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Evaluation of toxicity of essential oils palmarosa, citronella, lemongrass and vetiver in human lymphocytes

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ABSTRACT

The present investigation was undertaken to study the cytotoxic and genotoxic potential of the essential oils (palmarosa, citronella, lemongrass and vetiver) and monoterpenoids (citral and geraniol) in human lymphocytes. Trypan blue dye exclusion and MTT test was used to evaluate cytotoxicity. The genotoxicity studies were carried out by comet and DNA diffusion assays. Apoptosis was confirmed by Annexin/PI double staining. In addition, generation of reactive oxygen species was evaluated by DCFH-DA staining using flow cytometry. The results demonstrated that the four essential oils and citral induced cytotoxicity and genotoxicity at higher concentrations. The essential oils were found to induce oxidative stress evidenced by the generation of reactive oxygen species. With the exception of geraniol, induction of apoptosis was confirmed at higher concentrations of the test substances. Based on the results, the four essential oils are considered safe for human consumption at low concentrations.

1. Introduction

Essential oils extracted from the aromatic grasses like *Cymbopogon martini*, *Cymbopogon winterianus*, *Cymbopogon citratus* and *Vetiveria zizanioides* are of enormous commercial value for environmental, agricultural, food and medical applications as well as in perfumery and aromatherapy. Essential oils are complex natural mixtures containing about 20–60 components of different concentrations. They are characterized by one or two major components at high concentrations (20–70%) compared to the others components present in trace amounts (Bakkali et al., 2008). Phytochemical analysis of the essential oil of *C. martini* (palmarosa) indicated the presence of geraniol (65–85%) and geranyl acetate (5–20%) as major components (Raina et al., 2003). Geraniol (20–40%) is also the major constituent of *C. winterianus* Jowitt (java citronella) along with citronellal (20–30%) and citronellol (10–15%). Essential oil from *C. citratus* (lemongrass) is rich in citral (50–88%) with other components such as linalool, myrcene, geraniol, geranyl acetate and camphene (Anonymous, 2001; Ganjewala, 2009).

The greatest use of these essential oils and their major components (monoterpenoids) is in food as flavouring and preservative agents (Burt, 2004). The recent interest in 'green' consumerism has lead to the renewal of scientific interest in these substances. Palmarosa, citronella, lemongrass, vetiver, citral and geraniol are classified as GRAS (Generally recognized as safe) by the US FDA for their intended use. The essential oils from *Cymbopogon* and vetiver are used at levels of 5–40 ppm to impart flavour in alcoholic and non-alcoholic beverages, chewing gum, candies, dairy and baked food products (Council of Europe, 2000). In addition, they are used as preservatives for their antimicrobial (Hammer et al., 1999; Kim et al., 1995a,b; Onawunmi, 1989; Prashar et al., 2003), antifungal (Li et al., 2013) and antiprotozoal (Monzote et al., 2012) properties.

In vitro studies have demonstrated antibacterial activity of the essential oils at levels between 0.2 and 10 μl/ml and a number of their components has been identified having minimum inhibitory concentrations (MICs) of 0.05–5.0 μl/ml (Burt, 2004). To achieve the same antibacterial activity in foods, it has generally been found that a greater concentration of the essential oils or their component is used (Shelef, 1983; Smid and Gorris, 1999). The ratio has been approximately 50-fold in soup (Ultee and Smid, 2001) and 25- to 100-fold in soft cheese (Mendoza-Yepes et al., 1997). These concentrations are much higher than the Admissible Daily Intake (ADI) values. The ADI values of citral and geraniol is 0.5 mg/kg body weight/day (Council of Europe, 2000).

The random and inappropriate use of the essential oils may entail risks to human health due to mutational events, carcinogenic effects and genetic damages (Sousa et al., 2010). Moreover, there is a demand for their comprehensive safety evaluation and this issue is becoming more complicated as the use of the essential oils is not under any regulatory control in many countries.

The present study was undertaken to investigate the toxic effects of essential oils (palmarosa, citronella, lemongrass, vetiver acetate) and monoterpenoids (citral and geraniol) on human

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lymphocytes. Cytotoxicity and genotoxicity of test substances were evaluated by trypan blue dye exclusion test, MTT assay, single cell gel electrophoresis and DNA diffusion assay respectively. The reactive oxygen species generation and detection of apoptosis/necrosis was determined using flow cytometry.

2. Materials and methods

2.1. Chemicals

Methyl methanesulphonate (MMS), normal melting point agarose (NMA), low melting point agarose (LMPA), ethylenediaminetetraacetic acid (EDTA) disodium salt, ethidium bromide (EtBr), 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT), Trizma base, Spermine, Triton X-100, DMSO and 2′,7′-dichlorfluorescein-diacetate (DCFH-DA), trypan blue and Histopaque were also purchased from Sigma–Aldrich Chemical Co., Bengaluru, India. Phosphate-buffered saline (PBS; Ca^{2+}, Mg^{2+} free), and Roswell Park Memorial Institute media (RPMI-1640) were purchased from Hi Media, Mumbai, India.

2.2. Test substance

Palmarosa oil (CAS No. 8014-19-5; source-*C. martini*), citronella oil (CAS No. 8000-29-1; source-*C. winterianus*), lemongrass oil (CAS No. 8007-02-1; source-*C. citratus* Stapf), vetiver acetate (CAS No. 62563-80-8; source-Vetiver java), citral (CAS No. 5392-40-58) and geraniol (CAS No. 106-24-1), were purchased from Sigma–Aldrich Fine Chemicals, St. Louis, MO, USA.

2.3. Test system

Human peripheral blood was collected into heparinized vacutainers by vein puncture from healthy male volunteers ($n = 5$; 20–25 year old, non-smokers, non-alcohol consuming and not undergoing any medication) after obtaining written informed consent. The lymphocytes were isolated from fresh blood according to the method of Boyum (1976) with minor modifications. Fresh blood was diluted with equal volume of PBS (Ca^{2+}, Mg^{2+} free; pH 7.4) and layered over 2 ml of Histopaque and centrifuged at 1000 rpm for 40 min. The buffy coat was aspirated into 3–5 ml of PBS and centrifuged at 1000 rpm for 10 min. The pellet was resuspended in RPMI-1640 media (1×10^6 cells/ml) and viability was checked. Lymphocytes with 98% and above viability were used in the experiments (Tennant, 1964). All the experiments were done in triplicate and in accordance with the Institutional guidelines (Institutional Ethical Committee, University of Calcutta, India).

2.4. Cytotoxicity assays

2.4.1. Dose selection

The dose selection for the present study was based on initial cytotoxicity screening of the test substances using trypan blue dye exclusion method and MTT assay. Stock solutions of the test substances were prepared in DMSO and further dilutions were made with PBS. The cytotoxicity assays were carried out over a wide range of concentrations of 0–5000 μg/ml and the cut-off point was considered 70% cell viability as suggested by Henderson et al., 1997. Following the initial screening, the final treatment concentrations were selected. Palmarosa, citronella oils and geraniol were tested at different concentrations of 100, 200, 500, 1000, 1500 and 2000 μg/ml. Whereas lemongrass oil, citral and vetiver acetate oil have been tested at different concentrations of 25, 50, 100, 200, 400 and 800 μg/ml. Negative control in all the experiments was maintained as lymphocytes suspended in RPMI-1640 medium added with 0.01% DMSO. The detailed protocols of above mentioned cytotoxicity assays are described in the following sections.

2.4.2. Trypan blue dye exclusion method

The lymphocytes (1×10^6/ml) were incubated with different concentrations of the test substances. After treatment for 3 h at 37 °C, the lymphocytes were washed by centrifugation and fresh media was added (Tennant, 1964). The lymphocytes were stained with trypan blue dye (0.4% w/v) and the number of viable and dead cells were scored under the light microscope with Neubauer' hemocytometer.

2.4.3. MTT assay

Cytotoxicity of the test substances at concentrations of the test substances were also evaluated using MTT assay. The MTT assay is based on reduction of the yellow tetrazolium salt (MTT), to form a soluble blue formazan product by mitochondrial

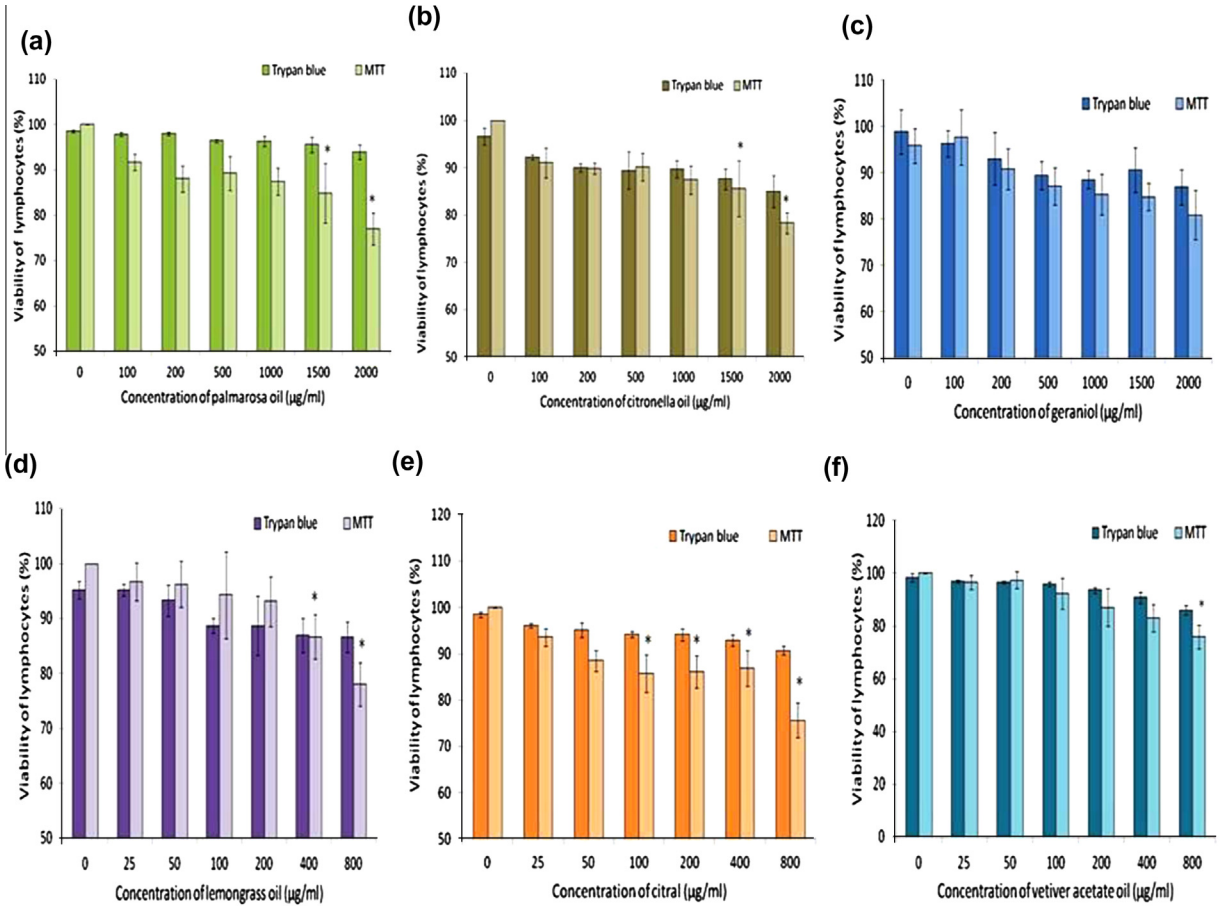

Fig. 1. Effect of the test substances on cell viability of human lymphocytes evaluated by trypan blue dye exclusion test and MTT assay; (a) palmarosa, (b) citronella, (c) geraniol, (d) lemongrass, (e) citral, and (f) vetiver acetate. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)