ORIGINAL ARTICLE

Mutagenicity evaluation with Ames test of hydro-alcoholic solution of terpenes

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Key words

Terpenes • Mutagenicity • Ames test

Summary

Mutagenic properties of terpenes (both synthesis and plant derived) have been tested, up to now, as a single molecule. A terpenes containing hydro-alcoholic solution deriving from frankincense and myrrh resins and hyssop essential oil was assayed for mutagenicity by means of Ames test. Extraction technique conducted with electromagnetic fields at room temperature enabled to obtain a solution of free active molecules that did not undergo thermal degradation and characterized by biocidal activity. In order to verify lack of mutagenic hazard in coming into contact with human, the solution was appropriately diluted and tested with Salmonella typhimurium TA98, TA1535 and YG1024 strains, both in absence and in presence of metabolic system S9. For none of the tested conditions a 2-fold increase of induced revertants, as regards to spontaneous, was registered. The ratio between induced and spontaneous His$^+$ revertants (Mutagenic Index) was around 1.00 in all the determinations and no statistically significant differences have been identified comparing the sample and the negative control. A similar result has been obtained for the dose-response curve. In conclusion, we verified that tested terpenes solution lacks of mutagenicity on Salmonella typhimurium with and without metabolic activator so this plant extract can be safely used as biocide.

Introduction

It has been since thousands of years that plants extracts have been used as essences, spices and fragrances and their properties for prevention and treatment of illnesses are known. Plant extracts contain a wide group of molecules, called terpenes, all sharing the same basic structural composition. Traditional methods of extraction, by means of hot temperatures, enable to obtain essential oil solutions (in ethanol or methanol), widely available on the market, where active principles are only partially free to be absorbed.

The hydro-alcoholic solution of terpenes considered in this work has been derived from extraction of *Boswellia sacra* (Burseraceae) resin, *Commiphora myrrae* (Burseraceae) resin and *Hyssopus decumbens* (Lamiaceae) essential oil. They all came from the Arabic peninsula. Obtained solution is the result of a new extraction procedure, called Electromagnetic Field Extraction (E.M.F.EX.).

The change in the extraction process consists in a system that does not employ neither toxic or dangerous chemical solvents nor high temperature or high pressure, as normally used for standard laboratory and industrial methods. Many natural products in particular terpenes, terpenoidis and terpene-like molecular structures are temperature and/or pressure sensitive. A large part of these compounds will have a critical temperature point between 50°C and 85°C. At these temperatures, the compounds can be inactivated or depolymerized forming very small, high reactive oligomers, so the integrity of the extraction will not be preserved. On the opposite, operating at room temperature (23°C) and using a high intensity variable magnetic field allows to obtain pure free molecules in a true solution. The outcome is a liquid without heavy metals and toxic or possible allergenic substances. So the terpenes, that are diluted into the solution, are in a free-like energetic form and totally vaporizable, having a very poor attraction force as these molecules have a very small molecular radius (ranging from 0.1 to 1 nanometre) and a low molecular weight (varying from 136 to 520 Daltons).

Preliminary tests allowed to establish that the hydro-alcoholic solution was characterized by antimicrobial power. Considering this finding and expecting a future employ that requires human exposure, it appeared necessary to verify its safety. In fact, this solution can be considered as a biocide and, before bringing it on the market, it has to fulfill the "precaution principle", as established by Directive 98/8/CE of European Parliament [1] acknowledged from the Italian legislation as the Legislative Decree 174/2000 [2]. Therefore, the efficacy of the solution and its lack of negative effects towards humans, animals and the environment must be verified at the same time. In particular, to establish complete harmless, the product has to be submitted to several assays with the aim of investigating its biological effects and exclude citotoxicity, mutagenicity, carcinogenicity and reproduction toxicity. Ames test is included among the assays to be performed to exclude mutagenicity. On

this basis, we evaluated possible mutagenic activity of the hydro-alcoholic solution of terpenes obtained by E.M.F.EX. extraction method by means of *Salmonella* microsome assay (Ames test) in absence and in presence of metabolic activator S9, verifying if it can exert mutagenic action towards three different strains of *Salmonella typhimurium*. Assessment of the lack of mutagenicity would represent a safety proof since, in the future, the extract could be used as a disinfectant.

Methods

Sample composition

Plant's extract was obtained from *Boswellia sacra* resin, *Commiphora myrrae* resin and *Hyssopus decumbens* essential oil applying E.M.F.EX. extraction method. The solvent was a mixture of distilled water and ethanol 96% (ratio 1:4). Quantification of terpenes was performed by GC-MS GC-FID, using a Hewlett-Packard gas-chromatograph.

Chemicals

Dimethylsulfoxide (DMSO) of high grade purity (99,9% from Merck) was used to dilute the sample and all positive controls. Sodium azide, 2-nitrofluorene, 1,8 dinitropirene, benzo-[*a*]-pyrene, 2-aminoanthracene and aminofluorene, chosen as positive controls, were all purchased from Sigma (Milan, Italy). Purity of all chemicals used in the assays was higher than 95%.

Sample

The sample was a plant's extract. Knowing antimicrobial effect of the solution, preliminary assays have been conducted in order to verify that chosen doses did not inhibit bacterial growth. Briefly, paper discs soaked with different doses of the solution were placed on agar medium uniformly seeded with the different bacterial strains that have been considered in this study. The plates were incubated at 37°C and checked at 24, 48 and 72 hours, in order to verify the absence of inhibition halo. This procedure permitted the identification of a wide range of dilutions starting from 1:750 to 1:100, lacking antimicrobial activity, in order to evaluate possible mutagenicity avoiding toxic or biocidal action towards *Salmonella typhimurium*. The solvent was always DMSO.

Metabolic activation system (S9 mixture)

Lyophilized rat liver S9 fraction induced by Aroclor 1254 was obtained from Moltox (Molecular Toxicology Inc., Boone, NC, USA). The 10% S9 mixture was freshly prepared as described in details by Maron and Ames [3].

Bacterial strains

Mutagenicity testing was performed considering TA98, TA1535 and YG1024 *Salmonella typhimurium* strains. For all assays, an overnight culture in Oxoid Nutrient Broth No. 2 incubated at 37°C was obtained.

Mutagenicity testing

Mutagenic activity of the sample was determined using the plate incorporation technique which preincubation on the *Salmonella typhimurium* strains in the presence or absence of metabolic activation (S9 500 µl/plate) [3]. Briefly, 100 µl of an overnight grown culture, 100 µl of sample (diluted in DMSO), 500 µl of sodium-phosphate buffer (S9-) or 500 µl of S9 mix (S9+), after 30 minutes of preincubation at 37°C, were mixed with 2 ml of top agar which was then poured onto a minimal glucose plate. In each assays, a positive and a negative control were included. Negative controls were assayed with the same volume of the vehicle. The positive controls in the absence of metabolic activator were: 2- nitrofluorene (1 µg/plate) for TA98; sodium azide (1 µg/plate) for TA1535 and 1,8 dinitropirene (1 µg/plate) for YG1024. The positive controls in the presence of metabolic activator were: benzo-[*a*]-pyrene (5 µg/plate) for TA98; 2-aminoanthracene (1 µg/plate) for TA1535 and aminofluorene (0,2 µg/plate) for YG1024. Each determination was made in triplicate and at least two independent experiments have been carried out to confirm the results [4]. Plates were incubated at 37°C for 72 hours in the dark and then revertant His^+ bacteria colonies were scored. The Mutagenic Index (M.I.) was calculated as the ratio between number of histidine revertants induced per plate of the test sample and spontaneous revertants of the negative control. His^+ values obtained for positive and negative control of each strain confirm the results previously achieved in our laboratory [5]. Mutagenic activity results were considered as positive when the following requirements were fulfilled [6, 7]: a) at least for one of tested doses, the ratio between induced and control His^+ is equal or higher than 2; b) a significant response for analysis of variance (ANOVA, $p < 0.05$) could be reached; c) induced revertants versus tested doses created a positive and representative ($p < 0.01$) dose-response curve. Mutagenic activity is expressed as number of His^+ induced revertants (mean ± standard deviation) for all tested doses. In addition, the ratio between induced and spontaneous revertants was calculated as Mutagenic Index.

Statistical analysis

Comparisons between His^+ revertants induced by the sample and spontaneous revertants were performed with ANOVA test for analysis of variance and coefficient regression of dose-response curve. Both tests were performed with Stat View Statistical Software version 5.1 (SAS Institute Inc., USA).

Results

Hydro-alcoholic solution of plants deriving extracts contains, on the whole, 31.847 mg/L of terpenes, mainly monoterpenes (13.889 mg/L) and triterpenes (8.323 mg/L). Main components are listed in Table I.

Sample mutagenicity analyzed according to Ames test on three different *Salmonella typhimurium* strains is shown in Tables II-IV. Considered doses were chosen

Tab. I. Chemical composition of sample (main components determined by GC/MS – GC FID analysis).

Solvent		
	Ethanol	80%
	Distilled water	20%
TERPENES		31.847 mg/L
Monoterpenes	1,8 cineole P-mentanol Linalool α-terpinol α-terpinene Lynalil acetate Isopulegol	13.889 mg/L
Diterpenes	Incensole oxide Incensole acetates (n-octanol and n-octylacetates) Verticiol	4.042 mg/L
Triterpenes	α- β - γ commiphoric acids α-amirin α-boswellic acid Acetyl-β-boswellic acid 11-ketoboswellic acid Acetyl- 11-keto-bosewellacid (AKBA)	8.323 mg/L
Sesquiterpenes	1,3 furanodiene 1,4 furanodiene 2-metossifuranodiene	5.593 mg/L

The solution contains citric acid (15.000 mg/L) as excipient, stabilizer and terpenes synergist.

Tab. II. Mutagenic activity tested with Ames test on TA98 strain in the absence and in the presence of metabolic activator (S9).

TA98	**Induction of revertants**			
Dose	**-S9**		**+S9**	
	Mean ± SD	**Mutagenicity Index (M.I.)**	**Mean ± SD**	**Mutagenicity Index (M.I.)**
0	27 ± 6	–	39 ± 5	–
1:750	27 ± 4	1.00	35 ± 6	0.89
1:500	25 ± 4	0.94	36 ± 8	0.92
1:250	24 ± 2	0.88	42 ± 10	1.08
1:100	23 ± 2	0.84	39 ± 4	1.00
p	0.228		0.324	

Dose 0 (negative control): 100 µl DMSO. Positive controls were 2-nitrofluorene (1 µg/plate): 108 ± 10.8 for S9- and benzo[*a*]pyrene (5 µg/plate): 327 ± 28.4 for S9+. Mutagenicity Index (M.I.) represents ratio of His^+ revertants induced by the sample/spontaneous His^+ revertants induced by negative control.

Tab. III. Mutagenic activity tested with Ames test on TA1535 strain in the absence and in the presence of metabolic activator (S9).

TA1535	**Induction of revertants**			
Dose	**-S9**		**+S9**	
	Mean ± SD	**Mutagenicity Index (M.I.)**	**Mean ± SD**	**Mutagenicity Index (M.I.)**
0	24 ± 6	–	18 ± 3	–
1:750	24 ± 7	1.00	19 ± 6	1.05
1:500	19 ± 8	0.79	17 ± 4	0.95
1:250	24 ± 9	1.00	20 ± 3	1.12
1:100	20 ± 5	0.83	21 ± 4	1.21
p	0.484		0.291	

Dose 0 (negative control): 100 µl DMSO. Positive controls were sodium azide (1 µg/plate): 190 ± 9.3 for S9- and 2-aminoanthracene (1 µg/plate): 196 ± 7.2 for S9+. Mutagenicity Index (M.I.) represents ratio of His^+ revertants induced by the sample/spontaneous His^+ revertants induced by negative control.

Tab. IV. Mutagenic activity tested with Ames test on YG1024 strain in the absence and in the presence of metabolic activator (S9).

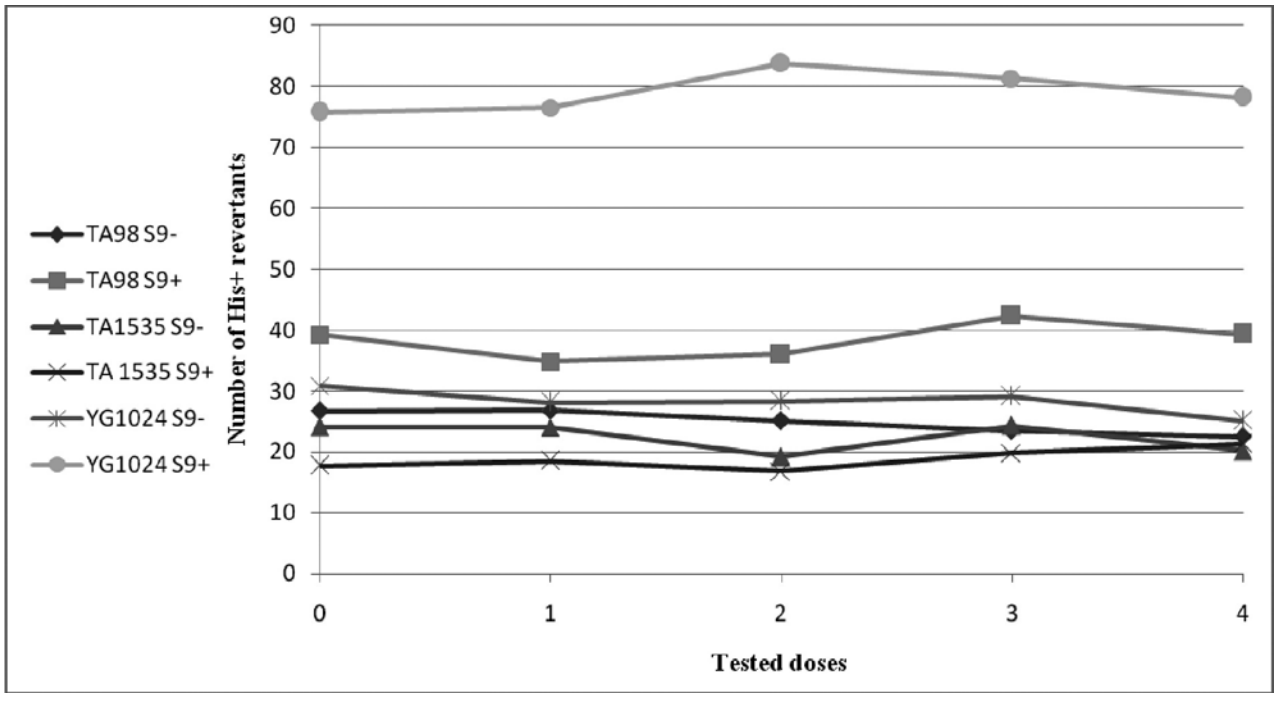
YG1024	Induction of revertants			
Dose	-S9		+S9	
	Mean ± SD	Mutagenicity Index (M.I.)	Mean ± SD	Mutagenicity Index (M.I.)
0	31 ± 3	–	76 ± 11	–
1:750	28 ± 5	0.91	77 ± 13	1.01
1:500	28 ± 5	0.92	84 ± 9	1.11
1:250	29 ± 6	0.95	81 ± 6	1.07
1:100	25 ± 4	0.82	78 ± 8	1.03
p	0.160		0.516	

Dose 0 (negative control): 100 µl DMSO. Positive controls were 1,8 dinitropirene (1 µg/plate): 285 ± 12.4 for S9- and aminofluorene (0,2 µg/plate): 211 ± 16.9 for S9+. Mutagenicity Index (M.I.) represents ratio of His^+ revertants induced by the sample/spontaneous His^+ revertants induced by negative control.

in order to test possible mutagenic activity on bacterial strains excluding high doses that could exert antimicrobial action. In all assays, no toxicity occurred: no reduction in the number of His^+ revertant bacterial colonies and/or thinning of auxotrophic background growth have been noticed. Table II reports mutagenicity tested on *Salmonella typhimurium* TA98 strain. When the sample was assayed without metabolic activator, the number of revertants was always similar or slightly lower the negative control. Likewise, the doses that have been tested in presence of S9 metabolic system exhibited a number of His^+ induced revertants equivalent. In both cases, no statistically significant difference could be found ($p = 0.228$ and $p = 0.324$, respectively). Mutagenicity Index was about 1.00. Mutagenicity assayed on TA1535 *Salmonella typhimurium* strain confirmed similar results, as described in Table III. Direct mutagenic activity was absent, as the number of induced revertants was equal or slightly lower than revertants induced by dose zero ($p = 0.484$). A comparable outcome was provided when the sample has been tested in presence of metabolic activator ($p = 0.291$). No significant differences between revertant's values have been established also for this strain, but, in this case, as indicated by the Mutagenicity Index, values resulted the same or imperceptibly higher than 1.00.

Assessment of mutagenicity on YG1024 *Salmonella typhimurium* strain is presented in Table IV. Sample tested in absence of metabolic activator never reached values of induced His^+ revertants equal or higher as spontaneous, as shown by the Mutagenicity Index which was inferior than 1.00 in all the determinations.

Fig. 1. Dose-dependent mutagenicity towards TA98, TA1535 and YG1024 *Salmonella typhimurium* strains with and without metabolic activator (S9).



As it has been already pointed out for other strains, the presence of metabolic system generated a number of revertant colonies slightly more elevated than only solvent. The comparison between spontaneous and induced revertants failed to achieve a statistically significant difference ($p = 0.160$ without S9 and $p = 0.516$ with S9). In all the conditions that have been tested in our laboratory, no increase in the number of His^+ revertants compared with the solvent was observed, while positive controls always induced a clear mutagenic response. When considering the dose-response curve (Fig. 1), all the tested conditions showed the same flat trend, although mean mutagenicity levels showed differences related to the strain and metabolic system activator presence.

Discussion and conclusios

A solution containing terpenes extracted from *Boswellia sacra* resin, *Commiphora myrrae* resin and *Hyssopus decumbens* essential oil without using hot temperature technique has been assayed for mutagenic potential. The new extraction method allows to obtain molecules that, lacking of bonds, are free to react and exert their activities. This ability enhances its biological effects, in which the killing of microorganisms is included, so resulting particularly interesting regarding future human applications, given the absence of negative outcomes and its favorable action, potentially at higher levels than extracts at present available on the market.

Our study clearly shows that the hydro-alcoholic solution we considered has no mutagenicity in *Salmonella* microsome assay on TA98, TA1535 and YG1024 strains, with or without metabolic activator system. Other recent studies on the same issue reported negative results when investigating mutagenic activity of molecules belonging to terpenes as well. In most cases, the analysis considered a single synthetic molecule belonging to monoterpenes [8] or sesquiterpenes [9, 10] but similar results have been reached. Assay of natural extracts, as the case of triterpenes resulting from bamboo shaving powder [11] or basil essential oil containing mainly the monoterpene linalool [12] achieved similar negative results.

In summary, our data show that the evaluated hydro-alcoholic solution obtained from *Boswellia sacra* resin, *Commiphora myrrae* resin and *Hyssopus decumbens* essential oil applying E.M.F.EX. extraction method has not mutagenic properties in the *Salmonella* assay, confirming lack of mutagenicity for this class of molecules.

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