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Rosmarinus officinalis L.: Chemical Modifications of the Essential oil and Evaluation of Antioxidant and Antimicrobial Activity

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Rosmarinus officinalis essential oil was separated into its hydrocarbon and oxygenated fractions. The major compounds in the hydrocarbon fraction were α-pinene (44.2%), camphene (24.5%), and limonene (11.7%), while in the oxygenated fraction they were 1,8-cineole (37.6%), camphor (16.5%), and bornyl acetate (21.4%). The hydrocarbon fraction was submitted to a hydroformylation process and the antioxidant activity of the product was screened by the DPPH and β-carotene/linoleic acid tests. The hydroformylated fraction maintained the antioxidant activity of the whole oil. The MIC (minimal inhibitory concentration) and the MBC (minimal bactericidal concentration) of the essential oil, hydrocarbon, oxygenated and hydroformylated fractions were also tested on several microorganisms. *Aeromonas sobria* and *Candida* strains were the most susceptible micro-organisms. The hydroformylated fraction exhibited a MBC against *Candida* strains resistant to the other fractions.

Keywords: *Rosmarinus officinalis*, hydrocarbon fraction, oxygenated fraction, hydroformylation, antioxidant, antimicrobial.

The essential oil of *Rosmarinus officinalis* L. has been the subject of several studies and there are many research papers dealing with its chemical composition [1], biological and pharmacological activities [2]. The utilization of this plant is well known in traditional medicine as a tonic, astringent and diuretic. Also important are the antioxidant [3] and antimicrobial properties of the oil [2].

In consideration of the relatively high abundance of this shrub in Sardinia, our research group has performed several studies on the qualitative and quantitative composition of the essential oil with respect to the seasonal collection time, altitude and geographical distribution in the island [4].

The oil was separated into two fractions, the hydrocarbon fraction and the oxygenated fraction. These were characterized and the hydrocarbon phase was submitted to a hydroformylation process, with the aim of comparing the biological activities of the unmodified oil with those of the transformed one.

Hydrodistillation of the plant material gave yellowish oil with a yield of 0.65%. Twenty –four compounds (12 in the oxygenated fraction and 12 in the hydrocarbon fraction) were identified. The compositions of these fractions are given in Table 1, the compounds being listed in order of their elution from an HP-5 column.

The major compounds in the hydrocarbon fraction were α-pinene (44.2%), camphene (24.5%) and limonene (11.7%), whereas in the oxygenated fraction the major compounds were 1,8-cineole (37.6%), bornyl acetate (21.4%), and camphor (16.5%).

Table 1: Percentage composition of the oil of *Rosmarinus officinalis*.

Hydrocarbon Fraction			*Oxygenated Fraction*		
Compound*	**RI****	**Area%**	**Compound***	**RI****	**Area%**
α-Pinene	941	44.2	1,8-Cineole	1032	37.6
Camphene	952	24.5	Linalool	1109	2.5
β-Pinene	983	6.3	α-Campholenal	1132	0.3
Myrcene	1002	3.8	Camphor	1145	16.5
α-Phellandrene	1010	0.8	*trans*-Pinocamphone	1164	0.4
α-Terpinene	1012	0.7	Borneol	1168	9.3
o-Cymene	1022	3.8	Isopinocamphone	1174	2
Limonene	1033	11.7	Terpinen-4-ol	1180	2
γ-Terpinene	1064	0.9	α-Terpineol	1193	2.3
Terpinolene	1093	0.7	Myrtenol	1198	0.5
(*Z*)-Caryophyllene	1409	1.9	Verbenone	1215	5.4
Humulene	1456	0.7	Bornyl acetate	1284	21.2
Total		**100.0**	**Total**		**100.0**

*compounds listed in order of elution; **retention indices relative to *n*-alkane series on a HP-5 column

Table 2: Results of the hydroformylation.

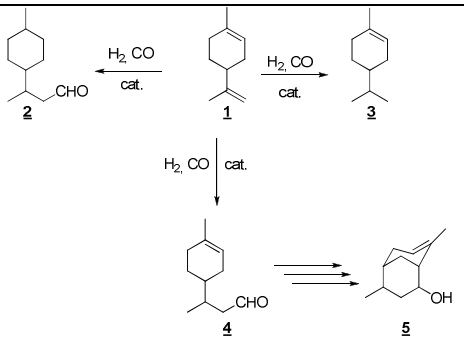
Components before hydroformylation		Components after hydroformylation		
Compound*	**Area%**	**Compound***		**Area%**
1 – Limonene	11.7	**2**	3-(4-Methylcyclo-hexyl)butanal	9.6
		3	*p*-1-Menthene	1.0
		5	4,8-Dimethylbicyclo [3.3.1]non-7-en-2-ol	0.4
6 – β-Pinene	6.3	**7 + 8**	2-((1*S*,2*R*,5*S*)-6,6-Dimethylbicyclo[3.1.1]heptan-2-yl)-ethanal (**7**) and 2-((1*S*,2*S*,5*S*)-6,6-Dimethylbicy-clo[3.1.1]heptan-2-yl)ethanal (**8**)	4.1
		9	2-((1*S*,5*S*)-6,6-Di-methylbicyclo[3.1.1]heptan-2-yl)ethanol	0.7
10 – α-Phellandrene	0.8	**11**	2-(4-Isopropylcyclo-hexyl)ethanal	0.6
12 – Myrcene	3.8	**13**	4,8-Dimethylnon-7-enal	1.0
		14	8-Methyl-4-methyl-enenon-7-enal	1.2
		15	(*Z*)-4,8-Dimethyl-nona-3,7-dienal	0.9
		17	4-(4-Methylpent-3-enyl)cyclopent-1-enecarbaldehyde	0.4
18 – Camphene	24.5	**19**	2-((1*R*,2*S*,4*S*)-3,3-Dimethylbicyclo[2.2.1]heptan-2-yl)ethanal	11.0
		20	2-((1*R*,2*R*,4*S*)-3,3-Dimethylbicyclo[2.2.1]heptan-2-yl)ethanal	10.9
21 – Humulene	0.7	**22**	(1*E*,5*E*)-1,5,8,8-Tetramethylcycloundeca-1,5-diene	0.3
23 – (*Z*)-Caryophyllene	1.9	**24**	(*Z*)-2-(6,10,10-Tri-methylbicyclo[7.2.0]undec-5-en-2-yl)-ethanal	0.9
25 – α-Pinene	44.2	**26**	*cis*-Pinane	0.3
Total	**93.9**	**Total**		**43.3**

The hydroformylation reaction on the hydrocarbon fraction gave an outcome of several compounds, as summarized in schemes 1-8.

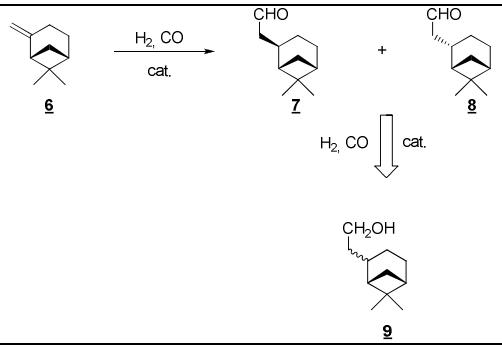
From the results it is evident that external double bonds were more reactive than internal ones. In addition, we observed that hydrogenation of double bonds and reductions of aldehydes occurred under hydroformylation conditions. Scheme 1 shows the outcome of the hydroformylation reaction for limonene (**1**), a component of the hydrocarbon fraction of the oil. Only the most reactive double bond of the molecule (the external one) was transformed to give an aldehyde (**4**), while the internal double bond was preferentially hydrogenated to mentene (**3**). The aldehyde (**4**) underwent rearrangement to (**5**) under hydroformylation conditions. β-Pinene (**6**), in scheme 2, under hydroformylation conditions gave aldehydes (**7**) and (**8**), which were partially reduced to alcohol (**9**).

α-Phellandrene (**10**) was hydroformylated and hydrogenated (scheme 3) at the same time to give the aldehyde (**11**). Hydroformylation occurred at the external double bond and hydrogenation at the double bond in the ring.

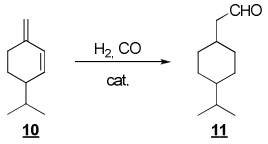
Myrcene (**12**) (scheme 4) underwent hydroformylation to give aldehydes (**13**), (**14**) and (**15**). The aldehyde (**13**) is the result of hydrogenation of the methylene group at position 3, and hydroformylation of the double bond at position 1. This effect is well known and occurs in conjugated dienes with two external double bonds. The aldehyde (**14**) is produced by hydroformylation of myrcene (**12**) at position 1, without hydrogenation of the methylene group at position 3. As an outcome of this reaction, we found an isomerization product (**15**) derived from hydroformylation of the double bond at position 1 and isomerization of the double bond at position 3. Aldehyde (**16**) is the result of hydroformylation of the aldehyde (**14**) at the external double bond. This product is highly reactive and, under hydroformylation conditions, cyclization occurs to form (**17**). Hydroformylation of camphene (**18**) (scheme 5) gave aldehydes (**19**) and (**20**), derived from the exocyclic double bond.



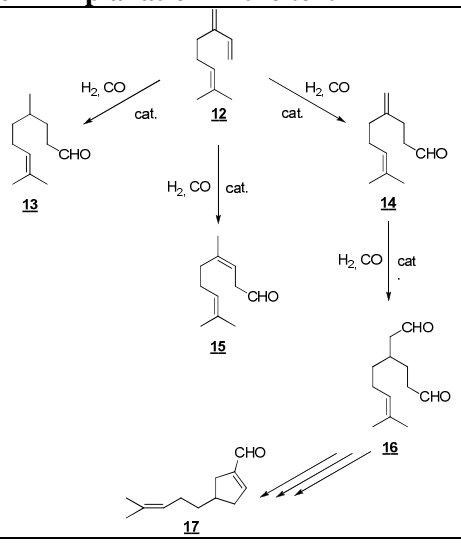
Scheme 1- Explanation in the text



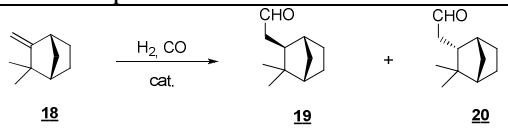
Scheme 2- Explanation in the text



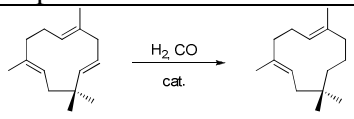
Scheme 3 - Explanation in the text



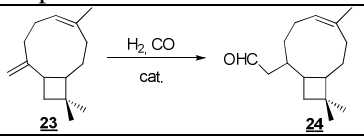
Scheme 4 - Explanation in the text



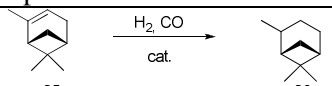
Scheme 5 - Explanation in the text



Scheme 6 - Explanation in the text



Scheme 7 - Explanation in the text



Scheme 8 - Explanation in the text

Partial hydrogenation occurred when humulene (**21**) was hydroformylated (scheme 6) to give the hydrocarbon (**22**), but no trace of aldehydes was detected. Caryophyllene (**23**) (scheme 7), when hydroformylated, gave only one aldehyde (**24**), derived from the reaction of the exocyclic double bond. β-Pinene (**25**), under hydroformylation conditions (scheme 8), gave only the hydrogenated product (**26**) and no aldehydes.

The samples were screened for their antioxidant activity by two complementary test systems: DPPH free radical-scavenging and β-carotene/linoleic acid systems. *R. officinalis* essential oil and its hydroformylated fraction notably reduced the concentration of DPPH free radicals, with no significant difference between them ($P < 0.001$). The values ranged from 55.3 ± 6.5 to 61.1 ± 5.7, respectively and were double the value of Trolox (30.2 ± 3.6).

The lipid peroxidation inhibitory activity of the essential oils and the hydroformylated fraction in the β-carotene bleaching test were consistent with data obtained in the DPPH test. *R. officinalis* essential oil (75.5 ± 8.7) and its hydroformylated fraction (70.3 ± 7.5) performed almost as well as BHT (66.5 ± 5.8).

Table 3: Antimicrobial activity expressed as minimal inhibitory concentration (MIC) and minimal bactericida concentration (MBC).

Microorganism	origin	Essential oil		Hydrocarbon fraction		Oxygenated fraction		Hydroformylated fraction	
		MIC %	MBC%	MIC %	MBC%	MIC %	MBC%	MIC %	MBC%
Aeromonas hydrophila	Fp	2.5^{e}	2.5^{e}	0.15^{a}	>2.5	2.5^{e}	2.5^{e}	2.5^{e}	>2.5
Aeromonas sobria	Fp	0.6^{bc}	>2.5	>2.5	>2.5	0.3^{a}	0.3^{a}	0.3^{a}	0.3^{a}
Candida albicans	Fp	1.25^{d}	1.25^{d}	0.15^{a}	>2.5	0.15^{a}	0.15^{a}	2.5^{e}	2.5^{e}
Candida glabrata	Fp	0.15^{a}	>2.5	0.15^{a}	>2.5	0.15^{a}	0.6^{bc}	2.5^{e}	2.5^{e}
Candida parapsilosis	Fp	2.5^{e}	2.5^{e}	2.5^{e}	2.5^{e}	2.5^{e}	2.5^{e}	2.5^{e}	>2.5
Candida tropicalis	Fp	0.15^{a}	>2.5	0.15^{a}	>2.5	0.15^{a}	1.25^{d}	2.5^{e}	2.5^{e}
Enterococcus faecalis	ATCC 24212	>2.5	>2.5	>2.5	>2.5	2.5^{e}	2.5^{e}	>2.5	>2.5
Escherichia coli	ATCC 35218	>2.5	>2.5	>2.5	>2.5	0.6^{bc}	2.5^{e}	2.5	>2.5
Pseudomonas aeruginosa	Fp	>2.5	>2.5	>2.5	>2.5	0.6^{bc}	2.5^{e}	2.5	>2.5
Salmonella tiphymurium	ATCC 14028	1.25^{d}	>2.5	>2.5	>2.5	>2.5	>2.5	2.5	>2.5
Staphylococcus aureus	ATCC 43300	0.6^{bc}	2.5^{e}	>2.5	>2.5	0.6^{bc}	2.5^{e}	2.5^{e}	2.5^{e}
Staphylococcus epidermidis	Fp	2.5^{e}	>2.5	>2.5	>2.5	1.25^{d}	2.5^{e}	2.5^{e}	2.5^{e}
Vibrio parahaemolyticus	Sb	>2.5	>2.5	>2.5	>2.5	1.25^{d}	2.5^{e}	>2.5	>2.5
Candida albicans s1221	Fp	1.25^{d}	1.25^{d}	1.25^{d}	>2.5	0.5^{ab}	2.5^{e}	0.5^{ab}	2.5^{e}
Candida albicans s 1234	Fp	1.25^{d}	1.25^{d}	>2.5	>2.5	0.5^{ab}	0.5^{ab}	0.5^{ab}	1.25^{d}
Candida albicans s 2314	Fp	1.25^{d}	1.25^{d}	0.15^{a}	>2.5	0.15^{a}	0.15^{a}	>2.5	>2.5
Candida glabrata s 1256	Fp	1.25^{d}	1.25^{d}	>2.5	>2.5	0.5^{ab}	1.25^{d}	0.5^{ab}	1.25^{d}
Candida glabrata s 1324	Fp	1.25^{d}	1.25^{d}	>2.5	>2.5	0.5^{ab}	2.5^{e}	0.5^{ab}	0.5^{ab}
Candida glabrata s 2167	Fp	0.15^{a}	2.5^{e}	0.15^{a}	>2.5	0.15^{a}	0.6^{bc}	2.5^{e}	2.5^{e}
Candida parapsilosis s 4323	Fp	1.25^{d}	1.25^{d}	>2.5	>2.5	0.5^{ab}	1.25^{d}	0.5^{ab}	0.5^{ab}
Candida parapsilosis s 4454	Fp	1.25^{d}	1.25^{d}	>2.5	>2.5	0.5^{ab}	1.25^{d}	0.5^{ab}	1.25^{d}
Candida parapsilosis s 4563	Fp	0.9^{c}	1.5^{d}	0.9^{c}	>2.5	0.5^{ab}	0.5^{ab}	0.5^{ab}	1.25^{d}
Candida parapsilosis s 4578	Fp	2.5^{e}	2.5^{e}	2.5^{e}	>2.5	2.5^{e}	>2.5	2.5^{e}	>2.5
Candida tropicalis s 6651	Fp	1.25^{d}	1.25^{d}	>2.5	>2.5	0.5^{ab}	>2.5	0.5^{ab}	>2.5
Candida tropicalis s 6834	Fp	1.25^{d}	1.25^{d}	>2.5	>2.5	0.5^{ab}	2.5^{e}	0.5^{ab}	1.25^{d}
Candida tropicalis s 8456	Fp	0.9^{c}	2.5^{e}	0.9^{c}	>2.5	0.5^{ab}	>2.5	0.5^{ab}	>2.5
Candida tropicalis s 8790	Fp	0.15^{a}	2.5^{e}	0.15^{a}	>2.5	0.15^{a}	1.25^{d}	2.5^{e}	2.5^{e}

Values having different letters are significantly different from each other using corrected Tukey's LSD test (P<005). Fp = from patients. Sb = soil-borne
The s number were our archieve number.

The mean values for antimicrobial activity are summarized in Table 3. The oil of *R. officinalis* exhibited good bacteriostatic effects against *C. glabrata* (s 2167) and *C. tropicalis* (s 8456) (MIC = 0.15%), *A. sobria* and *S. aureus* (MIC = 0.6%), and *C. parapsilosis* and *C. tropicalis* (MIC = 0.9%). The oil exhibited poor bactericidal activity in general, but there was good bactericidal activity against *Candida* strains, ranging from 1.25 to 2.5%. The hydrocarbon oil fraction exhibited good bacteriostatic effects against *A. hydrophila* (0.15%) and some *Candida* strains (from 0.9% to 2.5%), but the oil had no bactericidal activity.

The oxygenated oil fraction showed bacterostatic effects against all the micro-organisms tested, except *S. typhymurium*. The most susceptible was *A. sobria* (0.3%). Even the bactericidal activity was very good against *A. sobria* (0.3%) and most *Candida* strains. The hydroformylated fraction was less active than the oxygenated oil fraction, but more active than the hydrocarbon fraction. The most susceptible microorganism was *A. sobria* (MIC and MBC = 0.3%). This fraction exhibited positive MBCs for all the microorganisms tested, although some *Candida* strains were resistant. The hydroformylated oil fraction exhibited higher bactericidal activities against some *Candida* strains than either the oil or the untreated fraction tested, such as *C glabrata* (s 1324) (MBC = 0.5 %) and *C. parapsilosis* (s 4563) (MBC = 0.5 %). Hydroformylation of the hydrocarbon oil fraction could, therefore, improve the bactericidal and bacteriostatic action of *R. officinalis* essential oil against some strains otherwise resistant.

Experimental

Oil distillation and yield: *R. officinalis* L. was collected in May and June 2008 in Sardinia (Monte Doglia) near Alghero. Voucher specimens have been authenticated by Prof. Pintore and deposited in the Herbarium SASSA of the Department of Drug Science, University of Sassari under the accession codes 1091.

Fresh plant material was submitted to hydrodistillation using a Clevenger-type apparatus for 1.5 h, yielding a mean of 0.65% of oil calculated on the dry weight. The oil was dried over anhydrous sodium sulfate and stored in sealed vials under

refrigeration prior to analysis. A fraction of the oil was submitted to column chromatography using silica gel as stationary phase. This separation was performed in order to collect the two component groups of the oil, the hydrocarbon fraction and the oxygenated fraction. Each elution was made using 1.5 g of oil eluting with 300 mL of *n*-hexane first and then with 300 mL of ethyl acetate. Subsequent evaporation of the solvent gave the two oil fractions.

Hydroformylation: In a typical run [5-6], the glass vial inside the autoclave was filled under nitrogen purge with the catalytic complex (molar ratio substrate/catalyst=500/1) $Rh(CO)_2(acac)$ with PPh_3 (ratio catalyst/PPh_3 = 0.5) and the substrate (5.0 mmol referred to limonene) in toluene (10 mL). The reactor was closed and pressurized with syngas ($p(CO) = p(H_2)$ = 40 atm) and heated to 80°C for 6 h. After cooling at 25°C, the residual gases were released and the reaction products were characterized, as described elsewhere.

GC analyses: *R. officinalis* essential oil was analyzed using a Varian 3300 instrument equipped with a FID and either an HP-InnoWax capillary column (30 m x 0.25 mm, film thickness 0.17 μm), working from 60°C (3 min) to 210°C (15 min) at 4°C/min, or a HP-5 capillary column (30 m x 0.25 mm, film thickness 0.25 μm) working from 60°C (3 min) to 300°C (15 min) at 4°C/min; injector and detector temperatures, 250°C; 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°C and 280°C, respectively. Helium was used as the carrier gas; flow rate 1 mL/min; split ratio, 1:10.

The identification of the components was made for both columns by comparison of their retention time with respect to *n*-alkanes (C_6-C_{22}). The MS and RI were compared with those of commercial (NIST 98 and WILEY) and home-made library MS built up from pure compounds and MS literature data. The percentage composition of the oil was obtained by the normalization method from the GC peak areas, without using correction factors.

DPPH assay: The hydrogen atom- or electron-donation abilities of the samples were measured by the bleaching of a purple-colored methanol solution of DPPH. This spectrophotometric assay uses the stable radical, 2,2'-diphenylpicrylhydrazyl (DPPH), as a reagent [7,8]. Trolox (1 mM) was used as a synthetic reference. Inhibition of free radical DPPH in percent (I %) was calculated in the following way:

$$I\ \% = (A_{blank} - A_{sample}/ A_{blank}) \times 100,$$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound. Tests were carried out in triplicate.

β-Carotene/linoleic acid assay: In this assay, antioxidant capacity was determined by measuring the inhibition of the volatile organic compounds and the conjugated diene hydroperoxides arising from linoleic acid oxidation [9]. Butylated hydroxytoluene (BHT) was used as positive control. Antioxidative capacities of the extracts were compared with those of the BHT and blank.

Antioxidant activity was calculated as percent inhibition of oxidation versus control sample without sample added, using the equation:

$$\%\ \text{antioxidant activity} = 100 \times [1 - (A_s^0 - A_s^{120})/(A_c^0 - A_c^{120})]$$

where A_s^0 was the absorbance of sample at 0 min, A_s^{120} was the absorbance of sample at 120 min, A_c^0 was the absorbance of control sample at 0 min, and A_c^{120} was the absorbance of control sample at 120 min. Tests were carried out in triplicate.

Micro-organisms: Antimicrobial screening was performed using the general qualitative assay described by Barry [10-11]. A total of 27 strains of bacteria and yeast were investigated; 22 were isolated from patients, one from environmental sources and 4 from the American Type Culture Collection. The bacteriostatic and bactericidal activities were determined by measuring the minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC) of the essential oil performed in microtiter plates using a bacterial inoculum (taken from Luria Berani broth after overnight culture) with a turbidity equivalent to 0.5 MacFarland standard. Values of MICs and MBCs are expressed as percent v/v of total oil and culture medium used as diluent. All micro-organism species were tested in triplicate.

Bacterial species were: *Aeromonas hydrophila* (from patients) (Fp), *Aeromonas sobria* (Fp), *Candida albicans* (Fp), *C. glabrata* (Fp), *C. parapsilosis* (Fp), *C. tropicalis* (Fp), *Enterococcus faecalis* (ATCC 24212), *Escherichia coli* (ATCC 35218), *Pseudomonas aeruginosa* (Fp), *Salmonella typhymurium* (ATCC 14028), *Staphylococcus aureus* (ATCC 43300), *S. epidermidis* (Fp), and *Vibrio parahaemolyticus* (soil-borne). The follow strains of *Candida* were also tested: *C. albicans* (s 1221, s 1234 and s 2314), *C. glabrata* (s 1256, s 1324 and s 2167), *C. parapsilosis* (s 4323, s 4454, s 4563 and s 4578), *C. tropicalis* (s 6651, s 6834, s 8456 ands 8790).

Statistical analysis: Analyses of variance (Anova) followed by LSD post hoc determination were performed. All computations utilized the statistical software SPSS v. 13.

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