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Journal of Natural Sciences Research
ISSN 2224-3186 (Paper) ISSN 2225-0921 (Online)
Vol.4, No.17, 2014
www.iiste.org

Study the Anticancer Effect of *Lepidium sativum* Leaves Extract on Squamous Cell Carcinoma (CAL-27) Cell Lines

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Abstract
Leaf aqueous extracts of *Lepidium sativum* was investigated for anticancer activity on human tongue squamous carcinoma (CAL-27). The results showed that the plant extract inhibit the growth of CAL-27 cells in a dose-dependent manner (70, 100, and 150 µg/ml respectively). The toxic effect of *L. sativum* extract cause significantly ($p < 0.05$ and $p < 0.01$) damage to DNA and increase up the percentage of apoptotic nuclei to reach (30% and 60%) at concentrations of (100 and 150 µg/ml, respectively). Our results also showed that the *L. sativum* generate reactive oxygen species (ROS) in the mitochondria of CAL-27 cells compared to untreated control. The aqueous extract of the leaves of *L. sativum* holds great promise for the development of effective drugs for oral cancer treatment strategies.
Keywords: *Lepidium sativum*, Anticancer activities, Medicinal plants, Cell lines

1. Introduction

Cancer is a major public health distributed in both developed and developing countries that can lead to death. Searching for natural products with anticancer activity is our goal. Cancer cells usually invade and destroy normal cells. These cells are born due to imbalance in the body and by correcting this imbalance, the cancer may be treated. Billions of dollars have been spent on cancer research and yet the world do not understand exactly what cancer is (Madhuri & Govind 2009). Every year, millions of people are diagnosed with cancer, leading to death. The percentage of deaths arising from cancer constitute 2–3% of the annual deaths recorded worldwide. Thus cancer kills about 3500 million people annually all over the world. Several chemo preventive agents are used to treat cancer, but they cause toxicity with associated side effects that restricts their usage (Om *et al*. 2013) Natural therapies including plants extract are being beneficial to combat cancer. Plants, since ancient time, are using for health benefits by all cultures as well as source of medicines. It has been estimated that about 80-85% of global population rely on traditional medicines for their primarily health care needs and it is assumed that a major part of traditional therapy involves the use of plant extracts or their active principles (Elujoba *et al*. 2005). Although a lot of recent investigations have been carried out for advancements in the treatment and control of cancer progression, significant work for improvement remain.

Lepidium sativum or garden cress (family Brassicaceae) has been used widely in different parts of the world for its wide therapeutic application (Sharma & Agarwal 2011). A number of recent studies pointed out the traditional uses of *L. sativum* seeds extract in controlling many clinical problems (Gill & MacLeod 1980), and Ahmed *et al*. (2013) revealed that *L. sativum* seeds with high nutritional value can be exploited as a functional food ingredient. *Lepidium sativum* is usually cultivated for its leaves, which are used in salad, sandwiches (Eddouks *et al*. 2005).

The leaves and seedpods have a peppery taste, and has been reported to have enormous biological activities (Sarikami & Yanmaz 2011). It is documented to possess, tocopherol, phenolic compounds, nitrogen compounds, terpenoids, and some other endogenous metabolites, which are rich in antioxidant activity (Kumar *et al*. 2014). Vitamin E is an strong antioxidant, it was first isolated from green leafy vegetables (Aggarwal *et al*. 2010). Several studies demonstrated that vitamin E could reduce risks of infertility, neurological disorders, inflammation, cardiovascular diseases, diabetes, and certain types of cancers in humans (Kulie *et al*. 2009). Therefore, the objective of the present study was conducted to evaluate the anticancer effects of *L. sativum* leaves extract on oral cancer cell lines.

2. Materials and Methods

2.1 Cell lines and cell cultures

Human tongue squamous cell carcinoma (CAL-27) cell lines and normal human gingival fibroblast (HGF) cells [CAL 27 (ATCC® CRL-2095™)] generously provided by (ATCC USA) were used in this study. The cells were grown in DMEM supplemented with 10% FBS, 50 U/ml penicillin G, and 50 mg/ml streptomycin sulphate. The cultures were maintained at 37°C in a humidified atmosphere of 5% CO in air. Exponentially growing cells were used for all the experiments.

2.2 Preparation of Lepidium sativum leaf extract

For aqueous extract preparation, *L. sativum* leaves used in this study were collected in winter 2013 from Al Muthanna province/ Iraq. Leaves were washed to remove impurities such as dust and then dried in an air oven

for 2 days. Then, they were ground by grinder and sterilized with Tendalization method. One litter water was added to 50 grams powder obtained from leaves and put on the shaker to be solved thoroughly. The obtained solution was passed through filter (Mobasher *et al*. 2006).

2.3 Cytotoxicity assay

The MTT assay was used to detect the cytotoxicity. It based on the reduction of MTT by mitochondrial dehydrogenases of viable cells to a purple formazon product (Mosmann 1983). Briefly, cells were diluted in growth medium and seeded in 24-well plates (5×10^4 cells/well). After overnight growth, the growth medium was replaced with exposure medium (DMEM without FBS) containing indicated doses (10, 30, 50,70,100, and 150 µg/ml) of *L. sativum* extract. After 24 h, the cells in each well were washed with 200 ml of PBS, and incubated with 100 ml of 500 µg /ml MTT in PBS at 37°C for 3 h. The MTT-formazon product dissolved in 200 ml of DMSO was estimated by measuring the absorbance at 570 nm in an ELISA plate reader. Cell survival was expressed as percentage of viable cells of treated samples to control samples. All the dietary agents were tested in triplicates and the experiments were repeated at least three times.

2.4 Determination of ROS generation

To assess the generation of intracellular ROS, the oxidation-sensitive fluorescent probe DCFH-DA was used. Briefly, after treatment, CAL-27 cells were harvested and suspended in 0.5 ml PBS containing 10 mM DCFH-DA for 15 min at 37°C in the dark. DCFH-DA was taken up by the cells and deacetylated by cellular esterase to form a non-fluorescent product DCFH, which was converted to a green fluorescent product DCF by intracellular ROS produced by treated CAL-27 cells. The intensity of DCF fluorescence was measured by flow cytometry with excitation and emission settings of 488 and 530 nm respectively (Potikha *et al*. 1999). A total of 10^4 events were counted and the histograms were analysed using Cell Quest software and compared with histograms of control untreated cells.

2.5 Nuclear morphology

human tongue squamous carcinoma (CAL-27) cells were plated at a density of 5×10^4 cells/well into 6-well chamber slides. After 80% confluence, CAL-27 cells were treated with dietary agents for 24 h. The cells were then washed with PBS, fixed in methanol: acetic acid (3:1, v/v) for 10 min and stained with 50 µg /ml propidium iodide for 20 min. Nuclear morphology of apoptotic cells with condensed/fragmented nuclei was examined under a fluorescent microscope and at least 1×10^3 cells were counted for assessing apoptotic cell death (Keum *et al*. 2002).

2.6 Statistical analysis

Cytotoxicity data are presented as mean percentages of control $\pm$ S.D and linear regression analysis was used to calculate the IC values. Statistical analysis on the data for cytotoxicity of *L. sativum* on CAL-27 cells was done using analysis of variance (ANOVA).

3. Results

3.1 Cytotoxicity assay

Our results was focused on the examination the effects of different concentrations of *L. sativum* on the growth of CAL-27 cells (Figure 1). The leaf extract of *L. sativum* showed dose-dependent cytotoxic effects on CAL-27 cells with IC_{50} values of 70,100, and 150 µg/ml respectively compared to human gingival fibroblast (HGF) cells (control) at significantly level ($p < 0.05$ and $p < 0.01$ respectively). Overall, the results of MTT assay suggest that dietary agents showed preferential cytotoxic effects on CAL-27 cells. Since the dietary agents preferentially inhibited the growth of CAL-27 cells.

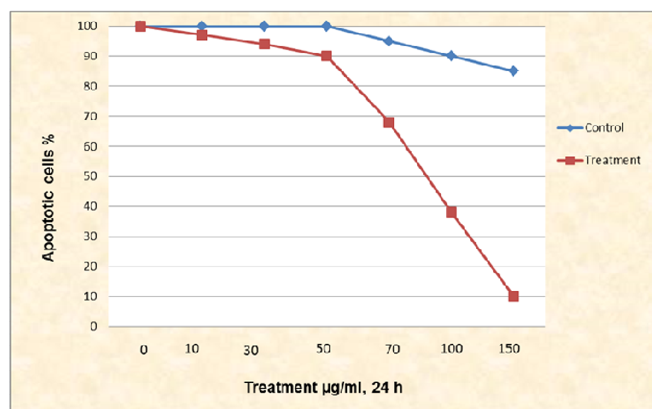


Figure 1. Effects of *L. sativum* on CAL-27 and HGF cell viability. Cell survival was measured by using MTT assay and expressed as percentage of viable cells of treated samples to control samples. Data are represented as mean + SD of two independent experiments each performed in triplicate.

3.2 Effect of L. sativum extract on intracellular ROS generation

To investigate whether ROS is involved in mediating apoptosis induced by the L. sativum (LE) extract, we measured the intracellular generation of ROS using the fluorescent probe DCFH-DA. Treatment with dietary agents significantly increased ROS generation in CAL-27 treated cells compared to untreated control. Our results showed that the mean of DCF fluorescence increased from Ma = 377 (control) to Mb = 955, Mc = 1248, and Md = 1320 for LE treated samples at concentrations (70, 100, and 150µg/ml respectively) (Figure 2).

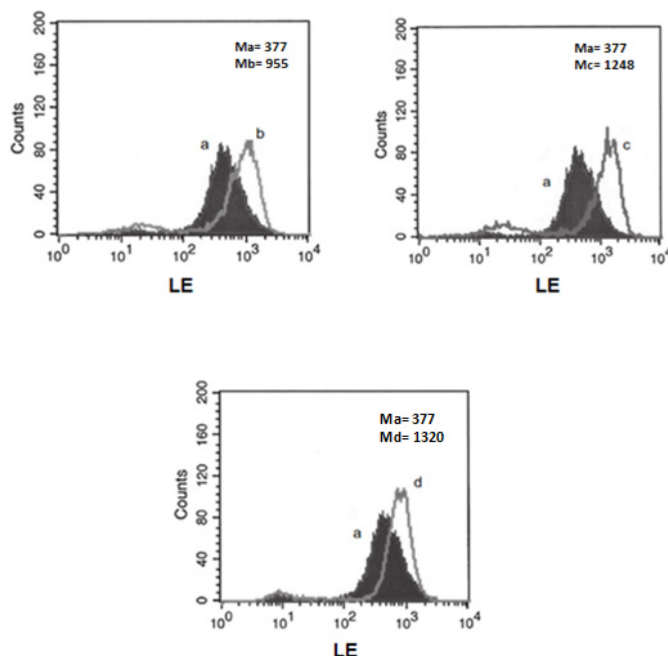
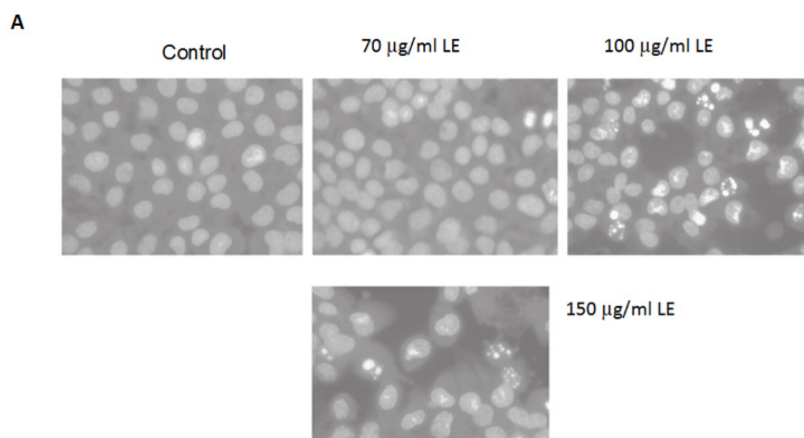


Figure 2. Induction of ROS generation in CAL-27 cells by dietary agent *L. sativum* (LE) extract . (a) Untreated control; (b) LE (70 µg/ml); (c) LE (100 µg/ml) ; (d) LB (150 µg/ml).

3.3 Nuclear morphology changes

To detect the cytotoxic role of LE in inducing apoptosis, nuclear morphology was observed using the fluorescent DNA-binding agent propidium iodide. Incubation of CAL-27 cells with the LE for 24 h significantly increased the number of apoptotic cells compared to control as evidenced by nuclear fragmentation and condensation (Figure 3). The cytotoxic effect and DNA damage observed through the formation nuclear fragments in LE treated CAL-27 cells (70, 100, and 150 µg/ml). The percentage of apoptotic nuclei was increased to 30% and 60% at (100 and 150 µg/ml, respectively). The obtained results was significant ($p < 0.05$ and $p < 0.01$, respectively) compared to control (Figure 3).



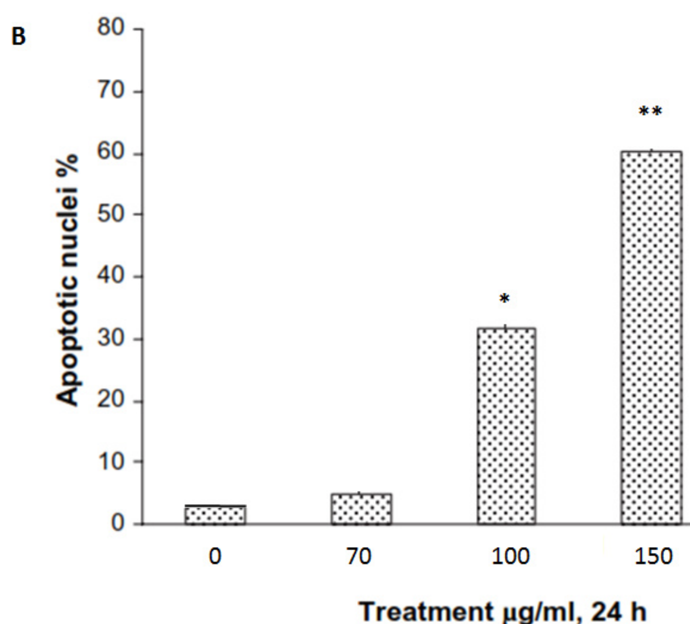


Figure 3. Nuclear morphological changes (A); The number of apoptotic nuclei formed after treatment with *L. sativum* for 24 h (B). * and ** indicates significantly increased ($p < 0.05$ and $p < 0.01$, respectively) compared to control.

4. Discussion

The results from our study demonstrate that *L. sativum* leaf extract preferentially exert cytotoxic effects on human tongue squamous carcinoma (CAL-27) cells in a dose dependent manner. In previous study *L. sativum* was found to have chemoprotective effects (Kassie *et al*. 2002). On the other hand, *L. sativum* is an ingredient in Carctol, which is a herbal dietary supplement marketed with claims it is based on traditional ayurvedic medicine. Its ingredients include *Hemidesmus indicus*, *Tribulus terrestris*, *Piper cubeba*, *Ammani vesicatoria*, *Lepidium sativum*, *Blepharis edulis*, *Smilax china* and *Rheum emodi* (Ernst 2009).

Lepidium sativum extract is rich with essential oil composition, imidazole alkaloids, flavonoids, and polyphenols (Mirza & Navaei 2006; Oszmiański *et al*. 2013). Polyphenolic compounds are known to mediate their anticancer properties by induction of apoptosis (Nandi *et al*. 2007). The present results also demonstrate that *L. sativum* inhibit the growth of CAL-27 cells by inducing apoptosis as revealed by characteristic changes in nuclear morphology. This support the evidence that apoptosis induced by chemopreventive or chemotherapeutic agents is associated with perturbation of a specific phase of the cell cycle (Kuno *et al*. 2012; Nair *et al*. 2014).

Moreover, our results demonstrate that incubation of CAL-27 cells with *L. sativum* extract increased ROS generation at significant level. Recent study has provided evidence for the involvement of *L. sativum* extract in ROS generation (Mahassni & Al-Reemi 2013). Increased caspase-dependent apoptosis, reactive oxygen species (ROS) generation and mitochondrial damage are phenomena, which can be frequently observed altogether in cells subjected to anticancer drugs treatment, that is, accumulation of ROS inside the cell often signalizes apoptosis or terminal differentiation (Watson *et al*. 2011).

Morphological changes are distinct hallmarks during apoptosis and the effector *L. sativum* have been implicated to play a major role in these processes. Our results showed that CAL-27 treated cells displayed a distinct nuclear morphological change in the presence of *L. sativum* extract and this is caused due to the strong toxicity of plant extract leads to higher degree of damage in DNA molecule.

5. Conclusion

The results of our study demonstrate that *L. sativum* exert strong antiproliferative effects against CAL-27 cells that is mediated through apoptosis. The data indicate a key role for ROS in mitochondrial mediated apoptosis. The study also emphasizes that *L. sativum* extract at higher concentrations lead to damage DNA and form clearly nuclear morphology changes. The results of the present study have opened the way for identifying the role of mitochondria in apoptosis. Additional work need in order to explain the molecular role of *L. sativum* extract on genes responsible for cell cycle control and apoptosis.

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