Antioxidative Action of *Citrus limonum* Essential Oil on Skin

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ABSTRACT

Aims: The purpose of this study was to investigate the action of *Citrus limonum* essential oil to control free radical-induced lipid peroxidation and preventing tissue damage in skin.

Place and Duration of Study: Department of Internal Medicine (University of Roma "Tor Vergata) and A.R.P.A (Aging Research, Prevention and Therapy Association, www.anti-aging.it), between January 2010 and June 2011.

Methodology: The essential oil was subjected to GC-MS analysis. The superoxide anion scavenging activity of essential oil was evaluated by the enzymatic hypoxanthine/xanthine oxidase system. The same oil diluted in DMSO or grape-seed oil was spread on the face of human volunteers after UV exposition. A sample of skin lipids was collected and the presence of peroxyl radicals was detected based on the measurement of light emitted (chemiluminescence) when the excited carbonyl and singlet oxygen decay to ground state.

Results: Our data demonstrate that the lemon essential oil is more active than α-tocopherol against $^{*}O_2^{-}$ and peroxide free radical inhibition at 1:100 dilution. A protocol for controlling free radical-induced lipid peroxidation in human skin was thus proposed.

Conclusion: The scavenging action of lemon essential oil could have a practical application for treating human skin against oxidative damage.

Keywords: anti-aging, GC-MS, grape seed oil, superoxide anion scavenging.

1. INTRODUCTION

The inhibition of lipid oxidation by essential oils such as *Origanum* spp., *Thymus* spp., *Satureja* spp., and *Rosmarinus officinalis*, have already been reported in literature (Estevez, M., Cava, R. (2006); Kulisic et al., (2005); Nakatsu et al., (2000)).

All the essential oils studied have shown a strong phenolic profile characterized by the presence of phenolic monoterpenes which are believed to be the active component of the essential oils (Teissedre, P.L.,Waterhouse, A.L. (2000); Angelini, P. et al., (2006); Angelini,

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P. et al., (2008); Angelini, P. et al., (2009); Pagiotti, R. et al., (2011); Tirillini, B. et al., (2009)). *Citrus* essential oil has also been reported to have antioxidative activities against linoleic acid oxidation (Song, H.S. et al., (2001)) and to induce both Cu^{2+}-induced and 2,2'-azobis(2-aminopropane)hydrochloride-induced oxidation of human low-density lipoprotein *in vitro* (Takahashi, Y. et al., (2003)). Among the compounds tested in *Citrus* essential oil, γ-terpinene had the strongest antioxidant effect (Takahashi, Y. et al., (2003)), but no clear relationship could be shown between the antioxidant activity and the essential oil composition of the extracts (Di Vaio, C. et al., (2010)). When skin is exposed to air that is irradiated by ultraviolet (UV) light consisting of UVA (320-400 nm) and UVB (290-320 nm), reactive oxygen species (ROS) including superoxide anion radical ($*O^{2-}$), hydrogen peroxide (H_2O_2), hydroxyl radical (*OH), singlet oxygen ($*O_2$), lipid peroxides (LOOH), and their radicals (LOO*) are formed. These in turn induce skin aging, phototoxicity, inflammation and malignant tumors (Bech-Thomsen, N., Wulf, H.C. (1995); Kligman, A.M. (1969); Oikarinen,A. et al., (1985); Sakurai, H., et al., (2005); Watson, R.E.B., Griffiths, C.E.M. (2005)).
Recently, consumer interest and the media have focused specifically on products that use natural ingredients, such as plant extracts. There is some evidence that these ingredients could have possible i*n vitro* anti-aging activity, but the question remains whether it is possible to deliver adequate doses to the skin *in vivo*. Lemon oil, traditionally used for its aromatic properties, has recently been investigated for its effects on skin (Chiu, A., Kimbal, A.B. (2003)). The purpose of this study was to investigate the effectiveness of *Citrus limonum* Risso essential oil in controlling free radical-induced lipid peroxidation and preventing tissue damage in skin.

2. MATERIALS AND METHODS

2.1 Plant material

The *Citrus limonum* (lemon) essential oil used in this study was obtained from cold pressed oil extracted from the peel of the fruit according to the methods of Sawamura and Kuriyama (1988).The cold pressed oil was then hydrodistilled for 1h in an all-glass Clevenger apparatus.

2.2 GC and GC-MS Analysis

The GC analyses were carried out using a Varian 3300 instrument equipped with an FID and an HP-InnoWax capillary column (30 m x 0.25 mm, film thickness 0.17 µm), working from 60℃ (3 min) to 210℃ (15 min) at 4℃/min or an HP- 5 capillary column (30 m x 0.25 mm, film thickness 0.25 µm) working from 60℃ (3 min) to 300℃ (15 min) at 4℃/min; The injector and detector temperature was 250℃. Helium was used as the carrier gas, with a flow rate of 1 ml/min, and the split ratio was 1 : 10.
GC-MS analyses were carried out with a Hewlett Packard 5890 GC-MS system operating in the EI mode at 70 eV, using the two above-mentioned columns. The operating conditions were analogous to those reported in the GC analyses section. The injector and transfer line temperatures were 220℃ and 280℃, respectively. He lium was used as the carrier gas, with a flow rate of 1 ml/min, and the split ratio was 1 : 10.

2.3 Identification of the components

The components were identified by matching the spectra with those from mass spectral libraries; the identity of each component was confirmed by comparing the retention indices, from both columns, relative to the C6-C22 n-alkanes, with those from the literature (Adams, R.P. (2001); Davies, N.W. (1990); Heller, S.R., Milne, G.W.A. (1983); Jennings, W.G., Shibamoto, T. (1980); McLafferty, F.W., Staufer, D.B. (1989)). When reported, co-elution gas

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chromatography with reference compounds was also used for an additional confirmation of the compound identity.
The percentage composition of the essential oil was obtained by the normalization method from the GC peak areas, without using correction factors.

2.4 Superoxide anion scavenging ($*O_2^-$)

Superoxide anion was generated by a hypoxanthine-xanthine oxidase system (Arouma, O. et al., (1989)). Reaction mixtures with 100 µl EDTA (30 mmol/l), 10 µl hypoxanthine (30 mmol/l), 100 µl cytochrome c (3 mmol/l) or nitroblue tetrazolium (3 mmol/l) were added to 150 µl of lemon essential oil (solubilized in DMSO 10%) at various concentrations in a final volume of 3 ml buffered in KH_2PO_4 (50 mmol/l), pH 7.4 (Gressier, B. et al., (1993)). The reaction was started by adding 200 µl xanthine oxidase (1U/ml) and the rate of reduced cytochrome c or nitroblue tetrazolium was measured at 550 nm, and 560 nm, respectively, against a reference. The amount of $*O_2^-$ generated was calculated using the extinction coefficient $\varepsilon_{550} = 2.1 \times 10^{-2}$ µmol $^{-1}$ per cm and the $*O_2^-$ inhibition was expressed as percentage values. The sample tested did not interfere with the xanthine oxidase activity (measured at 290 nm). The positive response was tested using α-tocopherol. Ten repetitions were carried out.

2.5 Randomized controlled trial

2.5.1. Subjects

Subjects were selected from among men aged 18 to 52 (mean 33±11) years who were found to have no serious illness on physical checkup at A.R.P.A (Aging Research, Prevention and Therapy Association), www.anti-aging.it (Civita Castellana, VT, Italy). Eighty volunteers (average age: 33±11 years) who gave their written consent to participate in the test were selected as subjects from January 2010 to June 2011.

2.5.2. Extraction of skin lipids from healthy volunteers

Skin lipids were collected with acetone-wetted cotton swabs from the forehead over a 9 cm^2 area from healthy volunteers (80 men, 18–52 years old–mean 33±11) in the morning for 7 days. The sampling procedure was identical for all the subjects. The volunteers were randomly divided into four groups (A, B, C, D). In group A the forehead was treated with α-tocopherol in ethanol (20%), group B with lemon essential oil solubilized in DMSO (1:100), group C with lemon essential oil solubilized in grape-seed oil (1:100), and group D was left untreated. In accordance with the European norm EN 60335-2-27 and under medical supervision, volunteers were irradiated with UVA and UVB of 0.3 W/m^2/mm from sunlamps for 7 min at each session. The participants were asked not to expose themselves to direct sunlight and to avoid the use of face creams or hair lotions for the entire duration of the experiment. Twenty-four hours after the last treatment, the skin lipids were collected.
Extracts were taken twice from the wet cotton swabs using 3 ml of chloroform/methanol (1:2.5) for two hours (10 µg heneicosanoic acid was used for the recovery test). The raw extracts were partitioned between 1% NaCl in 0.01 M HCl and chloroform. The chloroform extracts were washed with methanol/water (1:1) and dried under N_2 stream. The samples were stored at –20C° in 3 ml of chloroform/ethanol (2:1).

2.6 Lipid peroxidation analyzed by chemiluminescence

Chemiluminescence is an index of oxidative stress that quantifies lipid peroxidation and was measured according to the method of Gonzalez-Flecha, B. et al., (1991). This method is

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based on the measurement of light emitted (chemiluminescence) when the excited carbonyl and singlet oxygen produced by peroxyl radicals decay to ground state. This light is due to the generation of reactive oxygen species in whole lipids. Skin lipids were incubated with 3 mM t-BHP for 60 min at 37 C. Lipid peroxidation was initiated by adding a small amount of stock solution of t-butyl hydroperoxide (80 mM) to each vial which was then maintained at 37 C, and measured by monitoring light emission (Wright et al., 1979) with a liquid scintillation analyzer Packard 1900 TR. Chemiluminescence was measured over a 60 min period and recorded as counts per minute (cpm) every 12 min. Each reaction was terminated by adding 5 ml chloroform/methanol (2:1, v/v) containing 0.01% butylated hydroxytoluene (BHT). This also inhibited any further oxidation during the lipid extraction. The DMSO had no antioxidative action and gave a chemiluminescence curve that could be superimposed on to that of the control.

Statistical analysis

Analysis of variance, significances, correlations and other statistical analysis were performed using GraphPad Prism version 5.00, (GraphPad Software, San Diego, California, USA).

3. RESULTS AND DISCUSSION

3.1 Chemical composition of the essential oil

Citrus oils are a mixture of volatile compounds and consist mainly of monoterpene hydrocarbons (Dugo, P. et al., (1998)). Citrus essential oils, on the other hand, generally contain some amount of coumarins or furanocoumarins (Dellacassa, E. et al., (1997)), flavonoids (Miyake, Y. et al., (1997)) and tocopherols (Waters, R.D. et al., (1976)) in the non-volatile fractions of citrus oils. Coumarins and furanocoumarins may have an important role in skin photosensitization. Hydrodistillation of the cold pressed oil prevents this hazard.

Nineteen compounds were identified in the GC and GC/MS analyses. The percentage composition of *Citrus limonum* essential oil is shown in Table 1. The components are listed in the order of elution from the HP-5 column. The main component was limonene (54.6 %) followed by γ-terpinene (19.1 %) and β-pinene (14.5 %). The monoterpene hydrocarbons (87.7 %) constituted the main fraction of lemon oil. This oil composition, as reported in the literature, is similar to other volatile fractions characterized by the high content of limonene (Espina, L. et al., (2011)).

Table 1. Percentage composition of the essential oil from *C. limonum*.

Compound	RI[a]	%
α-pinene	938	3,9
β-pinene	978	14,5
myrcene	993	1,5
α-terpinene	1019	0,3
p-cymene	1024	0,1
limonene	1028	54,6
γ-terpinene	1061	19,1

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terpinolene	1090	0,8
linalool	1098	0,1
citronellal	1154	0,1
terpinen-4-ol	1176	0,1
α-terpineol	1189	0,3
citronellol	1225	0,1
nerol	1230	0,1
neral	1239	1,1
geraniol	1252	0,1
linalyl acetate	1258	0,1
geranial	1269	2,3
geranyl acetate	1383	0,8

[a] *Retention index, relative to C_9-C_{22} n-alkanes on the HP-5 column.*

3.2. *In vitro* and *in vivo* free radical scavenging activity of essential oil

The superoxide anion scavenging activity of *Citrus limonum* essential oils was evaluated using the enzymatic hypoxanthine/xanthine oxidase system. Among the concentrations tested (Fig.1), the 1:100 dilution of lemon essential oil in DMSO had an $*O_2^-$ inhibition that was comparable to that of α-tocopherol. The 1:1000 dilution inhibited $*O_2^-$ less than α-tocopherol but the level of inhibition was about 76% and 65% of the α-tocopherol activity on cytochrome c and tetrazolium nitroblue, respectively.

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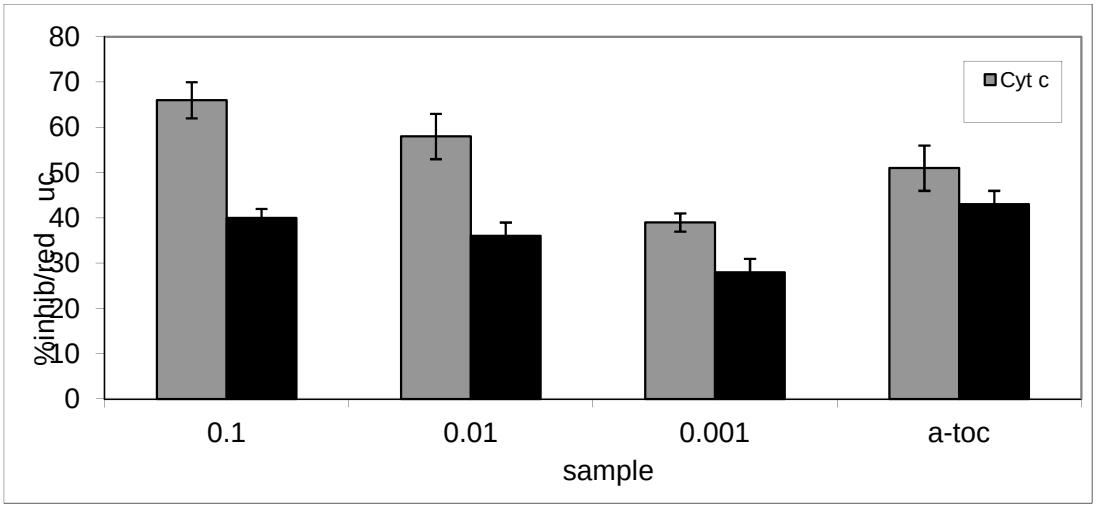

Fig.1. Percentage cytochrome c inhibition and percentage nitroblue tetrazolium reduction. *Test significant from normal control (P < 0.05). Mean ± S.E.M of ten experiments.*

The peroxidation data as evidenced by the light emission are shown in Fig 2.

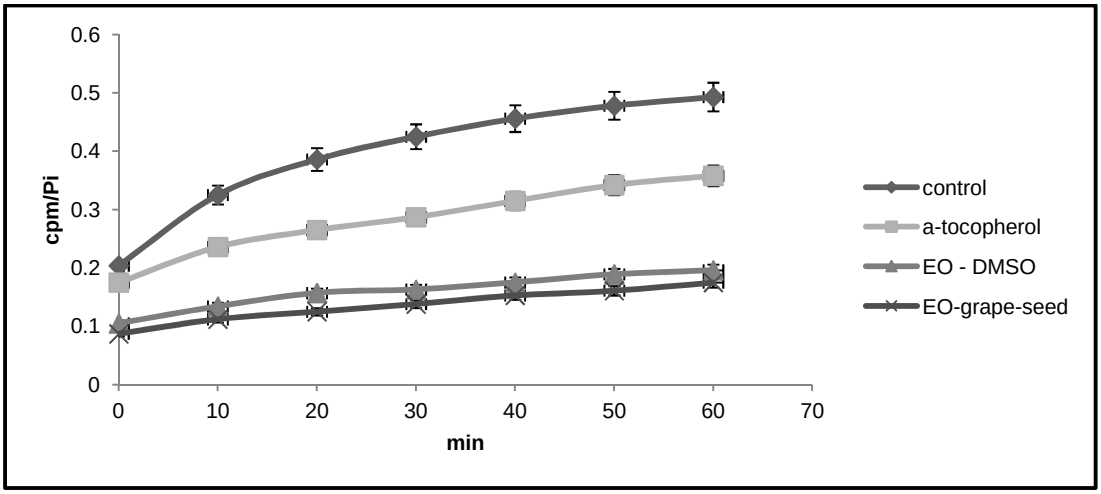

Fig. 2. Chemiluminescence over time in four groups of volunteers. α-tocopherol = group A; EO-DMSO = group B ; EO-grapeseed = group C; control = group D. *Test significant from normal control (P < 0.05). Mean ± S.E.M of twenty experiments*

The lipids from untreated volunteers showed the highest chemiluminescence and are considered to be the normal response to the peroxyl radical action. Lower emissions were recorded for the lipids from volunteers treated with antioxidative substances and the lemon essential oil was more effective than α-tocopherol as an antioxidant. The grape-seed oil showed a slightly higher antioxidative action that was added to the action of lemon essential oil; the chemiluminescence curve is a little lower than that of the lemon essential oil dissolved in DMSO, but the data belong to the same set according to the one-way ANOVA. These results show that these two oils had a similar scavenging action against peroxide free radicals *in vitro* and *in vivo* (Ahn, H.S. et al.,(2002)).

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The exposure of human skin to UV radiation can generate ROS in both the epidermis and dermis. The depth of penetration of UV radiation, as well as its damaging potential in deeper skin cells, have been demonstrated (Katiyar, S.K. et al., (2001)). Among the scavenging substances, α-tocopherol was chosen as a reference for comparing the scavenging action of lemon essential oil. The anti-oxidant activity of oil-in-water emulsion containing α-tocopherol has been reported over a wide range of conditions and test systems (Frankel, E.N. et al., (1994)). Our data demonstrate that the lemon essential oil is more active than α-tocopherol against $*O_2^-$ and peroxide free-radical inhibition at 1:100 dilution. Lemon essential oil is used instead of other lemon extracts, to avoid the toxic action that furanocoumarins have under UV exposure.

4. CONCLUSIONS

The results of this study suggest that lemon essential oil has properties that could benefit human skin as it undergoes environmental and chronological ageing.
The scavenging action of lemon essential oil solubilized in grape-seed oil could have a practical application in aesthetic medicine (a branch of medicine focused on satisfying the aesthetic desires and goals of patients) for treating human skin against oxidative damage. Therefore, continuous application of lemon essential oil solubilized in grape-seed oil might contribute to the prevention of lifestyle-related skin diseases by regulating the balance of oxidative stress.

COMPETING INTERESTS

The Authors declare that no competing interests exist.

AUTHORS' CONTRIBUTION

The work presented here was carried out with the collaboration of all the authors. GB and BT defined the research theme and designed the methods and experiments, analyzed the data, interpreted the results and wrote the paper. PA was involved in the writing process of the manuscript, RV co-designed the experiments, discussed the analyses, interpretation, and presentation of data. All authors have contributed to, seen and approved the manuscript.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

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