Antioxidative Action of *Citrus limonum* Essential Oil *In Vitro* and *In Vivo* on Humane Skin

G. Bertuzzi[1], B. Tirillini[2], P. Angelini [3*], R. Venanzoni[3]

(corresponding author: telephone number +39 0755856323, fax number +39 075 5856404, email paola.angelini@unipg.it)*

[1]Master in Aesthetic Medicine, Department of Medicina Interna, University of Roma "Tor Vergata", Via Montpellier, 1 – 00133 Roma (RM) – Italy

[2]Institute of Botany, University of Urbino, Via Bramante,28 - 61028 Urbino (PU) – Italy

[3]Dpt. of Applied Biology, University of Perugia, Borgo XX giugno 74 - 06121 Perugia (PG) - Italy

.

ABSTRACT

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

Place and Duration of Study: Department of Medicina Interna (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. A sample of skin lipids were collected and the presence of peroxyl radicals were detected based on the measurement of light emitted (chemiluminescence) when the excited carbonyl and singlet oxygen produced by decay to ground state.

Results: Our data demonstrate that the lemon essential oil is more active than □-tocopherol against $^{*}O_2^{-}$ and peroxide free radical inhibition at a 1:100 dilution. An extra activity could be obtained if the lemon essential oil is diluted in grape-seed oil. A protocol for controlling free radical-induced lipid peroxidation in human skin is 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 been reported (Estevez and Cava, 2006; Kulisic et al., 2005; Nakatsu et al., 2000).

* Tel.: +39 075 5856423; fax: +39 075 5856404.
E-mail address: paola.angelini@unipg.it.

All the essential oils studied have shown a strong phenolic profile characterized by the presence of phenolic monoterpenes that are believed to be the active component of the essential oils (Teissedre and Waterhouse, 2000; Angelini et al., 2006; Angelini et al., 2008; Angelini et al., 2009; Tirillini et al., 2009; Pagiotti et al., 2011). *Citrus* essential oil has also been reported to have antioxidative activities against linoleic acid oxidation (Song et al., 2001) and an inhibition of both Cu^{2+}-induced and 2,2'-azobis(2-aminopropane)hydrochloride-induced oxidation of human low-density lipoprotein *in vitro* (Takahashi et al., 2003). Among the compounds tested in *Citrus* essential oil, γ-terpinene had the strongest antioxidant effect (Takahashi et al., 2003). When skin is exposed to the 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 and Wulf, 1995; Kligman, 1969; Oikarinen et al., 1985; Sakurai et al., 2005; Watson and Griffiths, 2005).

Recently, consumer interest and the media have focused specifically on products that use natural ingredients, such plant extracts. There is some evidence that these ingredients could have possible i*n vitro* anti-ageing 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 and Kimbal, 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. MATERIAL AND METHODS

2.1 Plant material

The *Citrus limonum* (lemon) essential oil used in this study was isolated by mechanical pressure of the skin according to the methods of Sawamura and Kuriyama (1988).

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; injector and detector temperatures, 250℃; carrier gas, helium (1 ml/min); split ratio 1 : 10.

GC-MS analyses were carried out using 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. Injector and transfer line temperatures were 220℃ and 280℃, respectively. He lium was used as the carrier gas, flow rate 1 ml/min; split ratio, 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 their retention indices, from both columns, relative to the C6-C22 n-alkanes with those from the literature (Adams, 2001; Davies, 1990; Heller and Milne, 1983; Jennings and Shibamoto, 1980; McLafferty and Staufer, 1989). When reported, co-elution gas chromatography with reference compounds was also used for an additional confirmation of the compound identity.

* Tel.: +39 075 5856423; fax: +39 075 5856404.
E-mail address: paola.angelini@unipg.it.

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 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 et al., 1993). 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 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 (measure at 290 nm). Positive response was tested using α-tocopherol.

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). Forty-five 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 (45 men, 18–52 years old–mean 33±11) in the morning for 7 days. The sampling procedure was identical for all the subjects. A sample of skin lipids was collected before starting the experiment. The volunteers were randomly divided in three groups (A, B, C). In group A the forehead was treated with α-tocopherol in ethanol (20%), group B with lemon essential oil solubilized in DMSO (1:100), and group C with lemon essential oil solubilized in grape-seed oil (1:100). Twenty-four hours after the last treatment, the skin lipids were collected.
The wet cotton swabs were extracted twice with 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 et al. (1991). This method is 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 that was maintained at 37 C and was

* Tel.: +39 075 5856423; fax: +39 075 5856404.
E-mail address: paola.angelini@unipg.it.

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.

3. RESULTS AND DISCUSSION

Botanical extracts are currently among the most common ingredients added to over-the-counter antiageing cosmetic preparations. Various botanical extracts were initially popularized for their possible aromatherapeutic potentials. More recent claims focus on the antioxidant properties of these extracts and their ability to modulate certain types of environmental damage. In this study, *Citrus limonum* essential oil was investigated for its effects on skin.

3.1 Chemical composition of the essential oil

Nineteen compounds were identified in the GC and GC/MS analyses. The percentage composition of the *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.

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
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

* Tel.: +39 075 5856423; fax: +39 075 5856404.
E-mail address: paola.angelini@unipg.it.

geraniol	1252	0,1
linalyl acetate	1258	0,1
geranial	1269	2,3
geranyl acetate	1383	0,8

[a] Retention index, relative to C9-C22 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 were evaluated by 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.

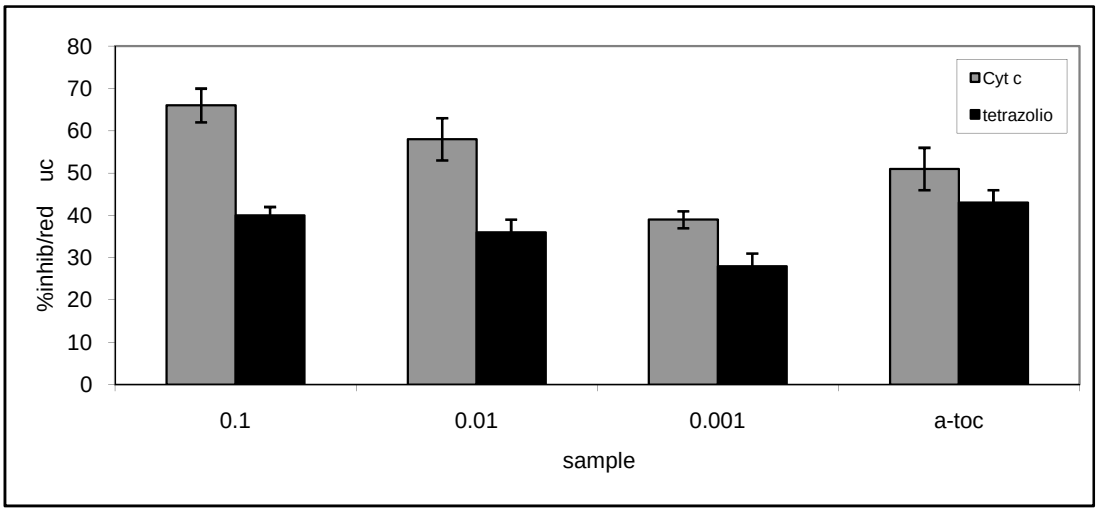

Fig.1. Percentage cytochrome c inhibition and percentage nitroblue tetrazolium reduction.

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

* Tel.: +39 075 5856423; fax: +39 075 5856404.
E-mail address: paola.angelini@unipg.it.

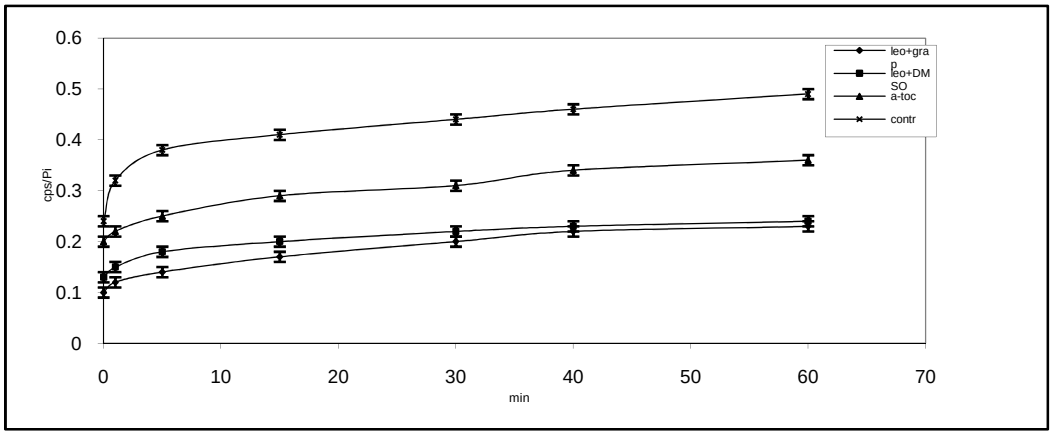

Fig. 2. Chemiluminescence over time

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 an extra 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. This result is in accord with the scavenging action of grape-seed oil tested against peroxide free radical i*n vitro* and *in vivo* (Ahn et al., 2002).

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 has been demonstrated (Katiyar 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 α-tocopherol has been reported over a wide range of conditions and test systems (Frankel 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. Extra activity is obtained if grape-seed oil is used to dilute lemon essential oil. Lemon essential oil is used instead of other lemon extracts, to avoid the toxic action that furanocoumarins have under UV exposure.

4. CONCLUSION

Natural products are in increasing demand from the manufacturers of cosmetics and pharmaceuticals. Thus the importance of conducting studies on essential oils lies not only in the chemical characterization but also in the possibility of linking the chemical contents with particular bioactive functional properties. The results of this study suggests that lemon essential oil have 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. A possible protocol could be: face cleaning with a non-alcoholic detergent, face peeling (salicylic acid and glycolic acid), washing to neutralise acid treatment, face wiping, application of lemon essential oil diluted 1:10 in grape-seed oil. Preliminary tests suggest a frequency of one application every seven days, for five applications and then once every fourteen for another five application. Therefore, continuous application of lemon essential oil

* Tel.: +39 075 5856423; fax: +39 075 5856404.
E-mail address: paola.angelini@unipg.it.

solubilized in grape-seed oil might contribute to the prevention of lifestyle-related diseases by regulating the balance of oxidative stress.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

AUTHORS' CONTRIBUTION

The work presented here was carried out in collaboration between all authors. GB and BT defined the research theme and designed 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 experiments, discusses analyses, interpretation, and presentation. 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.

REFERENCES

Adams, R.P. (2001). Identification of essential oil components by gas chromatography/quadrupole mass spectroscopy. Allured Publishing Corporation.

Ahn, H.S., Jeon, T.I., Lee, J.Y., Hwang, S.G., Lim, Y. ,Park, D.K. (2002). Antioxidative activity of persimmon and grape seed extract: *in vitro* and *in vivo*. Nutr. Res., 1265-1273.

Angelini, P., Pagiotti, R., Menghini, A., Vianello, B. (2006). A antimicrobial activities of various essential oils against foodborne pathogenic or spoilage moulds. Ann. Microb., 56, 65-69.

Angelini, P., Granetti, B., Pagiotti, R. (2008). Effect of antimicrobial activity of *Melaleuca alternifolia* essential oil on antagonistic potential of *Pleurotus* species against *Trichoderma harzianum* in dual culture. World J. Microb. Biotech., 24, 197-202.

Angelini, P., Pagiotti, R., Venanzoni, R., Granetti, B. (2009). Antifungal and allelopathic effects of Asafoetida against *Trichoderma harzianum* and *Pleurotus* spp. Allelopathy Journal, 23, 357-368

Arouma, O., Halliwell, B., Hoey, B.M., Butler, J. (1989). The antioxidant action of N-acetylcysteine: its reaction with H_2O_2, OH*, $OH^{*}_{2}^{-}$, HOCl. F.R.B.M., 6, 593-597.

Bech-Thomsen, N.,Wulf, H.C. (1995). Carcinogenic and melanogenic effects of a filtered metal halide UVA source and a tubular fluorescent UVA tanning source with or without additional solar-simulated UV radiation in hairless mice. Photochem. Photobiol., 62, 773-779.

Chiu, A., Kimball, A.B. (2003). Topical vitamins, minerals and botanical ingredients as modulators of environmental and chronological skin damage. Br. J. Dermatol., 149, 681-691.

Davies, N.W. (1990). Gas chromatographic retention indices of monoterpenes and sesquiterpenes on methyl silicone and Carbowax 20M phases. Cromatogr., 503, 1-24.

Estevez, M., Cava, R. (2006). Effectiveness of rosemary essential oil as an inhibitor of lipid and protein oxidation: contradictory effects in different types of frankfurters. Meat Sci., 72, 348-355.

* Tel.: +39 075 5856423; fax: +39 075 5856404.
E-mail address: paola.angelini@unipg.it.

291 Frankel, E.N., Huang, S.W., Kanner, J. ,German, J.B. (1994). Interfacial phenomena in the
292 evaluation of antioxidants: bulk oils vs emulsions. J. Agric. Food Chem., 42, 1054-
293 1059.
294 Gonzalez-Flecha, B., Llessuy, S.F., Boveris, A. (1991.) Hydroper- oxide-initiated
295 chemiluminescence: an assay for oxidative stress in biopsies of heart, liver and
296 muscle. Free Rad. Biol. Med., 10, 93–100.
297 Gressier, B., Cabanis, A., Lebegue, S., Brunet, C., Dine, T., Luyckx, M., Cazin, M., Cazin,
298 J.C. (1993). Decrease of hypochlorous acid and hydroxyl radical generated by
299 stimulated human neutrophils: comparison in vitro of some thiol-containing drugs.
300 Meth. Find. Exp. Clin. Pharmacol., 15, 101-105.
301 Heller, S.R., Milne, G.W.A. (1983). EPA/NIH mass spectral data base U. S. Government
302 Printing Office.
303 Jennings, W.G., Shibamoto, T. (1980). *Qualitative* analysis of flavour and fragrance volatiles
304 by glass capillary gas chromatography. Academic Press.
305 Katiyar, S.K., Afaq, F., Perez, A., Mukhtar, H. (2001). Green tea polyphenol (-)-
306 epigallocatechin-3-gallate treatment of human skin inhibits ultraviolet radiation-
307 induced oxidative stress. J. Carcinog., 22, 287-294.
308 Kligman, A.M. (1969). Early *destructive* effect of sunlight on human skin. J. Am. Med.
309 Assoc., 210, 2377-2380.
310 Kulisic, T., Radonic, A., Milos, M. (2005). Inhibition of lard oxidation by fractions of different
311 essential oils. Grasas y Aceites (Sevilla, Spain), 56, 284-291.
312 McLafferty, F.W., Staufer, D.B. (1989). The Wiley NBS registry of mass spectral data. John
313 Wiley and Sons.
314 Nakatsu, T., Lupo, A.T., Chinn, J.W., Kang, R.K.L. (2000). Biological activity of essential oils
315 and their constituents. Stud. Nat. Prod. Chem., 21, 571-631.
316 Oikarinen, A., Karvonen, J., Uitto, J., Hannuksela, M. (1985). Connective tissue alterations in
317 skin exposed to natural and therapeutic UV-radiation. Photoderm. J., 2, 15-26.
318 Pagiotti, R., Angelini, P., Rubini, A., Tirillini, B., Granetti, B., Venanzoni, R. (2011).
319 Identification and characterisation of human pathogenic filamentous fungi and
320 susceptibility to *Thymus schimperi* essential oil. Mycoses, 54(5):e364-76.
321 Sakurai, H., Yasui, H., Yamada, Y., Nishimura, H., Shigemoto, M. (2005). Detection of
322 reactive oxygen species in the skin of live mice and rats exposed to UVA light: a
323 research review on chemiluminescence and trials for UVA protection. Photochem.
324 Photobiol. Sci., 4, 715-720.
325 Sawamura, M., Kuriyama, T. (1988). Quantitative determination of volatile constituents in the
326 pummelo (*Citrus grandis* Osbeck forma Tosa-buntan). J. Agric. Food Chem., 36,
327 567–569
328 Song, H.-S., Ukeda, H., Sawamura, M. (2001). Antioxidative activities of citrus peel essential
329 oils and their components against linoleic acid oxidation. Food Sci. and Tech. Res.,
330 7, 50-56.
331 Takahashi, Y., Inaba, N., Kuwahara, S., Kuki, W. (2003). Antioxidative effect of citrus
332 essential oil components on human low-density lipoprotein in vitro. Biosc. Biotech.
333 Biochem., 67, 195-197.
334 Teissedre, P.L., Waterhouse, A.L. (2000). Inhibition of oxidation of human low-density
335 lipoproteins by phenolic substances in different essential oils varieties. J. Agric.
336 Food Chem., 48, 3801-3805.
337 Tirillini, B., Pagiotti, R., Angelini, P**.**, Pintore, G., Chessa, M., Menghini L. (2009). Chemical
338 composition and fungicidal activity of the essential oil of *Laserpitium garganicum*
339 from Italy. Chem. Nat. Comp., 45, 103-105.
340 Watson, R.E.B., Griffiths, C.E.M. (2005). Pathogenic aspects of cutaneous photoaging. J.
341 Cosm. Dermat., 4, 230-236.

* Tel.: +39 075 5856423; fax: +39 075 5856404.
E-mail address: paola.angelini@unipg.it.

Wright, J.R., Rumbaugh, R.C., Colby, H.D., Miles, P.R. (1979). The relationship between chemiluminescence and lipid peroxidation in rat hepatic microsomes. Arch. Biochem. Biophys., 192, 344-351.

* Tel.: +39 075 5856423; fax: +39 075 5856404.
E-mail address: paola.angelini@unipg.it.