SUPPLEMENT: SHORT COMMUNICATIONS

INHIBITION OF OXIDATIVE DNA DAMAGE BY PLANT ANTIOXIDANTS. Jasna Stanojević1, Jelena Knežević-Vukčević1 and George Miloshev2. 1Chair of Microbiology, Faculty of Biology, University of Belgrade, 11000 Belgrade, Serbia and Montenegro, 2Laboratory of Yeast Molecular Genetics, Institute of Molecular Biology, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria.

Reactive oxygen species (ROS), from both endogenous and exogenous sources, may be involved in aging and different human diseases such as atherosclerosis, cancer, diabetes, neurodegenerative diseases and acquired immunodeficiency syndrome (AIDS) (Olinski et al., 2002). Natural antioxidants contained in medicinal and aromatic plants, fruits and vegetables may be useful in preventing the deleterious consequences of oxidative damage caused by ROS and therefore they are considered as possible chemopreventive agents. They can possess a variety of biological activities e.g. anti-mutagenic, anti-carcinogenic, anti-proliferative, scavenging of free radicals or activated mutagens/carcinogens; they can modulate DNA repair and other enzyme activities or even regulate gene expression (Brigelius-Flohe and Traber, 1999; Craig, 1999; Heo et al., 2001; Mitić et al., 2001; Lazarova and Slamena, 2004; Nikolić et al., 2004). In our previous work, we have shown that terpenoids from sage (Salvia officinalis L.) and basil (Ocimum basilicum L.) possess antimutagenic potential in prokaryotic and lower eukaryotic test systems (Vuković-Gačić et al., 1993; Simić et al., 1998, 2000; Mitić et al., 2001). For estimation of their mechanism of action, it is very important to determine the potential to prevent oxidative DNA damage.

The Single Cell Gel Electrophoresis (SCGE) or Comet assay, a simple, rapid and sensitive technique for measuring DNA strand breaks (Collins, 2004), is also used in examination of protective effects of plant antioxidants (Heo et al., 2001; Russo et al., 2001; Labieniec et al., 2003). Recently Miloshev et al., (2002) have developed Yeast Comet Assay (YCA), an easy to perform and more sensitive method, applied on lower eukaryotic organism Saccharomyces cerevisiae. In this work YCA is applied for the detection of antioxidative activity of natural products.

As an oxidant we used hydrogen peroxide, a DNA damaging agent that produces base oxidation and single–strand breaks mediated by the highly reactive hydroxyl radicals generated in metal–catalyzed Haber–Weiss or Fenton reaction (Gille and Sigler, 1995; Horvatova et al., 1998). After validation with model antioxidants, vitamin E (α-tocopherol) and vitamin C (ascorbic acid), we have tested linalool, the major constituent of ethereal oil of basil (Ocimum basilicum L.

Saccharomyces cerevisiae strain 3A: a/a, gal 1, leu 2, ura 3–52 was cultivated in rich YPD medium at 30°C to middle logarithmic phase of growth (5 x 107 cells/ml). The cells were treated in vivo with antioxidant for 15 min at room temperature and, after washing, treated with H2O2 for 10 min at 4°C (pre-treatment). In the case of co-treatment oxidant and antioxidant were applied simultaneously for 10 min at 4°C. The spheroplasting with zymolyase was carried out in the gel. The cells were mixed with low melting agarose, spread on microscope slides and lysed by placing the slides in lysis solution. After unwinding the DNA in alkaline conditions, we subjected the slides to electrophoresis, dried the gels, stained and visualized the comets under the fluorescent microscope. The comets were scored visually at ten randomly selected fields for each slide and percentage of inhibition of oxidative damage was calculated as described by Wall et al., (1988). The presented values are from representative experiments.

In preliminary experiments different concentrations of oxidant hydrogen peroxide were tested: 0.5, 1, 5, 10, 50, 100, 150, 200, 250, 500, 1000 μM. Concentration which induces the strongest oxidant hydrogen peroxide were tested: 0.5, 1, 5, 10, 50, 100, 150, 200, 250, 500, 1000 μM. Concentration which induces the highest number of comets without effect on the number of cells was applied in further work. We have also investigated different concentrations of antioxidants (0.01, 0.05, 0.1, 0.5, 1, 5, 10, 50, 100 μM) in order to find and examine the concentration that does not induce oxidative damage. It is well known that antioxidants may act as antioxidants, they can exhibit pro-oxidative effect when applied in higher concentration (Singh, 1997; Labieniec et al., 2003). In the presence of transition metal ions such as iron or copper, they behave as reducing agents and provide components for Fenton reaction, or pro-oxidant effect may be due to the formation of pro-oxidant intermediates/end products via its biotransformation (Labieniec et al., 2003). The highest concentrations that did not induce strand breaks were 0.05 μM for vitamin E and C and 0.5 μM for linalool. Applied concentrations of vitamins are 500 to 1000 times lower than in mammalian Comet assay (Singh, 1997; Horvatova et al., 1998; Lazarova and Slamena, 2004), which indicates higher sensitivity of the method for detecting oxidative and antioxidative activity of different compounds. Ethanol, used as a solvent for vitamin E and linalool, was also applied in concentration that does not induce oxidative DNA damage (0.095 % or less).

Vitamin E, an antioxidant with strong antimutagenic potential (Nikolić et al., 2004), a potent scavenger of ROS, exhibited the protective effect against induced oxidative damage (Fig.1). For the applied concentration of 0.05 μM, the stronger
inhibitory effect was detected in pre-treatment (81%) in comparison to co-treatment (43%) (Fig. 4). The other model antioxidant, vitamin C (0.05 μM), a free radical scavenger, also displayed a significant protective capability against H₂O₂-induced DNA damage (Fig. 2). Percentage of inhibition reached the same value (65%) both for co-treatment and pre-treatment (Fig. 4). Linalool, the major constituent of ethereal oil of basil (69.2%), is the tertiary monoterpene alcohol. Concentration 0.5 μM exhibited very strong antioxidative activity observed as decreasing the level of H₂O₂-induced oxidative DNA damage (Fig. 3). Percentage of inhibition ranges from 55% for co-treatment to 70% for pre-treatment (Fig. 4).

These results put linalool as one of the substances with very strong antioxidative activity and recommend it for further antimutagenic/anticarcinogenic studies. We have also shown that alkaline Yeast Comet Assay, a sensitive method for detection of DNA damaging agents (oxidants and other agents which cause DNA strand breaks), is also suitable for investigation of protective effect of plant antioxidants.

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**References:**