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Full Length Research Paper

Dedifferentiation of leaf explants and antileukemia activity of an ethanolic extract of cell cultures of *Moringa oleifera*

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The present study was aimed at developing an efficient protocol for callus induction from the leaves of *Moringa oleifera* and to investigate its crude extract antileukemia activity on leukemia cells. Several secondary metabolites are present in *M. oleifera* as the plant serves as reservoirs for various bioactive compounds. Callus cultures of *M. oleifera* were induced from leaf explants incubated on MS medium supplemented with different concentrations of 2,4-dichloro-phenoxyacetic acid (2,4-D). The crude extracts of the callus were evaluated *in vitro* for their activity against leukemia cells and hepatocarcinoma. Among the different concentrations, 2,4-D at 0.1 mg/l induced highest frequencies of callus growth index (7.8) when compared with other concentrations. Ethanolic extracts killed about 36% of abnormal cells among primary cells harvested from 3 patients with acute myeloid leukemia (AML) and hepatocarcinoma cells HpG2. These results provide an *in vitro* evidence and support the traditional use of *M. oleifera* leaf as a potent source of anticancer. However, more researches are needed at phytochemical and clinical levels to confirm the traditional use of this plant as anticancer.

Key words: *Moinga olifera*, callus culture, antileukemia, hepatocarcinoma.

INTRODUCTION

Moringa (*Moringa* spp.) is a softwood perennial tree that belongs to the monogeneric family, Moringaceae (order Capparales). Among the 13 species of the genus *Moringa* (Ray et al., 2006), *Moringa oleifera* commonly known as drumstick tree or horseradish tree (Little and Wadsworth, 1964; Morton, 1991) is most commonly cultivated in South India, Ethiopia, Philippines, Sudan and other tropical countries (Fahey, 2005). It is a small fast growing evergreen or deciduous tree valued mainly for its edible fruits, leaves, flowers, roots and seed oil, and extensively used in traditional medicine throughout its native and introduced ranges (Booth and Wicken, 1988; Jahn et al., 1986; Morton, 1991; Nautiyal and Venhatarman, 1987).

Traditional medicine has a long history of serving peoples all over the world (Cheng, 2000). Medicinal plants are an important element of indigenous medical systems that has persisted in developing countries. The plant kingdom was estimated to produce over 500,000 natural products and about 40 to 80 thousand per plant species (Bhatt, 1995). Recently, the use of traditional medicine based on plants has received considerable interest (Han et al., 2002). There are national and indigenous rights over plant derived resources. Basic scientific investigations based on medicinal plants and indigenous medical systems have increased. It has been estimated that 1 to 10% of the large diversity of 250.000 to 500.000 plant

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Abbreviations: MTT, 3-(4,5-Dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide; **PBS,** phosphate buffered saline; **AML,** acute myeloid leukemia; **HCC,** hepatocellular carcinoma; **2,4-D,** 2,4-dichloro-phenoxyacetic acid.

species on the Earth have been studied chemically and pharmacologically for their medicinal properties (Farnsworth, 1991; Verpoorte, 2000). An examination of the phytochemicals of *Moringa* species affords the opportunity to examine a range of fairly unique compounds. In particular, this plant family is rich in compounds containing anticancer activity which include 4-(4'-O-acetyl--L-rhamnopyranosyloxy) benzyl isothiocy-anate (Abrams et al., 1993) and 4-(-L-rhamnopyranosyloxy) benzyl glucosinolate (Asres, 1995).

Different plants produce diverse products and their production is often related to a particular developmental stage, and is profoundly affected by seasonal variations (Srivastava et al., 2009). Cell cultures are attractive alternatives to whole plants for production of high value secondary metabolites due to consistency in quality and quantity of the desired product (Rao and Ravishankar, 2002). Moreover, it is well known that, *in vitro* culture is able to provide secondary metabolite, some times even in quantities that allow economically feasible production (Fujita et al., 1981; Fujita, 1988; Hara et al., 1987). Furthermore, tissue culture technique like callus culture could provide an alternative supply of compounds for use in medicine, stimulating the production or inducing the biosynthesis of novel compounds not found in the intact plant (Furmanowa and Glowniak, 1997; Zhao et al., 2001). In recognition of all these important properties of tissue culture, the present study is an attempt to develop an efficient protocol for callus induction from the leaf explant of *M. oleifera* and investigate its crude extract antilekumeia activity on leukemia cells harvested from 3 patients with acute myeloid leukemia (AML).

MATERIALS AND METHODS

Plant material

M. olifera seeds were collected from Botanical garden of Botany and Agricultural Biotechnology Department, Faculty of Agriculture, University of Khartoum, Sudan. Seeds were germinated in plastic bag containing mixture of soil and sand under green house conditions.

Explant surface sterilization and callus induction

Leaves explants (Figure 1a) obtained from 21 day-old seedlings were sterilized by sodium hypo-chloride (Clorex® v/v 10%) for 15 min, and then rinsed five times with sterile distilled water under aseptic conditions. The explants were then placed on MS (Murashige and Skoog, 1962) supplemented with different concentrations of 2,4-D (0.1, 0.5, 1.0, 2.0 and 3.0 mg/L) for callus induction. Cultures were incubated for six weeks at 25°C ± 2 under cool white fluorescent lamps with a 16 h photoperiod. Data were recorded after six weeks and callus growth index was recorded according to procedure described by Abdellatef and Khalafalla (2008).

Callus proliferation

For callus proliferation, small sized callus of 6 weeks-old were sub cultured on MS basal media that is hormone-free.

Samples preparation

The extraction used 1 g of freeze-dried, powdered callus from culture suspended in 1 ml of hot water, cold water, or 80% (v/v) ethanol. Extracts were stirred mechanically for 12 h at room temperature (25°C) except the hot water extract (80°C) that was made in 3 min. Solids were removed by centrifugation (4,000, 1 min) and the supernatant was collected. The resulting extracts were completely dried in a rotary evaporator at 40°C and the lyophilized extracts were stored at 4°C for further process.

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 viability of AML cells were calculated according to MTT assay (Selvakumaran et al., 2003). The MTT assay is a test of metabolic competence based upon assessment of mitochondrial performance relying on the conversion of yellow MTT to the purple formazan derivative by mitochondrial succinate dehydrogenase in viable cells. Mononuclear cells were separated from other blood cells by Ficoll hypaque density gradient (Pharmacia, Uppsala, Sweden), according to Harbeck et al. (1982). The cytotoxicity of each extract on AML cells was determined by the MTT assay (Selvakumaran et al., 2003). AML were diagnosed by peripheral blood and bone marrow examination, cytochemistry (and immunological markers in some 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 phosphate buffered saline (PBS). The cell counts were adjusted to 3×10^3 cell /well and plated in100 µl of medium/well in 96-well plates (Costar Corning. Rochester, NY). After overnight incubation, extracts were in various concentrations (10 and 20 µg/ml) with cytotoxicity to human normal myeloid cell line (reported in elsewhere); 3 wells were included in each concentration. After treatment with extracts for one day, 20 µl of 5 mg/ml MTT (pH 4.7) were added per well and cultivated for another 4 h, the supernatant fluid was removed, and then 100 µl dimethysulfoxide (DMSO) were added per well and shaken for 15 min. The absorbance at 570 nm was measured with a microplate reader (Bio-Rad, Richmond, CA), using wells without cells as blanks. All experiments were performed in triplicate.

Calculation

The effect of extracts on the proliferation of human AML cells was expressed as the percent cytoviability, using the following formula:

Cytoviability % = A57of treated cells / A57of control cells x 100

Viability of HpG2 cells

Hepatocellular carcinoma (HCC) cell line HpG2 was obtained from VACSERA, Egypt. The viability of HpG2 cells in culture was calculated by the MTT assay (Selvakumaran et al., 2003). As with the AML cells, the cell counts were adjusted to 3×10^3 cell /well and plated in 100 µl of medium/well in 96-well plates (Costar Corning. Rochester, NY). After overnight incubation, extracts were in various concentrations (10 and 20 µg/ml) with cytotoxicity to human normal myeloid cell line; 3 wells were included in each concentration. After

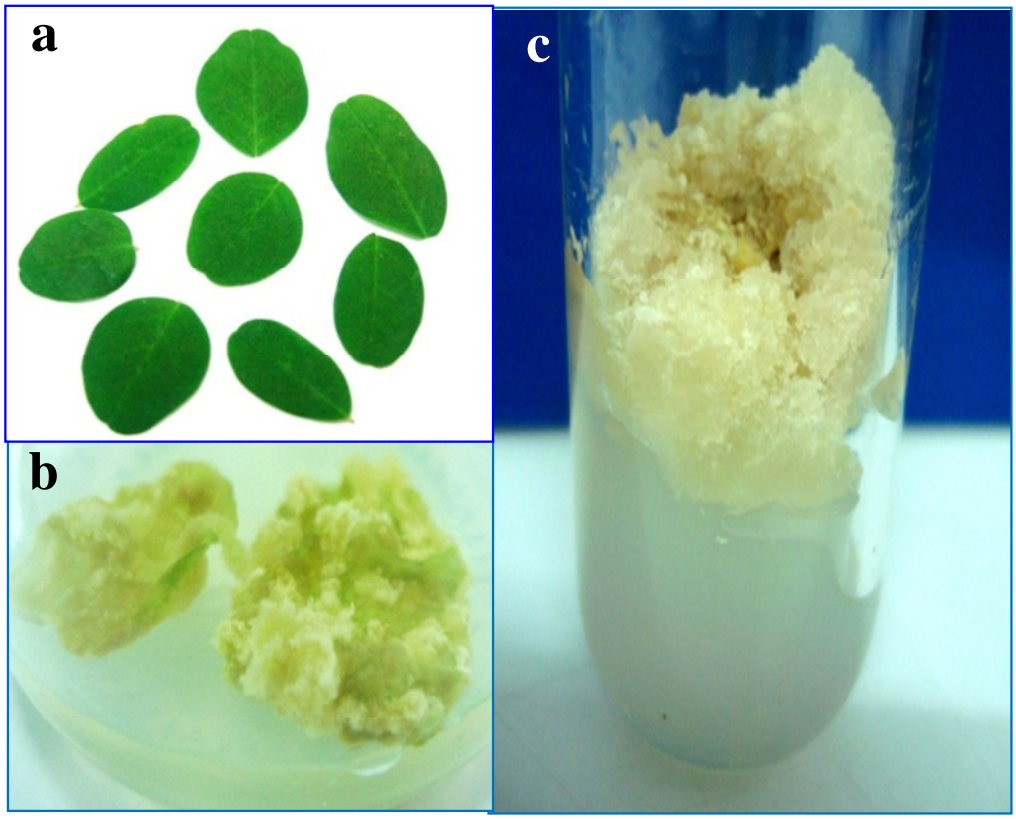

Figure 1. *M. olifera* callus induction from leaves explants. a, Leaves explants obtained from 21 day-old seedlings; b, yellowish green callus produced from leaves explants cultured on MS media supplemented with 0.1 mg/L 2,4-D after 6 weeks of culture; c, callus proliferation on MS free hormone media after 6 weeks of culture.

treatment with extracts for one day, 20 µl of 5 mg/ml MTT (pH 4.7) were added per well and cultivated for another 4 h, the supernatant fluid was removed, and then 100 µl DMSO were added per well and shaken for 15 min. The absorbance at 570 nm was measured with a microplate reader (Bio-Rad, Richmond, CA), using wells without cells as blanks. All experiments were performed in triplicate.

RESULTS AND DISCUSSION

Callus cultures were induced from *M. olifera* leaf explants incubated on MS medium supplemented with different concentrations of 2,4-dichloro-phenoxyacetic acid (2,4-D). The leaf explants induced callus in the presence of 2,4-D, but failed to produce callus in hormone free media. This showed that, the presence of 2,4-D was capable of inducing callus. Many researchers observed 2, 4- D as the best auxin for callus induction as common in monocot and even in dicot (Evans et al., 1984; Ho and Vasil, 1983; Jaiswal and Naryan, 1985; Chee, 1990; Mamun et al., 1996). The callusing responses, callusing degree and callus appearance are concentration dependant (Table 1). Among the different concentrations tested, 2,4-dichloro-phenoxyacetic acid (2,4-D) at 0.1mg/L induced the highest callusing rate (7.8 ± 0.1) and best callus appearance (Table 1) (Figure 1b). Several types of callus were distinguishable based on the physical appearance. Callus induced on MS medium containing 0.1 mg/L 2,4-D was initially healthy green yellowish and more granular when compared to other concentrations. *In vitro* derived callus were proliferated on MS-hormone free media (Figure 1c).

Recently, cell cultures have been used for the production of various groups of secondary metabolites (Alfermann and Petersen, 1995). Competence of undifferentiated callus cultures is critical for production of secondary metabolites (Wewetzer, 1998; Kuruvilla et al., 1999; Prakash et al., 2002). As plant cells are biosynthetically totipotent; that is, cells in culture retain complete genetic information, consequently, they are capable of producing metabolites found in the mother plant (Rao and Ravishankar, 2002). Heterogeneity in biochemical activity within a population of cells derived from the same plant species or even various explants from the same plant can be exploited to obtain highly productive cell lines (Evans et al., 1984). After 24 h incubation of the mononuclear AML cells with callus extract, ethanolic extract at 60 µg /ml score the highest cell death (36%) when compared to cold water extract and hot water extract at the same concentration which gave 9.6%

Table 1. Effect of 2,4-D on callus induction from leaves explants obtained from 21 days old seedlings of *M. olifera* after 6 weeks of culture.

2,4-D (mg/L)	Callusing response (%)	Callus growth index (Mean ±SE)	Callus color
0.1	100	7.8±0.1	Green yellowish
0.5	100	7.0±0.1	Green yellowish
1	100	6.0±0.1	Yellow
2	100	5.5±0.0	Yellow
3	84	5.3±0.1	Yellow

Table 2. The effect of *Moringa* callus extracts on AML cells after incubation for 24 h.

Extracts	Concentration (µg/ml)		
	20	40	60
	Dead (%)	Dead (%)	Dead (%)
Ethanolic (80%)	20.4	28.6	36.7
Hot water	2.9	5.3	3.7
Cold water	3.4	6.4	9.6

Table 3. The effect of *Moringa* callus extract on HpG2 cells after incubation for 24 h.

Extracts	Concentration (µg/ml)		
	20	40	60
	Dead (%)	Dead (%)	Dead (%)
Ethanolic (80%)	12.6	16.8	28.8
Hot water	10.9	26.3	11.6
Cold water	19.4	22.6	22.3

and 3.6% cell death, respectively (Table 2). In a similar way, the viability of HpG2 cells, after incubation with extracts was affected. Ethanol extracts at 60 µg /ml gave the best result (28.8%) (Table 3). From this observation, it is clear that the antitumor activity of the leaf callus was mostly due to compounds that were ethanol soluble. A previous report of plant derived antileukemia treatment showed that allamandin derivatives that were extracted with ethanol from *Allamanda catharica* (Apocynaceae) had significant activity *in vivo* against the p-388 leukemia in the mouse (Kupchan et al., 1976). In this study, the major destructive effect on AML cells was obtained by ethanol fractions. The phenolic compounds, mostly glycosides, dissolve in ethanol solutions (Bravo, 1988). Therefore, these groups of compounds may contain the major active components for the destruction of leukemia and carcinoma cells (El-Shemy et al., 2007; Khalafalla et al., 2009). A number of food components have been identified that inhibit the initiation and progression of cancer or otherwise influence the potential for disease outcome (Hu et al., 1997). For example, some epidemiological studies showed a close association between low incidence of coronary heart disease and breast cancer (Renaud and Lorgeril, 1992).

In conclusion, the active ingredients that were easily dissolved in ethanol from drumstick tree (*M. oleifera*) leaf callus could be used as natural antitumor medicines. They were active against leukemia and hepatocarcinoma cells *in vitro*. The metabolites within the extract and their role in killing of cancer cells will be identified. Also, the result substantiates the value of callus cultures as source of high value metabolites.

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