Evaluation of the anticancer properties of the methanol leaf extract of *Chromolaena odorata* on HT-29 cell line

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Abstract

Plant materials have been used for medicinal purposes since ancient time and scientific works are being carried out so as to discover/develop lead agents which can be used to meet the numerous health challenges of man. The plant used in this study is *Chromolaena odorata*. The effect of methanol leaf extract of *Chromolaena odorata* (MLECO) on Human Colorectal Adenocarcinoma Cell lines HT-29 (ATCC® HTB-38™) proliferation was investigated using the Cell Titer 96 MTT Proliferation Assay where the viable cells were seeded at a density of 5 × 10⁴ (100 μL/well). Varying log concentrations of extract (100-700 μg/mL) were added and incubated for 24, 48, and 72 h time points. Incubation of the extract in the presence of VEGF and ET-1 was also conducted at different times.

MTT assay showed that after 72 hours, the extract caused marked inhibitory effects on the cancer cell lines with lower concentration showing greater effect. When the plant extract was incubated alone with cancer cell lines at 200 and 800 μg/mL, the results showed that the latter concentrations was more potent at cell inhibition but when incubated with VEGF and ET-1, the 200 μg/mL +ET-1 was more potent at 24 hours.

The result showed that the methanol leaf extract of *Chromolaena odorata* alone caused marked inhibition of HT-29 cell lines after 72 hours but in the presence of the mitogens (VEGF and ET-1), the effect on the cell line was that of proliferation showing that the mitogens interfered with the plant extract's ability to cause inhibition of cell line.

Keywords: *Chromolaena odorata*, anticancer properties, HT-29 cell line, MTT assay, VEGF, ET-1

1. Introduction

Plant materials have been used for medicinal purposes since ancient times because cost, availability, accessibility and effectiveness are some of the reasons attributable for its widespread use in modern times. Researches on plant based scientific works are being carried out so as to discover/develop lead agents which can be used to meet the numerous health challenges of man (Ijioma et al., 2014) [26].

A lot of research efforts are directed over the years on the anticancer therapies; nevertheless, cancer mortality rates are still on the increase. For instance among the women folks, the highest number of cancer-related deaths are caused by cancers of the breast, lungs, stomach, colon and/or rectum, and cervix, while among men cancers of the lung, stomach, liver, colon and/or rectum, esophagus, and prostate result in the highest mortality (Thomasset et al., 2007, Jemal et al., 2011, Lewandoska et al., 2013) [54, 27].

Chemotherapy, despite its many side effects, is still the most popular way of treating cancer (Kordek et al., 2007) [31]. It is for this reason a lot of attention is being paid on the natural products especially from plant to ameliorate cancer treatment. Plants such as *Abrus precatorius*, *Azadirachta indica*, *Brassica oleracea*, *Cinnamomum zeylanicum*, *Curcuma longa*, *Piper longum*, *Plantago major*, *Ginkgo biloba*, *Podophyllum emodi*, *Saussurea lappa*, *Solanum nigrum*, *Caesalpinia bonducella* and *Terminalia catappa* are some well-known medicinal plants with anti-cancer property (Kathiresan et al., 2006) [28].

Chemoprevention, which consists in using synthetic, semi-synthetic or natural agents to inhibit or reverse the process of carcinogenesis, particularly in individuals with a high risk of developing cancer may be another alternative way of combating cancer. In fact epidemiological studies showed that incidence of some cancers is low among the Asians than their American and European counterparts may be due in part to the fact that their diet is...
markedly richer in health beneficial plant-derived polyphenols (Mohammad et al., 2006; Khan and Mukhtar, 2008) [38, 30]. Phenolic compounds are secondary metabolites of plant origin which carry one (phenol) or several (polyphenols) hydroxyl moieties in their aromatic ring (Stalikas, 2010) [52]. Phenolic compounds consist of approximately 8,000 naturally occurring metabolites which are divided into the flavonoids and the non-flavonoids. Phenolic compounds undertake antioxidant, pro-oxidant, anti-inflammatory activities and also exert great influence on the bioavailability of nitric oxide in humans (Li, 2011) [53]. Plants rich in phenols could therefore be explored for their anticancer properties. *Chromolaena odorata* is being evaluated for their anticancer properties in this study. *Chromolaena odorata* (L) King and Robinson regarded as an invasive weed, has been used in Indonesia, Thailand, Malaysia, Vietnam and parts of Africa including Nigeria as a hemostatic (Schoonjans et al., 1996; Murphy et al., 2000, Rangwala et al., 2004) [50, 40, 48] wound healing (Bohllmann et al., 1982a, 1982b, Hwang et al., 2002) [7, 8, 25] anti-inflammatory drug (Agrawal, 1989; Braca et al., 1999) [1, 9] and for intestinal diseases and burns (Thang et al., 2001) [53]. Studies about its antioxidant, antibacterial, and anti-gonorrea effects have been reported (Dat et al., 2009) [10]. Its phytochemical investigations revealed the presence of flavonoids, alkaloids, and terpenoids (Thang et al., 2001, Dat et al., 2009) [53, 13]. In this study, cell viability and other properties of this plant alone and in the presence of VEGF and ET-1 on HT-29 cell lines were examined.

2. Materials and Methods

2.1 Plant collection and extract preparation

Fresh leaves of *Chromolaena odorata* were collected from the campus of the University of Ibadan, Ibadan, Nigeria. The leaves were dried under shade for about 10 days after which they were ground to powder using an electric blender. 200 g of the powdered material was soaked in 1 litre of methanol and shaken vigorously. The sample was then filtered after 3 days using a Buckner funnel and Whatman No. 1 filtered paper. The extract was further concentrated using a water bath. The weight of the extract was 12.8 g.

2.2 Cell Culture: HT-29 colorectal cancer cell lines were supplied by Dr. Yakubu of the Vascular Biology Unit, Center for Cardiovascular Diseases, Texas Southern University. Cells were cultured in RPMI 1640 medium (GIBCO, Grand Island, NY, USA) containing 10% fetal bovine serum and antibiotics. Cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ in 95% air. HT-29 cells were treated with 100 nM of phorbol myristate acetate (PMA, Sigma-Aldrich Co., St. Louis, MO, USA) for 72 h to induce differentiation into macrophages. After differentiation, nonattached cells were removed by aspiration and adherent macrophages were washed with RPMI 1640 medium three times and then incubated in cell culture medium at 37°C.

2.3 Cell viability: The effect of methanol leaf extract of *Chromolaena odorata* (MLECO) on cell proliferation in HT29 cells was investigated using the using the Cell Titer 96 MTT Proliferation Assay. The viable cells were seeded at a density of 5 × 10^4 (100 μL/well) in 96-well plates and incubated in a humidified atmosphere of 5% CO₂ and 95% air at 37°C for 24h to form a cell monolayer. After 24h, the supernatant on the monolayer was aspirated and 100 μL of medium and varying log concentrations of extract (100-700 μg/mL) were incubated for 24, 48, and 72 h time points. After the specific times of exposure to the extract, 20 μL of 5 mg/mL MTT in PBS was added to each well and incubated for 3 h at 37°C in a 5% CO₂ atmosphere. Supernatants were removed and 150 μL of isopropanol was added and the plates were gently shaken for 15 min to solubilize the formazan crystals and absorbance was measured at 560 nm using Bio Tex ELX 800 plate reader. In another study, the effects of *Chromolaena odorata* alone and with mitogens (VEGF and ET-1) on colorectal cancer cell lines (HT29) viability after 24 and 48 hours were investigated.

3. Results

MTT assay showed that after 72 hours, the extract caused marked inhibitory effects on the cancer cell lines with lower concentration showing greater effect. When the plant extract was incubated alone with cancer cell lines at 200 and 800 μg/mL, the results showed that the latter concentrations was more potent at cell inhibition but when incubated with VEGF and ET-1, the 200 μg/mL +ET-1 was more potent at 24 hours. After 48 hours, 200 μg/mL alone was more potent than the 800 μg/mL but in the presence of the mitogens, the effects of the plant extract at both concentrations were significantly different from the control and in this case caused significant proliferation. There was no significant difference between all the groups (Figures 1-7).

4. Discussion

The result showed that the methanol leaf extract of *Chromolaena odorata* alone caused marked inhibition of HT-29 cells after 72 hours but in the presence of the mitogens (VEGF and ET-1), the effect on the cell line was that of proliferation showing that the mitogens interfered with the plant extract’s ability to cause inhibition of cell line. The inhibition of cancer cell line in this study by the methanol leaf extract of *Chromolaena odorata* may have lend credence to the fact that natural product especially from plant hold the key in cancer therapy. It has been stated that plants rich in phenols are of great medicinal value because phenols are potential antioxidants because there is relation between antioxidant activity and presence of phenols in common vegetables and fruits (Cai et al., 2004; Fu et al., 2011) [10, 22]. In fact, a positive linear correlation between antioxidant capacities and total phenolic contents may indicate that phenolic compounds in some tested 50 medicinal plants could be the main components contributing to the observed activities and could therefore be rich potential sources of natural antioxidants (Gan et al., 2010) [23]. Most phenolic antioxidants are flavonoids, such as catechins, of different structures and antioxidant activities (Pokorny, 2000) [47].

Phytochemical analysis of *C. odorata* showed that phenolics, alkaloids, terpenoids and cardiac glycosides were compounds detected in the extracts of this plant and these compounds have been documented to possess medicinal properties as well as health-promoting effects (Salah et al., 1995; Del-Rio et al., 1997; Okwu, 2004; Liu, 2004) [49, 14, 43, 36]. It may thus be safe to say that the methanol extract of *C. odorata* has anti-oxidant potential (Akinmoladun et al., 2007; Vijayaraghavan et al., 2013; Bhargava et al., 2013; Archana et al., 2015) [2, 55, 6, 3]. Although a lot of attention has been focused on anticancer therapies for many years with much progress being made, nevertheless, cancer mortality rates are still on the increase. Cancers of the breast, lungs, stomach, colon and/or rectum, and cervix, are the highest number of cancer-related deaths among women while cancers of the lung, stomach, liver, colon
and/or rectum, esophagus, and prostate resulted in the highest mortality in men (Thomasset et al., 2007; Jemal et al., 2011) [54, 27]. It is thought that increase in industrialization, environmental pollution, together with life style (smoking, excessive consumption of highly processed food, long-term stress) are all looked upon as some of the causes of this phenomenon. Many chemical compounds present in air, water, food, synthetic materials, and other products act as carcinogens (Huff and LaDou 2007; Loeb and Harris 2008; Parzefall, 2008) [24, 37, 44].

In this study, the methanol leaf extract of Chromolaena odorata caused dose-dependent suppression of cell viability especially at 72 hours. The interesting thing is that the lowest concentration (200 µg/ml) caused the highest suppression of cell viability with the highest concentration causing the least. It thus showed that at higher concentration cell proliferation was encouraged and vice-versa. In the presence of VEGF and ET-1, the cell antiproliferative property of this extract was reversed.

VEGF is the primary stimulus for angiogenesis in tumours. Angiogenesis is the process whereby new blood vessels sprout in response to local stimuli is essential for the development, progression, and metastasis of malignant tumors (Folkman, 1995; Ferrara, 2002) [20, 17]. In the absence of angiogenesis, tumors cannot grow beyond 1–2 mm³ in size (Bergers and Benjamin, 2003) [5]. Vascular endothelial growth factor (VEGF) as the primary stimulus of angiogenesis in tumors carry out this functions through binding to VEGF receptor-2 (VEGFR2; also known as flk/kdr) and VEGFR1 (also known as flt) expressed on endothelial cells (Ferrara et al., 2003) [10]. The stimuli primarily consist of the release of angiogenic factors, activation of metalloproteases to break down extracellular matrix, and subsequent remodeling. The switch to the angiogenic phenotype is crucial in both tumor progression and metastasis (Fidler and Ellis, 1994) [19]. In fact the key factor involved in nearly all human tumors is vascular endothelial growth factor (Senger et al., 1993; Dvorak et al., 1995) [91, 15]. As a matter of fact, overexpression of VEGFR in the endothelial cells of tumor vasculature further attests to the significance of VEGF in tumor angiogenesis (Leung et al., 1997; Chan et al., 1998) [33, 11]. VEGF also acts as a survival or an anti-apoptotic factor and has been shown to induce Bel-2 in endothelial cells as well as in breast cancer cells (Pidgeon et al. 2001) [48]. The VEGF family of ligands includes VEGF-A, -B, -C, -D, -E, and placenta growth factor (PIGF). These ligands bind to three tyrosine kinase receptors: VEGFR-1 (Flt-1), VEGFR-2 (KDR, or the murine homolog Flk-1), and VEGFR-3(Flt-4), all of which have been well characterized on endothelial cells (Fan et al., 2005) [16].

VEGF-C has been implicated in the response to ROS in prostate cancer cell lines (Muders et al., 2009) [39]. Wang et al. (2014) demonstrated that VEGF-C does protect breast cancer cells from ROS-induced cell death because an antioxidant factor—SOD3—served as a downstream effector of VEGF-C. In this case SOD3 is at least in part responsible for the ability of VEGF-C to protect against ROS-induced cell death and to mediate breast tumor progression. It thus means that any agent with anti-oxidant property may in a way be promoting the activity of VEGF in tumor angiogenesis. Could this have been responsible for the results obtained in this study in which antiproliferative effect of this extract was abolished in the presence of VEGF especially that this plant is said to have anti-oxidant property?

Endothelin-1 (ET-1), on the other hand is an endothelial cell–derived vasoconstrictor peptide, an important member of the endothelin family (Yanagisawa et al., 1988a, Yanagisawa et al, 1988b) [57, 58] with myriad developmental, physiological, and pathological functions (Kedzierski and Yanagisawa, 2001) [29]. ET-1 is a potent vasoconstrictor involved in the development of cardiovascular diseases and is an important regulator of heart development (Chen et al., 2010) [12]. The endothelin axis as it is so-called consists of three similar small peptides, ET-1, ET-2, and ET-3, two G protein–coupled receptors, ETAR and ETBR, and two membrane bound proteases, the ET-converting enzymes, ECE-1 and ECE-2 (Kedzierski and Yanagisawa, 2001) [29], that activate the secreted pro-forms of the peptide. ET-1 production is stimulated by a variety of cytokines and growth factors, hypoxia, and shear stress, while ETAR activation triggers signaling networks involved in cell proliferation, new vessel formation, inflammation, and metastatic spread (Kedzierski and Yanagisawa, 2001; Nelson et al., 2003; Bagnato et al., 2005) [29, 42, 4]. ET-1 is secreted by human carcinoma cell lines and detected in malignant tissue (Kuhlhaar et al., 1990; Nakagawa et al., 1990) [32, 45].

A study has shown that the fresh leaves and extract of Chromolaena odorata are used as a traditional herbal treatment of burns, soft tissue wounds and skin infections in developing countries (Phan et al., 2000) [45], where it has been established that the extract had an effect on the growth and proliferation of keratinocytes and fibroblasts (Foster et al., 1990; Nakagawa et al., 1990) [45]. As a matter of fact, overexpression of VEGF in the endothelial cells of tumor vasculature further attests to the significance of VEGF in tumor angiogenesis (Leung et al., 1997; Chan et al., 1998) [33, 11]. VEGF also acts as a survival or an anti-apoptotic factor and has been shown to induce Bel-2 in endothelial cells as well as in breast cancer cells (Pidgeon et al. 2001) [48]. The VEGF family of ligands includes VEGF-A, -B, -C, -D, -E, and placenta growth factor (PIGF). These ligands bind to three tyrosine kinase receptors: VEGFR-1 (Flt-1), VEGFR-2 (KDR, or the murine homolog Flk-1), and VEGFR-3(Flt-4), all of which have been well characterized on endothelial cells (Fan et al., 2005) [16].

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Fig 3: MTT assay on HT29 cell line after 72 hours

Fig 4: Effects of *Chromolaena odorata* alone and with mitogens (VEGF and ET-1) alone on colorectal cancer cell line (HT-29) viability after 24 hours. Superscripts “a” indicates significant difference compared with control; (b) indicates significant difference compared with VEGF and (c) compared with ET-1 respectively. Abbreviations: VEGF (vascular endothelial growth factor), ET-1 (endothelin-1).

Fig 5: Effects of *Chromolaena odorata* on mitogens (VEGF and ET-1) induced-colorectal cancer cell lines (HT-29) proliferation after 24 hours. Superscripts “a” indicates significant difference compared with control; (b) indicates significant difference compared with VEGF and (c) compared with ET-1 respectively.

Fig 6: Effects of *Chromolaena odorata* alone and with mitogens (VEGF and ET-1) alone on colorectal cancer cell lines (HT-29) viability after 48 hours. Superscripts “a” indicates significant difference compared with control; (b) indicates significant difference compared with VEGF and (c) compared with ET-1 respectively.

Fig 7: Effects of *Chromolaena odorata* on mitogens (VEGF and ET-1) induced-colorectal cancer cell lines (HT-29) on proliferation after 48 hours. Superscripts “a” indicates significant difference compared with control; (b) indicates significant difference compared with VEGF and (c) compared with ET-1 respectively.

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