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Effect of ethyl acetate aroma on viability of human breast cancer and normal kidney epithelial cells *in vitro*

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ABSTRACT

Background: Aromatherapy is used in clinical settings for patients suffering from several chronic and critical diseases such as cancer. Ethyl acetate (EA) is a colorless liquid with a characteristic fruity smell and is naturally present in fruits and wines.
Methods: In the present study, the effect of the aroma of EA was evaluated on human breast cancer cell line MDA-MB-231 and normal cell line, Vero. Cell line viability and mechanism of EA cytotoxicity were determined by Trypan blue dye exclusion assay and phase contrast microscopy.
Results: It was found that EA at a concentration of 0.026 M was effective in causing considerable cytotoxicity in breast cancer cells (without even coming in contact with the culture medium and cells), while showing no effect on normal cells. Mechanism of action of EA on cancer and Vero cells was investigated by DNA fragmentation and dye binding assays using agarose gel electrophoresis (AGE) and fluorescence microscopy/cytometry, respectively. It was found that EA aroma induced predominantly necrosis in the cancer cells exposed to it.
Conclusion: A study such as this has not been attempted before and results need further investigation before EA aroma can be used as a complementary therapy.

1. Introduction

In aromatherapy, essential oils (also known as volatile oils) are extracted from various aromatic plants for use in palliative care of patients suffering from serious illness in order to improve quality of life.[1,2] The main mode of action of essential oils is through alteration of physiological processes. The volatile constituents of the essential oils are taken up by the body through topical, oral, vaginal, rectal, or olfactory

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routes.[3] Olfactory receptors in the nose respond to the volatile chemical constituents present in the aroma of these oils by sending chemical messages to the limbic system of the brain via neurons.[3] This causes a change in mood and emotions by creating a sedative effect for reduction of stress and anxiety, which leads to reduced pain perception.[2]

Aromatherapy has been demonstrated to be effective in alleviating and relieving the symptoms of stress, anxiety, depression, and nausea in patients undergoing radiotherapy and chemotherapy for cancer.[4] Some cancer patients ultimately experience a better quality of life when the pain is controlled through aromatherapy. The National Cancer Institute has also reported the benefits of inhalation of vapors of peppermint, ginger, and cardamom oil on alleviation of the harmful side effects of radiation and chemotherapy.[4] Recently, the ability of essential oils to kill cancer cells has been demonstrated with no harm to healthy cells.[5]

The sense of smell is indispensable to life.[6] Our sense of smell usually serves as a warning regarding the safety of food.[6] Pleasant aromas also make food more palatable to eat. Esters are a family of organic compounds responsible for many of the pleasant odors of fruits. Esters are formed from carboxylic acids by replacing the acidic hydrogen by an alkyl or aryl group.[6] The most common ester, ethyl acetate (EA) occurs as a colorless volatile liquid at room temperature with a pleasant fruity smell, having a boiling point of 77 °C (www.chm.bris.ac.uk). EA is used for flavoring confectionery, ice creams, and cakes, as well as in artificial fruit essences and aroma enhancers and perfume manufacturing industries.[6] This colorless liquid has a characteristic sweet smell, similar to pear drops. EA is present in confectionery and perfumes, as well as fruits such as apples. Because of its light and fruity odor, EA is used in perfumes since it confers a fruity smell (as do many esters) and is highly volatile, producing a cooling sensation and leaving the scent of the perfume on the skin.[7]

EA is inhaled via the nasal route into the body where it is broken down to ethyl alcohol and acetic acid, which are metabolized further before being excreted (www.onlinelibrary.wiley.com). The median lethal dose (LD_{50}) for rats is 5620 mg/kg, which is indicative of low toxicity (www.hazard.com). Since the ester naturally occurs in many organisms, there is little possibility of toxicity. Exposure to concentrations > 1500 mL/m^3 causes irritation of the upper respiratory tract and eyes. EA behaves as a narcotic at high concentrations and causes lung, kidney, and liver damage.[8] In animal experiments, after repeated exposure to EA at subnarcotic concentrations, the organs most likely to be affected are the lungs, liver, kidneys, and spleen. If applied repeatedly and occlusively to the skin, EA, in its undiluted form causes irritation. It is questionable whether EA causes sensitization.

At high concentrations and under certain culture conditions, EA induces aneuploidy in *Saccharomyces cerevisiae*.[8] Chromosomal aberrations have been reported in the fibroblasts of the Chinese hamsters on EA exposure.[8] *Salmonella* mutagenicity tests and two *in vivo* micronucleus tests have yielded negative results.[8]

There are no scientific reports available on the mechanism of action of EA. However, the rapid metabolism of EA to ethanol and acetic acid deserves attention for toxicological evaluation. It has been found that topical application of acetic acid (parent compound) on mucosal or serosal surface causes necrosis of tumor in mouse model of gastric cancer.[9] Substituted derivatives of acetic acid like flavone acetic acid (LM975) and flavone acetic acid ester (LM985) are known to exhibit anticancer activities.[10,11] In the present study, the effect of aroma of EA on the viability and survival of breast cancer cells has been studied *in vitro*. Promising results have indicated that the cytotoxic effect of EA aroma should be studied further with respect to other cancer cell lines and the mechanism of action of EA-induced cytotoxicity *in vitro* should be investigated in detail.

2. Methods

2.1. Reagents

Trypan blue (0.4%), phosphate-buffered saline (PBS; pH 7.2, 1×), 0.25% trypsin–EDTA (1×), Dulbecco's Modified Eagle's Medium (DMEM/F-12) (1×) and antibiotic/antimycotic solution (100×) were obtained from Gibco, Life Technologies; EA was from Rankem, and fetal bovine serum (FBS) was from Himedia. Agarose, 2,7-dichlorofluorescein diacetate (DCFHDA), acridine orange, and ethidium bromide were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Tali Apoptosis AnnexinV/PI Staining Kit was from Molecular Probes™, Thermo Fisher. All other chemicals used in the study were of analytical grade.

2.2. Cell lines

MDA-MB-231 (human breast carcinoma, estrogen receptor negative, tumorigenic and invasive cell line) and Vero (ATCC-CCL-81 normal kidney epithelial cell line) cells were obtained from the National Centre for Cell Science (Pune, India), and were maintained by subculturing and passaging as monolayers in 25- and 75-cm^2 cell culture flasks (Nest; Tarsons) at 37 °C in the Tissue and Cell Culture Laboratory, Era's Medical College, Lucknow, India, in a 5% CO_2 incubator at 95% humidity for producing HCO_3 buffering capacity as reported previously.[12] The cells were maintained at pH 7.4 in DMEM containing phenol red as a pH indicator and supplemented with 5% FBS.[12] The medium, prior to being used in cell culture experiments was vacuum filtered using a Corning filtration system (Corning®, Sigma-Aldrich).

2.3. Experimental setup

Experiments were carried out in 25-cm^2 cell culture flasks. Square pieces of sterilized cotton enclosed in cotton mesh were cut and affixed to the inside of the upper boundary of the culture flask by means of a transparent cello tape as shown in Fig. 1. EA in pre-calculated concentrations (0.02 M, 0.026 M, 0.03 M, 0.034 M, 0.04 M, 0.05 M, 0.1 M, 0.2 M, and 0.5 M) was added on to the cotton swabs inside each flask by means of a micropipette. The flasks were immediately closed so as to allow vapors of EA to saturate the flask.

MDA cells were trypsinized and added to each flask at a density of 10^5 cells/mL and were allowed to attach. The flasks were placed in the incubator for the next 48 hours. After 24 hours and 48 hours, the cells in the flasks were observed