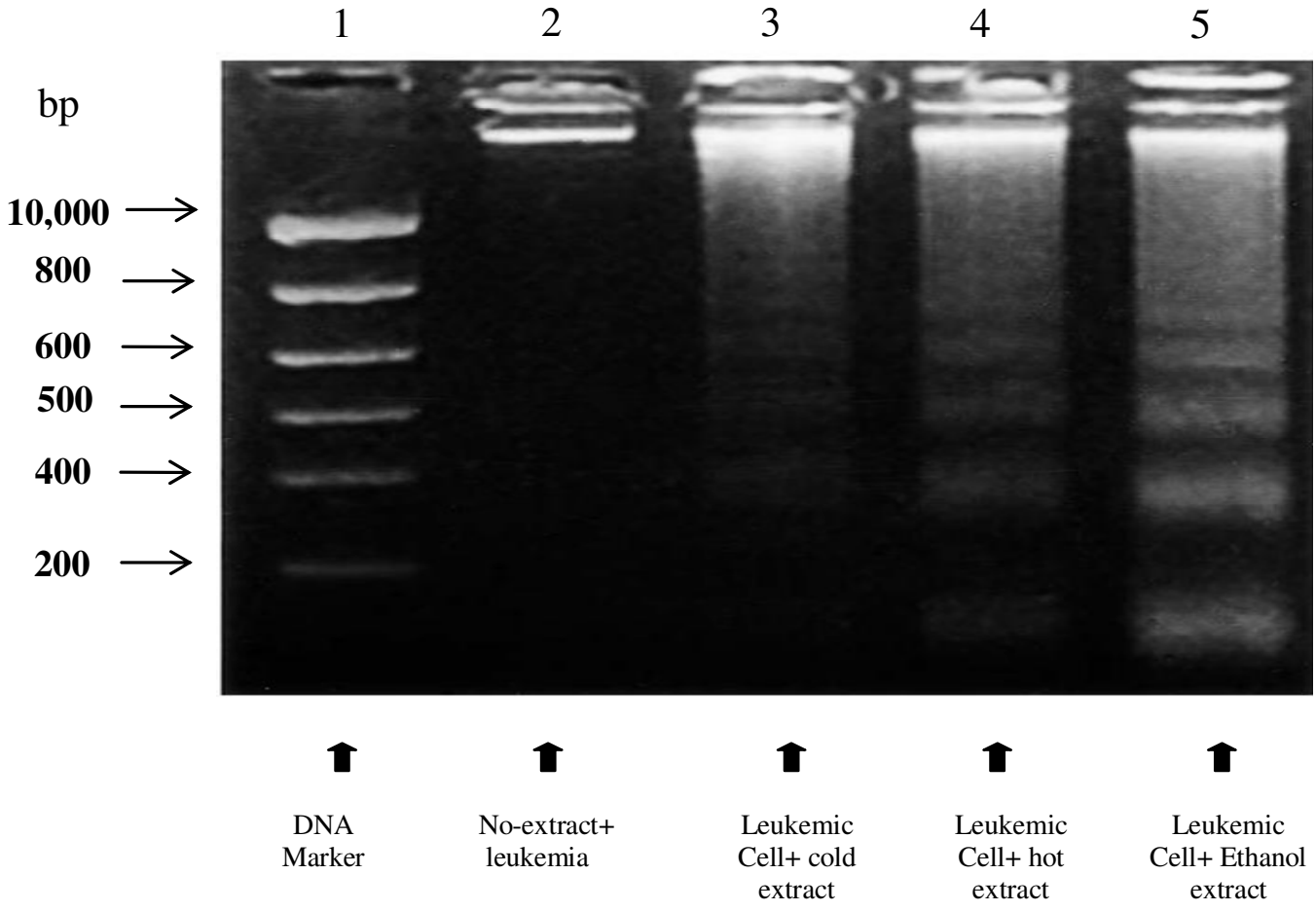

Figure 3. Agarose gel electrophoresis of DNA extracted from AML cells treated with plant extracts. Lane 1 shows a DNA ladder. Lane 2 shows AML cells treated with culture fluid but no extract. Lane 3 shows the cold water extract treatment effect. Lane 4 shows the hot water extract treatment effect. Lane 5 shows the ethanol extract treatment effect.

internucleosomal DNA fragmentation and the cleavage of procaspase-3 in resveratrol treated leukemic cells. Here, a major destructive effect on AML and ALL cells was obtained by the ethanol extract (Tables 2 and 3). That extract could be used as a natural antitumor medicine.

The active ingredient(s) may be phenolic compounds because most glycosides and many types of tannin will dissolve in ethanol solutions (Bravo, 1988). Whatever the active factor the extract appears to promote cell apoptosis since DNA damaged in leukemic cells incubated with ethanol extract (Figure 3).

In comparison to established treatments for leukemia, the extracts were equally effective in killing 80% of diseased cells within a few hours (Mishra et al., 2006). Therefore, this plant extract used alone or in combination with other extracts (El-Shemy et al., 2007) or other drugs (Mishra et al., 2006) has the potential to kill all leukemia cells whilst leaving healthy cells viable. The plant extracts described may provide low cost treatments for cancers and new treatments for drug resistant cancers.

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