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In Vitro Production of Callus Cultures of *Alpinea zerumbet* and Their Cytotoxicity on Cancer Cells.

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Alpinia zerumbet is a rhizomatous herb rich in essential oils and bioactive constituents. Rapid direct regeneration was obtained from the rhizome explants (15.66±0.57 shoots) on MS media supplemented with the combination of 6.65 µM of BAP and 2.3µM of kinetin. The callus cultures of *A. zerumbet* were initiated from in vitro primordial leaves of 8 weeks old initiated from rhizome explants on MS nutrients media supplemented with 2, 4-dichlorophenoxyacetic acid (2,4-D), 6-benzyladenine (BA), kinetin (Kn), in various concentrations and combinations. The crude methanolic extract was prepared by standard protocols. The anti-proliferative effect of the methanolic extract was evaluated in vitro using the MTT analysis. The strength of each extract concentration was calculated as a percentage of the reduction in viable HeLa cells compared to the control value. The extract showed dose-dependent antitumor activity. The MTT assay showed an anti-proliferative activity with (IC50) at 156 µg / ml, 44ug/ml and 38ug/ml and 13ug/ml of crude extracts which indicate that accumulate of bioactive compounds in callus obtained from rhizomes.

Keywords: *Alpinia zerumbet*, 2, 4-dichlorophenoxyacetic acid (2,4-D), The MTT assay.

INTRODUCTION

The high healing value of Alpinia and its use in food production make it vulnerable to wild species that place their survival in a normal habitat. Monocotyledons do not allow a wide range of explants (rhizomes and peaks) to spread, and the conventional propagation technique for rate separation is not fast enough to meet the requirements of commercial activity. Lightning explants are often contaminated with pathogens, such as the rhizome (Chan, et al., 2004) and leaf rot, in which case storage of germplasm storage becomes difficult for Zingiberaceae (Nayak, 2002). Biotechnological approaches to crop improvement require efficient regeneration of crop tissue crops. In vitro techniques are considered to be one of the best options that a large number of plant materials can give to planting companies and further research (Hamirah, et al., 2010)

The ability to modify the chemical profile of metabolism by manipulating the chemical or physical environment for the preparation of a valuable compound can be achieved by tissue culture techniques. (Kavyashree, 2009). The simplest way to induce somaclonal variations is by regenerating the shoots through the callus phase, which paved the way for improving the species (Das and Rout (2002).

Cancer is a severe community health problem, which represents the subsequent leading cause of

death (Siegel et al., 2016). It has been reported that natural products act directly or indirectly through multiple cellular signal pathways. Therefore, the combination of extracts and / or traditional chemotherapeutic drug isolates can provide an effective alternative for the treatment of cancer or to overcome himiorezisnostta (Mokhtari, 2017)**.** Among natural products, phenolic compounds together with flavonoids have established a range of pharmacological actions (Kristo et al, 2016). As anti-inflammatory, chemoprevention and chemotherapeutic agents for cancer (García-Lafuente et al., 2009; George et al., 2017).

The aim of the present study was to establish reproducible and promising protocols for callus production of primordial foliage explants and plantlets regenerated from Alpinia rhizome. Moreover, assessing the cytotoxic and apoptotic effect of mthanol crude extracts of *Alpinia zerumbet* on cervical cancer HeLa cell line.

MATERIALS AND METHODS

Plant material, sterilization and growth conditions

Mature rhizomes of *A. zerumbet* were collected at the Department of Medical and Aromatic Plants, Dokki, Cairo, Egypt. The identification of the plant was achieved by the morphological comparison with authentic herbarium samples held in the herb garden of the Orman Botanical Garden, Giza, Egypt.

Then the explants were carefully cleaned with 70% alcohol to remove loose contaminants and reduce the size by removing the outer layer. Then the explants were taken in 5.25 % of commercial sodium hypochlorite and washed further by vigorous shaking for about 20 minutes. This was followed by washing 5 times with sterile distilled water.

The standard medium for propagation of the stock and for all experiments was Murashige and Skoog (1962), supplemented with the combination of 6.65 µM of BAP, 2.3µM of kinetin as described by Rakkimuthu, et al., 2011, 3 % sucrose and 0.2% (w/v) Phytagel. In one experiment (Fig. 1A), the BAP concentration was 67 lowered to 3 µM BAP. The pH was adjusted to 5.8 before autoclaving (121°C for 20 min). The cultures were reserved at 19°C and a 16-h photoperiod (30 µmol m-2 s-1, TL 33 Philips). Plants were subcultured every 4 weeks by transfer rhizomes from which the bigger part of the aerial shoots had been cut off (leaving *ca.* 1 cm aerial stem). Lateral rhizomes were divided from the main rhizome when sufficiently big (> *ca.* 1 cm).

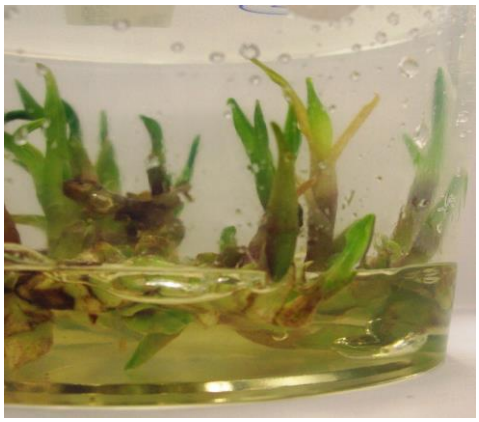

Figure 1: Regenerated *Alpinea zerumbet* on MS medium supplemented with the combination of 6.65 µM of BAP, 2.3µM of kinetin.

Influence of plant growth regulators on initiation of callus

Callus Induction

Callus cultures were initiated from in vitro primordial leaves of 8 weeks old plant cultures. The basal primordial leaves were cut into small segments (0.5-1.0 cm), almost even in size, and placed in glass beads (50 ml) containing MS solid medium, 3% sucrose and 0.2% phytagel added different concentrations of containing 2,4-D. and BAP as follows:

Preparation of methanol extract

One hundred g of fresh leaves, root and different callus treatments of *A. zerumbet* were crushed thoroughly, using mortar and pestle. The shattered plant parts were completely depleted by adding small amounts of methanol, followed by filtration to a final volume of 1 liter. The extract was then filtered and concentrated to dryness under controlled temperature (40 ° C - 50 ° C) in a rotary evaporator. To prepare the stock solution (100 mg / ml), the 100 mg extract was dissolved in 1 ml dimethylsulfoxide (DMSO).

In-vitro evaluation of anticancer activity by MTT assay Cell culture

The cell line of human cervical carcinoma (HeLa) was cultured in an essential medium from Eagles (himedia Laboratories Pvt. Ltd.) containing 10% fetal calf serum (himedia Laboratories Pvt. Ltd.). All cells were maintained at 37°C CO2, 95%

air. Cells were used in experiments during the linear growth phase.

Preparation of plant extracts

To 4.5 ml DMSO, 0.5 ml of stock extract of plant part (100 mg / ml) was added to a concentration of 10 mg / ml. Then, nine serial dilutions were prepared (extract 500 µl each in DMSO to the concentration of the extract of 0.0195-10 mg/ ml), as shown in Table 1. The diluted extracts were transferred to a 96 wells culture plate. To each well 500 µl of HeLa cell culture at a concentration of 105 cells / ml was added. Eight wells receive only cell suspension without extract and serve as a control. The plate was incubated for 72 hours in a humidified CO2 incubator at 37°C. The plate was examined under a microscope to determine the monolayer of the confluent cell, turbidity and toxicity

Table 1. Different hormone treatments for callus establishment

Medium No.	Treatment MS + GHRs
CT0	**Free hormone Ms medium**
CT1	Ms +9.1 µM 2, 4-D
CT2	Ms + 13.6 µM 2, 4D
CT3	Ms + 18.2 µM 2, 4D
CT4	Ms + 9.1µM 2, 4D+0.5 µM BAP
CT5	Ms + 13.6µM 2, 4D+0.5 µM BAP
CT6	Ms +18.2 µM 2, 4D+0.5 µM BAP
CT7	Ms +18.2 µM 2, 4D+0.5 µM BAP+ 9.3 µM Kin

Cytotoxicity Assay

MTT assay

The MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric assay was used to evaluate the anti-proliferative activity of methanol crude extract. MTT is indicative of metabolism and thus is a viable assay; i. e. can develop relationship between absorbance and concentration. This assay is based on metabolic reduction of soluble MTT by mitochondrial enzyme activity of viable tumor cells in an insoluble (dark purple) color formazan product, which can be measured spectrophotometrically after dissolution in dimethyl sulfoxide (DMSO). Cellular toxicity of the crude methanol extract of the *A. zerumbet* leaves, root and calli cultures on cultured cells was measured using this method. (Van Meerloo, Kaspers, and Cloos, 2011).

Statistical analysis

All experiments were conducted in triplicate and the data obtained were represented as mean ± standard error that was interpreted using Microsoft Office Excel 2007®.

RESULTS AND DISCUSSION

Callus Initiation

Treatments from table (1) and data in Figures (2–4) revealed that, MS medium fortified with 2,4-D in concentration of (18.2 µM) alone or in combination with 0.5 µM BAP and 9.3 µM Kin gave the greatest value of fresh weight of calluses: 3.35 and 3.87 g/explant, respectively, without significant difference between them but significantly different from the other treatments at (P<0.001). Conversely, the low concentration of 2,4-D (9.1µM) alone gave the lowest value of fresh mass of callus without significant difference between them. The obtained results were in accordance with those reported by Prakash et al., 2004 cleared that, MS medium supplemented with 2,4-D favored for callus initiation from leaf sheath explants of *Curcuma amada* and the maximum amount of callus was obtained with MS medium supplemented with 9.0 µM 2,4-D. While, Al-Jibouri and Al-Shamarri, 2008 reported that, plant parts of sugarcane cultured on the MS medium consist of 4 mg/L of 2,4-D was superior in fresh and dry weights of callus. Their results indicated that the fresh and dry weights of callus was decreased as concentration of 2,4-D increased more than 4 mg/L in the medium. In accordance of this result reached by Memon et al., 2010 those who had given it, when the amount of 2,4-D was increased (then 3 mg / L) in the culture culture medium, the rate of callus formation was reduced. Aazami and Hassan pouraghdam, 2009 reported that 1 mg / L 2, 4-D and 2 mg / L BAP gave the highest dry weight of calli in *Raphanus sativus* cultures. The callus obtained from *A. zerumbet* leaflet segments was very crispy with a creamy white crystalline appearance (Figures 2 and 3). The effectiveness of 2,4-D and BAP / Kn for callus induction of the internode and leafy plants of *Ceropegia candelabrum* L. was reported by Beena and Martin, 2003.

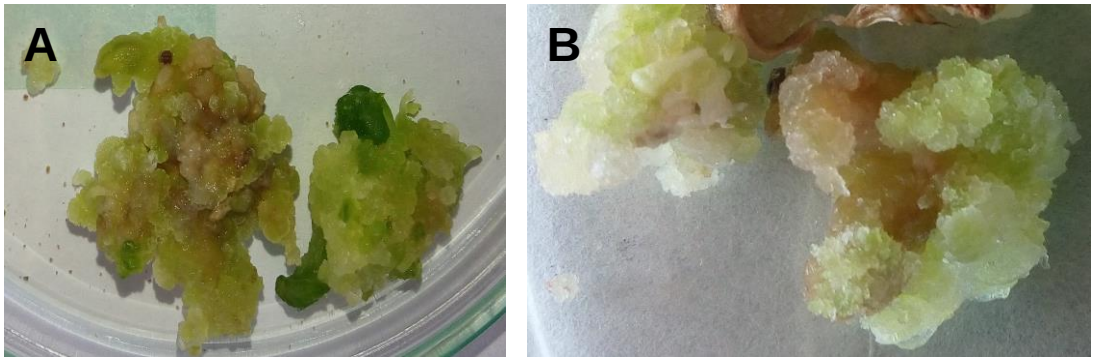

Figure 2: Callus induction from leaflet segments of in vitro Alpinea plantlets cultured on MS medium supplemented with A: 2,4-D (18.2 µM) + BAP (0.5 µM), B: 2,4-D (18.2 µM) after 8 weeks of cultivation and incubation under standard condition at 19 ± 1°C.

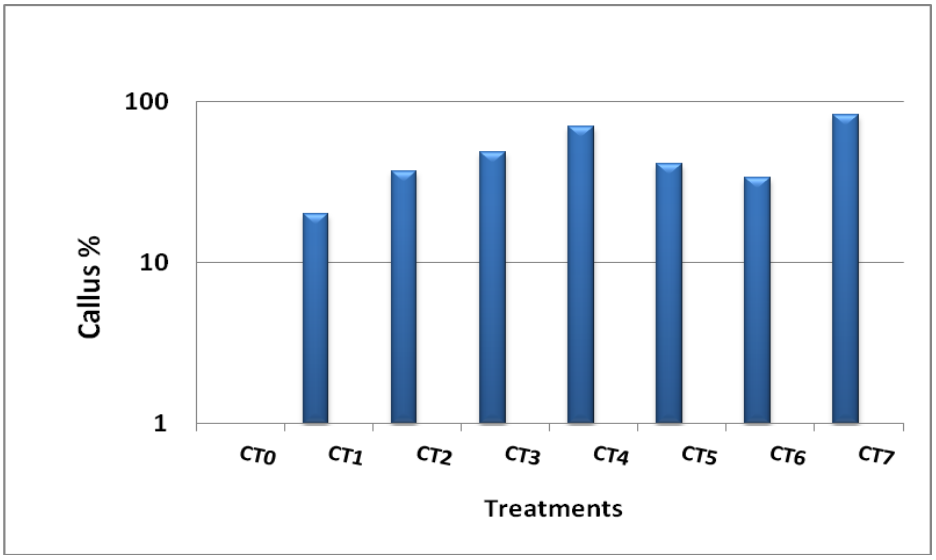

Figure 3: Effect of MS-medium supplemented with 2,4-D and BAP at different concentrations on calli growth parameter induced from in vitro leaves of *Alpinea zerumbet* after 8 weeks of cultivation under standard condition at 19 ± 1°C.

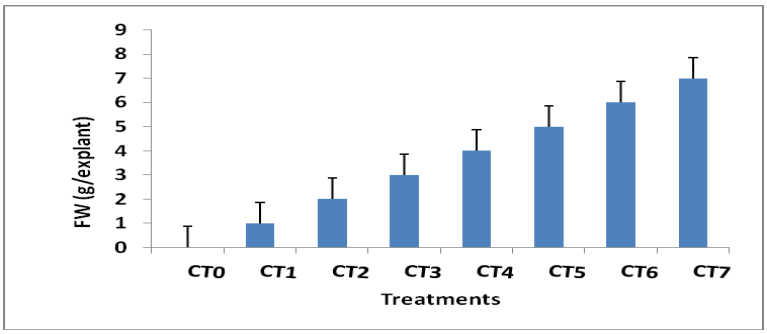

Figure 4: Influence of different hormone treatments added to MS medium on calli fresh weights obtained from in vitro leaves of *Alpinea zerumbet* rhizome after 8 weeks of cultivation. Data± standard error

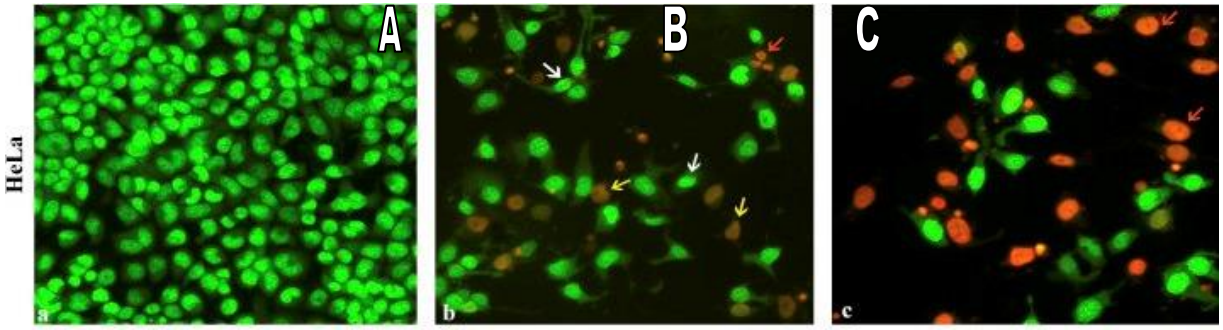

Figure 5: **AO/EB Staining. For HeLa cells A. Shows the cotrol HeLa cells (B) After treatment with CT4 (C) Treatment with CT7. Treated with the extract (48.25µg/ml) for 24hr**

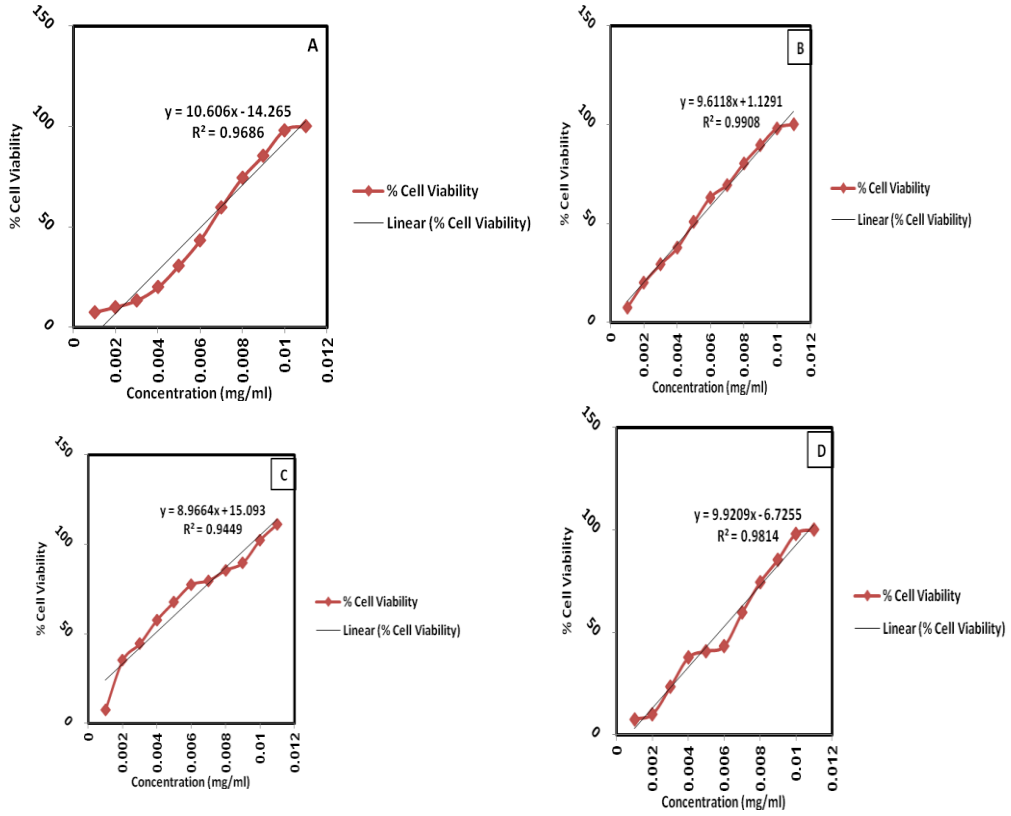

Figure (6): MTT analysis shows methanol-Alpinea extract inhibited HeLa cells in a dose-dependent manner with cytotoxicity of IC50 (48.25µg/ml). (A) Rhizome extract. (B) Leaf extract. (C) Callus extract on Ms medium + 9.1µM 2, 4D+0.5 µM BAP (CT4). (D) Callus extract on Ms medium + 9.1µM 2, 4D+0.5 µM BAP+ 9.3 µM Kin (CT7). Treated with the extract (48.25µg/ml) for 24hr

Cytotoxicity and Apoptosis study

Induction of apoptosis is the preferred approach in the development of anti-cancer drugs, as this is the most effective way to treat cancer. In this study, the objective was to investigate the cytotoxic effects and if the apoptotic mechanism

on HeLa cells is induced by *A. zerumbet* extract. The cytotoxicity of *A. zerumbet* extract on HeLa was evaluated by MTT assay based on percentage of cell viability. Methanol rhizome extract was found to have cytotoxic effect against HeLa, showing cell proliferation inhibition in a concentration-dependent manner. The IC50 for *A. zerumbet* crude rhizome extract was found to be 156µg/mL (Figure 6).

The results obtained from the present study showed that *A. zerumbet* has an anticancer activity. Apparently, true morphologies of apoptosis were observed in treated HeLa cells in double acridine oranges and ethidium bromide staining (Figure 5). Control HeLa cells that were elongate and spider-shaped (Fig. 5A) were deformed following *Alpinia zerumbet* extract treatment (Fig. 5B&C). Cellular accumulation with irregular shape, cytoplasmic condensation, nuclear pythosis and tremor followed by the appearance of apoptotic bodies resulted in a reduction in the number of viable cells after treatment with the extract for 24 hours. Nutritional depletion in growth media or contact inhibition that results in natural cell death can be the cause of the appearance of any apoptotic cells in the HeLa control.

In order to evaluate the cytotoxic effect of methanolic extracts of *Alpinia zerumbet*, a MTT assay with HeLa (human cervical cancer) cell line was performed. The extract was screened for its cytotoxicity at different crude extracts (rhizome, leaf, CT4 and CT7) to determine the IC50 (50% growth inhibition) value

A chart was plotted using the % cell viability in Y-axis and concentration of the plant extract in X-axis. Cell control was included in each assay to compare the full cell viability in cytotoxicity and antitumor activity assessments. The results are graphically represented in Figure 6.

Cytotoxicity effect of *Alpinia* leaves extract was evaluated by MTT assay. The results of the present study demonstrated inhibitory and cytotoxic manner Figure (6). The achieved IC50s were in mg/ml for *A. zerumbet* crude leaf, root, CT4, CT7, respectively. According to the results, crude CT7 and root extract showed high cytotoxic effect on proliferation of HeLa cells. So that, they are selected for further analysis. When HeLa cells were treated with the methanol extract of the roots of *A. zerumbet*, there was a concentration dependent cytotoxic effect. As the concentration increased from 19.5–10,000 µg/ml, percentage of inhibition increases from 11.9% - 72.3 %. The IC50 value was found to be 156µg/ml from the graph.

Traditionally, many medicinal plants, which have the ability to prevent or even expel the progress of cancer, are in use. Plants have certain chemicals, which have the ability to modify the cell's physiological function and therefore act as anti-cancer agents to stop the spread of cancer cells. The mechanism of action of the drugs is unknown, but we integrate our proven knowledge of plant properties and modern technological tools. Effective anti-cancer drugs can be derived from plant sources and their mechanism can be clarified.

CONCLUSION

Current need is to develop medications that can target cancer cells through their inherent difference with normal cells. The development of such drugs with differential effect will be very valuable in cancer chemotherapy without the observed side effects. The method includes the use of cancer cell lines to test the effect of plant extracts *in vitro.*

The potential use of *A. zerumbet* as therapeutic agent holds great promise as the isolation of one or more cytotoxic chemicals from crude extract and the judicious use of such chemicals can control the progression of cancer and also can prevent the formation of tumors in individuals who are highly susceptible to developing a tumors.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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