ANTIMUTAGENIC EFFECT OF ESSENTIAL OIL OF SAGE \textit{(Salvia officinalis} L.) AND ITS FRACTIONS AGAINST UV-INDUCED MUTATIONS IN BACTERIAL AND YEAST CELLS

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Abstract - The inhibition of spontaneous and UV-induced mutations by essential oil (EO) of sage (\textit{Salvia officinalis} L.) and its fractions F1-F5 containing different proportions of mono- and sesquiterpenes was studied with the \textit{Salmonella}/microsome, \textit{E. coli} K12, and \textit{S. cerevisiae} D7 reversion assays. The EO, F1, and F2 exhibited antimutagenic potential against UV-induced mutations in all tests. Fractions F3 and F4 produced a toxic, mutagenic, or antimutagenic response, depending on the test organism used. Reduction of spontaneous and UV-induced mutations by F5 was detected only in permeable strains of \textit{E. coli}. The obtained results demonstrate antimutagenic activity of volatile sage terpenes and recommend them for further antimutagenesis and anticarcinogenesis studies.

Key words: Sage, essential oil, UV-irradiation, antimutagenesis, \textit{Salmonella}/microsome, \textit{E. coli}, \textit{S. cerevisiae}

INTRODUCTION

Mutations are implicated in many important human diseases, including atherosclerosis, autoimmune and neurodegenerative diseases, some types of diabetes and cancer. There is increasing evidence that many plant extracts and their components can act as inhibitors of mutagenesis and carcinogenesis (Craig, 1999; Weisburger, 2001). Chemoprevention of mutation-related diseases and cancer is an important research field, and the dietary use of antimutagens and anticarcinogens has been proposed as the most promising approach to protection of human health (Wattenberg, 1985; Hayatsu et al. 1988; Ferguson, 1994).

For many years our research efforts have been focused on detection of antimutagenic properties of medicinal and aromatic plants of our region. For that purpose we designed and validated an \textit{Escherichia coli} K12 assay system and used it along with standard mutagenicity tests recommended by OECD, the \textit{Salmonella}/microsome mutation assay and the \textit{Saccharomyces cerevisiae} D7 mutation assay. Results of screening indicated that terpenoid fractions of wild and cultivated sage (\textit{Salvia officinalis} L.) possess antimutagenic potential against mutations induced by UV-C and ethidium bromide in \textit{E. coli} and \textit{S. typhimurium} (Vuković-Gačić and Simić, 1993; Simić et al. 1994,1997; Mitić et al. 2001). Interestingly, between differently prepared extracts, only the fractions from cultivated sage with high content of low-molecular-weight terpenes inhibited UV-induced mutations. The antimutagenic effect was lost when the same plant was steam distilled before extraction and fractionation (Simić et al. 1994, 1997, 1998). The obtained data led us to hypothesize that volatile terpenes from cultivated sage possess antimutagenic potential. An inhibitory effect of sage essential oil on chromosome aberrations induced by mitomycin C in mice was recently reported by Vujošević and Bлагојевић (2004).

To verify further the proposed antimutagenic potential of volatile terpenes from sage we investigated the inhibitory potential of essential oil of cultivated sage and its fractions containing different proportions of mono- and sesquiterpenes on UV-induced mutations. The study was conducted with two standard mutagenicity tests, namely the \textit{Salmonella} microsome and \textit{S. cerevisiae} D7 reversion assays, as well as with our \textit{E. coli} K12 reversion assay,
MATERIAL AND METHODS

Bacterial strains

Salmonella typhimurium TA102 (hisG428/pAQ1 rfa/pKM101) and Escherichia coli K12 SY252 (argE3) and IB103 (argE3 mutS215::Tn10) were used in prokaryotic mutagenicity and antimutagenicity assays (Maron and Ames, 1983; Simić et al. 1997). Permeable E. coli strains IB112 and IB113 were constructed for this work by selecting spontaneous mutants of SY252 and IB103 resistant to coliphage T7 (McCoy et al. 1985). Susceptibility to large molecules was ascertained by the demonstration of the increased sensitivity to crystal violet (Maron and Ames, 1983). The S. cerevisiae D7 diploid strain ade2-40/119 trp5-12/27 ilv1-92/92 was used in eukaryotic mutagenicity and antimutagenicity assays (Zimmermann et al. 1975). Fresh overnight cultures of all tester strains, to which glycerol was added as a cryoprotective agent, were stored at -20°C. The strains were

which was used in a previous study of ours on sage.
routinely checked to confirm genetic features.

Media and growth conditions

The bacteria were grown in LB medium (5 g NaCl, 10 g bacto tryptone, 5 g yeast extract, 1 L distilled water) at 37°C with aeration. Saccharomyces cerevisiae D7 was grown in YPD medium (10 g yeast extract, 20 g bacto-peptone, 20 g D-glucose, 1 L distilled water) at 30°C with aeration. All media for the S. typhimurium reversion assay were as described by Maron and Ames (1983). The Semi-enriched minimal medium (SEM) for the E. coli K12 reversion assay was a minimal agar medium supplemented with 3% (v/v) NB (Nikolić et al. 2004). The selective medium (4 g yeast nitrogen base without amino acids, 10 mg tryptophan, 20 g D-glucose, 15 g agar, 1 L distilled water) was used in the S. cerevisiae reversion assay.

The S9 fraction was isolated from the liver of albino Wister male rats (170-180 g) induced with pheno-barbital/β-naphtho flavone (Ong et al., 1980). The S9 mixture contained 4% (v/v) S9 fraction, 33 mM KCl, 8 mM MgCl₂, 5 mM glucose-6-phosphate, and 4 mM NADP in 0.1 M phosphate buffer (pH 7.4).

Preparation of essential oil of sage (Salvia officinalis L.) and its fractions

Sage (Salvia officinalis L.) was cultivated by the "Dr. Josif Pančić" Institute for Medicinal Plant Research in Pančevo. The essential oil was prepared according to Ph. Jug. IV by distillation of the dried aerial part (Salviae herba) in a 2 - m³ steam distiller (Hromil) for 2 hours at a pressure of 3-4 bars and temperature of 135-145°C. The essential oil was fractionated by vacuum rectification on a low-resolution column to yield five fractions, designated F1-F5 (Brkić et al. 1999).

The composition of essential oil and fractions was determined using analytical GC/FID and GC/MS techniques and the Wiley/NBS library of mass spectra (Marinković et al. 2002). The quality of essential oil meets the standards of Ph. Jug. IV and ISO9909. Essential oil and fractions were stored at 4°C and dissolved in 98% ethanol (1/10) just before use.

Ultraviolet irradiation

Ultraviolet irradiation was carried out with a germicidal lamp (Camag) having maximum output at 254 nm. Dose rates were measured with the Latarjet dosimeter (Latarjet et al. 1953). Cell suspensions in 0.01 M MgSO₄ were irradiated in glass Petri dishes at a thickness of less than 1 mm. Cell suspensions were kept in the dark to prevent photoreactivation.

Detection of mutagenic and antimutagenic potential in S. typhimurium

The overnight culture of S. typhimurium TA102 strain was washed by centrifugation, resuspended in the same volume of 0.01 M MgSO₄, and UV-irradiated. The UV dose was 24 J/m². Samples (0.1 mL) of un-irradiated and UV-irradiated cells were added to 2 mL of molten top agar with and without the S9 mixture (0.3 mL), mixed, and poured in duplicates onto minimal glucose agar plates with different concentrations of essential oil or fractions. ethanol was used as a negative control. After incubation at 37°C for 48 h, the number of His⁺ revertants was determined and the presence of the bacterial background lawn on all plates was inspected.

Detection of mutagenic and antimutagenic potential in E. coli and S. cerevisiae

Overnight cultures of E. coli strains SY252 and IB112 (wild type) and IB103 and IB113 (mutS) were washed by centrifugation and resuspended in the same volume of 0.01 M MgSO₄. Cell suspensions of SY252 and IB112 strains were UV-irradiated with a dose 28 J/m². Samples (0.1 mL) of un-irradiated and UV-irradiated cells, appropriately diluted for determination of cell survival and undiluted for determination of Arg⁺ revertants, were spread in duplicates onto 3% SEM plates with different concentrations of essential oil or fractions and incubated at 37°C for 48 h. Mutagenesis and antimutagenesis assay with S. cerevisiae D7 was performed in the same way, except that an exponential culture containing about 3 x 10⁷ cells/mL was used. The UV dose was 130 J/m². Cell survival was determined on YPD plates. Scoring of IIV⁺ revertants were scored on selective C medium. Plates were incubated at 30°C for 72 h. In all experiments ethanol was used as a negative control.

Statistical analysis

The Student t-test was employed for statistical analysis. Significance was tested at the P < 0.05 level. Experiments were repeated twice. The results presented in
The effect of EO and fractions on spontaneous and UV-induced mutations was compared in *S. typhimurium* TA102 and *E. coli* K12 SY252 and IB103 strains. All strains used contain point mutations leading to auxotrophy and can revert to prototrophy by base substitutions. The *S. typhimurium* TA102 strain is repair-proficient and contains an ochre hisG428 mutation, located on the multicopy plasmid pAQ1; the chromosomal copy of the hisG gene has been deleted. It also contains an rfa mutation conferring increased permeability to large molecules and carries the mutator plasmid pKM101 (M a r o n and A m e s, 1983). The repair proficient *E. coli* strain SY252 and its mutS counterpart IB103 are from our *E. coli* K12 assay system and carry the ochre chromosomal mutation argE3 (S i m i č et al. 1997). We have used these strains in the past for detection of antimutagenic activities of model antimutagens and different plant extracts and for assessing the mechanisms of antimutagenesis (S i m i č et al. 1997, 1998; N i k o l i č et al. 2004). *Escherichia coli* IB112 and IB113 strains with increased permeability to large molecules are derivatives of SY252 and IB103, respectively, constructed to match the permeability of *Salmonella* strains (B e r i č and B j e d o v, 2003).

Ultraviolet C radiation (254 nm) is one of the most extensively studied mutagens, shown to induce mutations in both prokaryotic and eukaryotic test systems. We used UV-C as a model mutagen because many chemical mutagens/carcinogens induce mutations by the same mechanism as UV-C (F r i e d b e r g et al. 1995). Since UV-C is not known to produce reactive oxygen species in addition to pyrimidine dimers (Y o u n g et al. 2003), possible chemical interaction between mutagen and antimutagen is prevented.

The composition of essential oil of sage (EO) and its fractions is shown in Table 1. Among 44 different terpenes identified in EO, the oxygen-containing monoterpenes α-thujone, 1,8-cineole, and camphor were dominant. Fractions F1 and F2 contain exclusively monoterpenes. The most abundant monoterpenes in F1 and F2 are 1,8-cineole and α-thujone, respectively. Fractions F3 and F4 contain a small proportion of sesquiterpenes in addition to monoterpenes. They both contain a very high proportion of α-thujone and camphor. Fraction F5 contains about 40% of sesquiterpenes, the most abundant being α-humulene.

### RESULTS AND DISCUSSION

**Salmonella typhimurium mutagenicity and antimutagenicity assays**

The effect of EO and fractions on spontaneous and UV-induced mutagenesis in *S. typhimurium* TA102 was tested in a range of concentrations (1-10 µL/plate), both with and without metabolic activation by S9 enzymes. Toxicity was determined by observing alteration of the bacterial background lawn and spontaneous revertant counts significantly decreased in comparison with the solvent control. Since similar results were obtained in the presence of S9 enzymes, only the results with S9 are presented.

No mutagenic or co-mutagenic potential of EO and fractions was detected in the range of concentrations applied; there was no increase in the number of spontaneous or UV-induced revertants compared with corresponding solvent controls. Moreover, the number of spontaneous and UV-induced revertants decreased in a concentration dependent manner on plates with EO and fractions. The indication for an antimutagenic effect, i.e., significant decrease of UV-induced revertants without any effect on spontaneous revertants, is seen with EO and F1-F3 (Fig. 1). The inhibition was 35-60% and decreased in the order of EO>F1>F2>F3. With F4 and F5, there was alteration of the bacterial background lawn and significant reduction of spontaneous revertants at all concentrations applied, indicating toxicity (data not shown).

**Escherichia coli mutagenicity assay**

The effect of EO and fractions on survival and spontaneous mutagenesis in *E. coli* SY252 and its permeable counterpart IB112 was tested. The range of concentrations to be used was determined by monitoring cell counts on plates with EO or fractions. The SY252 strain was about 10 times less sensitive than TA102 and IB112, owing to increased permeability of the latter strains.

There was no evidence of mutagenicity of EO and fractions F1, F2, and F5 at any of the concentrations tested (data not shown). However, on plates with F3 and F4 there was about two-fold increase in the spontaneous revertant counts, in both the non-permeable SY252 and permeable IB112 strain (Fig. 2), indicating a mild mutagenic effect of these fractions. Fractions F3 and F4 contain a high proportion of thujone, which has been reported to...
have antioxidative properties (Perry et al. 2001). It is known that antioxidants may act as oxidants and induce DNA damage (Labieniec et al. 2003).

It has been shown recently that mismatch repair cor-

Fig. 1. Effect of EO and F1-F3 of sage on spontaneous and UV-induced mutagenesis in the TA102 Salmonella/microsome assay. UV-dose 24 J/m². Number of revertants: spontaneous 287±11; UV-induced 1224±20. *p<0.05 compared with corresponding samples without EO and fractions.

Fig. 2. Effect of F3 and F4 of sage in SY252 (a,b) and in IB112 (c,d) of the E. coli mutagenicity assay. Survival (open symbols), mutagenesis (closed symbols). Number of spontaneous revertants: SY252 29±7; IB112 58±12. *p<0.05 compared with corresponding samples without fractions.
rects mismatches formed by oxidatively damaged bases (Wyrzykowski et al. 2003). To test the involvement of oxidative damage in the mutagenic effect of F3 and F4, we introduced mismatch repair deficient mutS strain (IB103) and its permeable counterpart (IB113) and compared the mutagenic potential of F3 and F4 in the wild type and mutS strain. There was no significant difference in the extent of mutation induction in SY252 (Fig. 2) and IB103 (Fig. 3a,c), indicating that oxidative DNA damage by F3 and F4 is not important.

Interestingly, in the permeable mutS derivative IB113, the mutagenic potential of F3 and F4 was lost and the F5 fraction showed antimutagenic potential (Fig. 3b,d,f). Fraction F5 contains a high proportion of sesquiterpenes which may possess antimutagenic properties.

It is interesting that no mutagenic effect of the F3 and F4 fractions was detected in the Salmonella/microsome assay. The fractions are complex mixtures of components, and it is possible that the E. coli and Salmonella strains used in this study are distinctly sensitive to their toxic, mutagenic, or antimutagenic effects. It is known, for example, that limonene, a monoterpene present in EO of sage and some other plants, is carcinogenic for male F344/N rats, but there is no evidence of carcinogenicity.
ANTIMUTAGENIC EFFECT OF ESSENTIAL OIL OF SAGE

169

in female F344/N rats or in B6c3F1 mice of both sexes (http://ntp-apps.niehs.nih.gov/). This indicates that even in preliminary screening, especially of complex mixtures, more than one test organism should be used.

Escherichia coli antimutagenicity assay

After UV-irradiation of wild type strain SY252, significant reduction of UV-induced revertants in the presence of EO, F1, and F2 was detected (Fig. 4). Mutagenesis inhibition was 40-70% and decreased in the order of EO>F1>F2, as in TA102. However, there was no antimutagenic effect of F3, F4, and F5 at any of the tested concentrations (Fig. 5a,c,e). In the permeable IB112 strain, F3 and F4 showed no antimutagenic effect, whereas F5 was mildly antimutagenic (Fig. 5b,d,f), confirming the antimutagenic potential of sesquiterpenes.

Saccharomyces cerevisiae mutagenicity and antimutagenicity assays

In order to obtain preliminary information about mutagenic and antimutagenic potential of EO and fractions in eukaryotic cells, we included the S. cerevisiae test system in our study. The diploid strain D7 contains the ilv1-92 mutation on both homologous chromosomes. revertants to Ilv prototrophy were formed by reverse mutations or specific suppressor mutations of the base substitution type. The strain is widely used because it permits simultaneous study of mitotic crossings-over and mitotic gene conversions in addition to point mutations (Zimmermann et al. 1975).

According to our results and the classification given by Wall et al. (1988), EO of sage and its fractions can be considered to contain substances with antimutagenic potential against UV-induced and possibly spontaneous mutations. In view of the composition of sage oil and fractions, obvious candidates are α-thujone, 1,8-cineole, camphor, and α-humulene. Available data on antigenotoxic features of these terpenes are limited. Goel et al. (1989) demonstrated that camphor antagonized γ-radiation-induced increase in SCE frequency in mice bone marrow cells. Kim et al. (1992) reported an antimutagenic effect of cineole and camphor against aflatoxin B1 in S. typhimurium TA100. The protective effect of EO of sage and its fractions against UV-C-induced mutations demonstrated in the present study, together with previously
reported data (Simić et al. 1994, 1997, 1998; Mićić et al. 2001; Vujošević et al. 2004), recommend sage terpenes for further antimutagenesis/anticarcinogenesis studies.

Acknowledgements: This research was supported by the Ministry of Science and Environmental Protection of the Republic of Serbia (Project No. 1502). The skilful assistance of Ljubica Lalić is gratefully acknowledged.

REFERENCES


Fig. 5. Effect of F3-F5 of sage on UV-induced mutagenesis in the SY252 and IB112 permeable strain of E. coli antimutagenicity assay. UV-dose 27 J/m². Survival (open symbols), mutagenesis (closed symbols). Number of UV-induced revertants 175±24. *p<0.05 compared with corresponding samples without fractions.


Fig. 6. Effect of EO and F3-F5 of sage on UV-induced mutagenesis in the *S. cerevisiae D7* antimutagenicity assay. UV-dose 130 J/m². Survival (open symbols), mutagenesis (closed symbols). Number of UV-induced revertants 95±14. *p<0.05 compared with corresponding samples without EO and fractions.
АНТИМУТАГЕНИ ЕФЕКАТ ЕТАРСКОГ УЉА ЖАЛФИЈЕ (Salvia officinalis L.) И ЊЕГОВИХ ФРАКЦИЈА НА UV-ИНДУКОВАНЕ МУТАЦИЈЕ У ЋЕЛИЈАМА БАКТЕРИЈА И КВАСЦА

ЈЕЛЕНА КНЕЋЕЋ-ВУКЧЕВИЋ

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Инхибиција спонтаних и UV-индукованих мутација етарским уљем (ЕО) жалфије (Salvia officinalis L.) и његовим фракцијама F1-F5, са различитим уделом моно- и сесквитерпена, испитивана је у Sal

monella/микрозом, E. coli K12 и S. cerevisiae D7 реверзним тестовима. У свим тестовима ЕО, F1 и F2 су показале антимутагени потенцијал према UV-индукованој мутагенези. У зависности од примењеног теста, фракције F3 и F4 су показале токсични, мутагени или антимутагени ефекат. F5 фракција је редуковала спонтану и UV-индуковану мутагенезу само у пропустљивим слојевима E. coli K12. Добијени резултати показују антимутагену активност испарљивих терпена из жалфије и препоручују их за даља истраживања у области антимутагенезе и антиканцерогенезе.