Royal jelly lipophilic fraction induces antiproliferative effects on SH-SY5Y human neuroblastoma cells

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Abstract. Royal jelly (RJ) is one the most important bee product because it strongly influences the larval development in the hive, including the queen bee. In literature, RJ is known for its antioxidant, immunoregulatory, antifungal, antibiotical, erythropoietic, hypoglycemic, anticholesteremic, antithyroidic, anti-osteoporotic and estrogenic properties. However, it is surprising how rare the scientific evidence about RJ antineoplastic capacity are. That being said, we investigated, for the first time, the *in vitro* bioactivity of six different RJs on the growth of three different mammalian cell lines: immortalized murine myoblasts (C2C12), human prostate cancer (PC3) and human neuroblastoma (SH-SY5Y). These studies were performed treating the cells with the only lipophilic, or hydrophilic, fraction of the RJs, a scientific approach never performed before. Moreover, chemical and protein profiles of all RJs were finely characterized, in qualitative and quantitative terms, by GC-MS and 1D-SDS-PAGE, respectively, in order to give a complete framework to the research. Despite the deep differences we found in the composition of each sample, unexpectedly, RJs showed comparable or very similar biological effects. In particular, our attention was captured by the extraordinary antiproliferative activity of the lipophilic extract of all RJs against SH-SY5Y cells, suggesting a potential medical application of this bee product to prevent the onset and slow down the growth of human neuroblastoma.

Introduction

Royal jelly (RJ) is an animal secretion produced by hypopharyngeal and mandibular glands of worker honeybees (*Apis mellifera* L.) that are from 5- to 14-day-old. It represents the principal food source for all the larvae in the hive, until the third day of age, and for the queen bee, for the whole life (1). As the queen honeybee is the only fertile organism in the hive, its life expectancies are the highest of the colony and its morphological traits are unique and very peculiar (i.e. big dimensions, reduction of faringeal and wax glands, absence of pollen pocket, growth of pheromonal glands and gonads), it appears clear that a RJ-based diet can strongly influence honeybee's development and destiny, acting on gene expression and metabolism (2).

RJ chemical composition varies according to honeybee species, physiological state of the colony, environmental conditions and production period. Generally, it contains water (50-60%), nitrogen compounds (18%), sugars (15%), lipids (3-6%), mineral salts (1.5%) and traces of vitamins (3). Among nitrogen compounds, free essential amino acids, several enzymes (i.e. glucose oxidases, phosphatases and cholinesterases) and five honeybee typical proteins (major royal jelly proteins, MRJP) were detected (4-7). Fructose is the main carbohydrate (50%), followed by glucose (33-43%), sucrose (6%) and other minor saccharides (8). Finally, although in low concentration, the lipid profile may be considered a fingerprint for RJ. This class of molecules includes short-chain (8C-10C) fatty acids, such as trans-10-hydroxy-2-decenoic acid (10-HDA), non-polar lipids, sterols, including cholesterol, and a non-saponifiable fraction of hydrocarbons (9-11).

Various studies document that RJ possesses many biological properties on murine and human cell systems. In particular, it was demonstrated to have antioxidant (12,13), anti-inflammatory (14), anticholesteremic (15), hypoglycemic, erythropoietic (16), antithyroidic (17), immunostimulatory (18,19), anti-osteoporotic (20), antifungal and antibiotic properties, especially against *Escherichia coli, Salmonella ssp., Proteus ssp., Bacillus subtilis* and *Staphylococcus aureus* (21,22). In addition, Taniguchi *et al* (23) and Shirzad *et al* (24) demonstrated that RJ had an important role in control and regression of murine fibrosarcoma tumors. Of note, Tamura *et al* (25) even suggested that RJ could exert a significant antiproliferative activity against slow-growing cancers but not towards fast-growing ones. However, it is surprising and unexpected to note the scarce scientific evidence reported on RJ antineoplastic power.

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To extend our knowledge on RJ medical potentialities, we investigated the *in vitro* bioactivity of six different RJs on the growth of three mammalian cell lines: immortalized murine myoblasts (C2C12), human prostate cancer (PC3) and human neuroblastoma (SH-SY5Y). In fact, no data are provided in literature on the effect of this matrix on these specific cells. Moreover, the originality of our study also consisted in performing separate analyses on the lipophilic and hydrophilic portions of the RJs, an approach never performed before, in order to better discriminate the biological role of both these fractions. Finally, to establish a complete framework, biochemical and protein profiles of the RJs were deeply characterized by gas chromatography-mass spectrometry (GC-MS) and one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (1D-SDS-PAGE), respectively.

Materials and methods

Sample material. Six different RJs were used in the current study. Three of them (P1, P2 and P3) were bought directly from local producers of RJ, while the others (G1, G2 and G3) were purchased from National Pharmaceutical Societies. The Honey Research Center of the University of Rome 'Tor Vergata' certified freshness, authenticity and quality (i.e. absence of pesticide and antibiotic contamination) of the samples. RJs were stored at 4˚C until their analysis.

GC-MS analysis. For GC-MS study, RJs were separated in two fractions, according to their lipophilicity, as described in Isidorov *et al* (26). Briefly, 500 mg of RJ were resuspended in 10 ml of diethyl ether and constantly mixed for 15 min at room temperature. Then, after centrifugation for 5 min at maximum speed (13.000 rpm) at 4˚C, the supernatant was recovered, filtered by a Millipore 0.45 μm sieve and conserved at 4˚C. On the other hand, the pellet, containing more polar and less lipophilic compounds, was subjected to methanol extraction, following the same identical steps previously performed in the procedure with diethyl ether. Finally, both samples were completely dried out under nitrogen flow and then resuspended with 500 μl of diethyl ether or methanol, respectively. Each extract (1 μl) was injected in a GC-MS instrument (QP2010 Shimadzu, Japan) and analyzed. The chromatographic separation was performed, in a DB-5 column (30 m x 0.25 mm x 0.25 μl; Agilent Technologies, Santa Clara, CA, USA), setting the GC oven as follows: 50˚C for 10 min, 150˚C (reached at a rate of 5˚C/min) for 10 min, 280˚C (reached at a rate of 1˚C/min) for 35 min. Helium was used as carrier gas at a constant flow of 2.1 ml/min. MS conditions and details about the identification of the molecules was the same of those reported in Gismondi *et al* (27). The amount of each compound was expressed as percentage of its relative abundance in the RJ, as described in Giovannini *et al* (28).

Protein study. Lipophilic and hydrophilic protein portions of RJs were purified according to the procedure of Li *et al* (29) adequately modified. In brief, 200 μl of 1X phosphate-buffered saline (PBS) were added to 100 mg of RJ. The solution was vortexed for 5 min, sonicated for further 5 min and centrifuged for 10 min at maximum speed at 4˚C. The supernatant, containing the hydrosoluble protein portion, was recovered, transferred into a new Eppendorf tube and stored at 4˚C. On the contrary, the pellet was resuspended with 200 μl of solubilization buffer (SB: 7 M urea; 2 M thiourea; 4% 3-[(3-cholamido propyl)-dimethylammonio]-1-propane sulfonate; 0.8% IPG-buffer pI 3-10 NL; 1% DTT) and subjected to the same previous passages. This second supernatant, including the liposoluble protein portion, was conserved at 4˚C, while the pellet was discarded. At this point, both the extracts were enriched with 22 μl of 100% trichloroacetic acid and put on ice for 10 min, in order to favour protein precipitation. Then, samples were centrifuged at 4˚C for 10 min at maximum speed. While the supernatants were discarded, the protein pellets, resuspended with 100 μl of SB, were stored at -20˚C until their analysis. Protein quantitation was carried out according to Bradford (30) method, using a specific dye reagent (Quick Start Bradford, Bio-Rad Laboratories, Milan, Italy) and bovine serum albumin as standard (Sigma-Aldrich, Milan, Italy). Finally, protein samples were fractionated through 1D-SDS-PAGE (12% concentrated) for 30 min at 80 V and further 60 min at 120 V. Gels were stained with Coomassie blue (G-250) for 2 h, destained three times for 30 min in methanol, acetic acid and water (2:3:5; v/v/v) and photographed by VersaDoc (Bio-Rad Laboratories) instrument associated to Quantity One software (Bio-Rad Laboratories).

Cell growth assay. Lipophilic and hydrophilic fractions of each RJ were purified as follows. RJ (250 mg) were resuspended in 1 ml of 1X PBS, vortexed for 30 min and centrifuged at maximum speed for 10 min at 4˚C. The supernatant, containing the hydrosoluble portion of RJ, was transferred into a new Eppendorf tube, while the pellet was resuspended in 1 ml of dimethyl sulfoxide (DMSO) and subjected to the same previous protocol, in order to obtain the liposoluble portion of RJ. Hence, the final pellet was discarded, while both the extracts were stored at 4˚C until their application as cell treatment. Tumoral (PC3 and SH-SY5Y) or health (C2C12) mammalian cell lines were cultivated as widely reported in Gismondi *et al* (31). In particular, PC3 and C2C12 were propagated in D-MEM (Dulbecco's modified Eagle's medium), while SH-SY5Y in D-MEM/Ham's F12 (1:1). Cells were equally distributed in 24-well plates and treated, for 24 and 48 h, with 1, 3 or 5 μl of hydrophilic or lipophilic RJ extract per ml of culture medium, which corresponded to the hydrosoluble or liposoluble molecules contained in 250, 750 and 1250 μg of RJ, respectively. Control cells (CNT) were treated with equal volumes of PBS 1X or DMSO for the same times. Cell growth was monitored using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay carried out exactly according to manufacturer's guidelines (Sigma-Aldrich). Results were expressed as percentage variation of the cell proliferation with respect to control (PBS or DMSO CNT), considered as unit (100%).

Statistics. All data were expressed as mean ± standard deviation (s.d.) of the relative results obtained in three independent replicates (n=3). The significance of the analyses was measured by One-way ANOVA test, using PAST software; p-values <0.05 (vs. control) were considered significant.

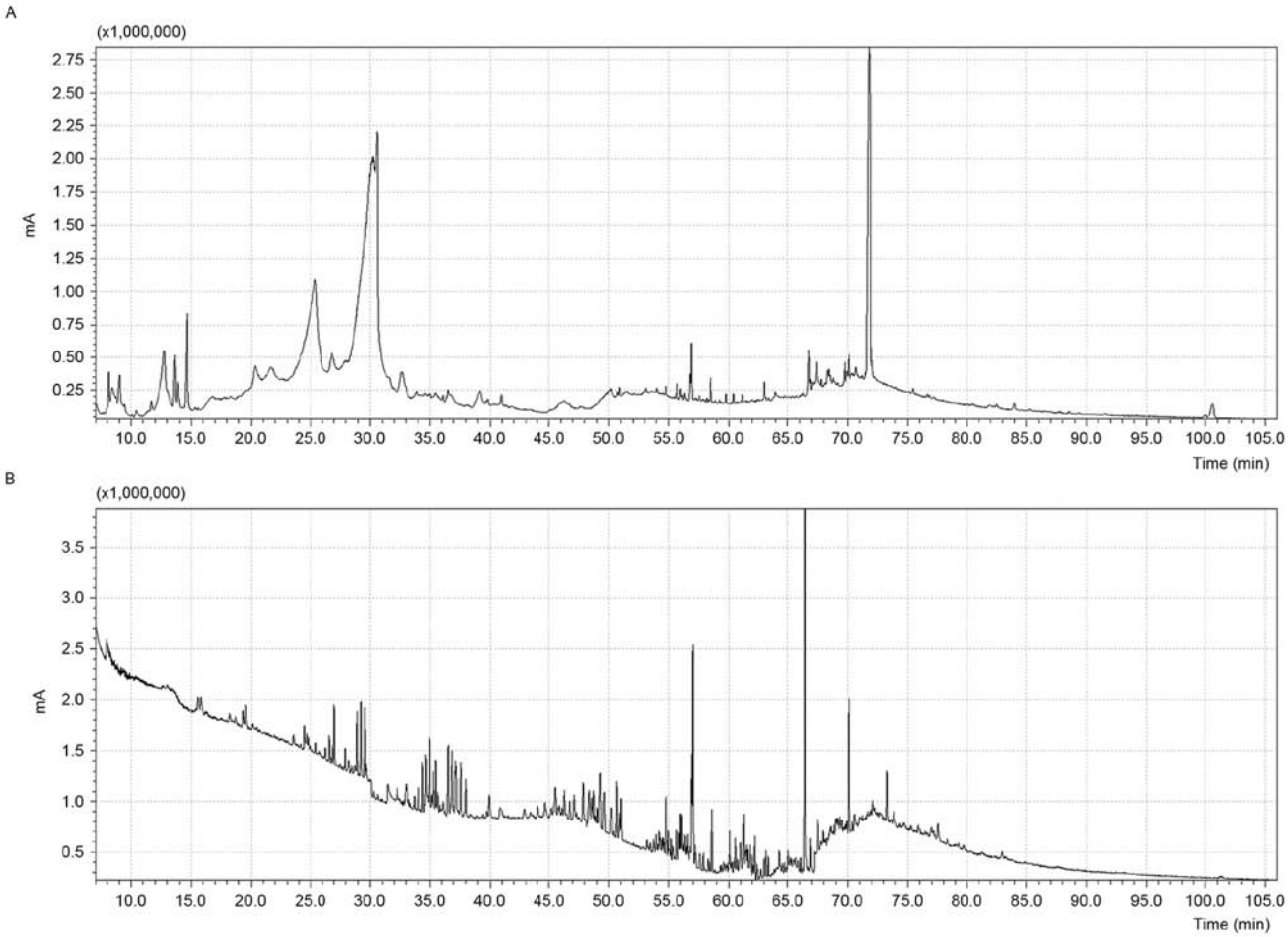

Figure 1. Gas-chromatographic profiles. Chromatograms reporting the chemical profiles of one representative RJ extracted both in diethyl ether (A) and in methanol (B) are shown. In the graphs, the x-axis represents the retention time of the molecules in the column (minutes), while the y-axis is the detected total ion current (milliAmpere, mA).

Results

GC-MS profiles of RJs. GC-MS analysis detected 276 molecules in the diethyl ether extracts of the RJs. On the contrary, the methanol extractions appeared to be richer than the previous ones, containing a total of 348 different compounds. In fact, as shown in Fig. 1, the GC profiles of the methanol extracts presented a higher number of peaks in comparison with the chromatograms obtained analyzing the diethyl ether extracts of the RJs.

The lists of molecules identified both in diethyl ether and in methanol extracts of each RJs are shown in Table I. In this table, the amount of each compound was reported as percentage of relative abundance (% RA) in the RJ. In order to resume our results, all the molecules revealed into the diethyl ether extracts were grouped in four classes of frequency: class 1, including compounds identified in all the RJs; class 2, containing molecules present, at least, in 4 RJs; class 3, presenting rare compounds detectable in 2 or 3 RJs; class 4, clustering all the substances which could be found only in one RJ. According to this classification, in the diethyl ether samples, we detected 4 molecules for the class 1 (eicosane; dodecane 2,6,11-trimethyl; octacosyl trifluoroacetate; phenantrenemetil-2-phenilcinnamato) and 13, 49 and 210 other compounds for the class 2, 3 and 4, respectively (Table I). Likewise, the chemical species identified in the methanol extracts were grouped in similar classes of frequency. In this case, no molecule could be included in class 1, while 15, 66 and 267 compounds were grouped in class 2, 3 and 4, respectively (Table I).

To better describe the mean composition of the RJs, we further classified all the molecules detected in the diethyl ether extracts in different molecular groups as follows: alkanes (25.27%), acids (including fatty acids; 19.92%), aromatic derivatives (17.02%), alcohols (15.94%), alkenes (7.60%), carbohydrates and sugary derivatives (1.81%), quinolinic derivatives (1.45%), oligopeptides and amino acids (1.08%), coumarins (0.72%) and others (i.e. aldehydes, ketones, nitrilic derivatives; 10.50%). In contrast, on the average, the methanol extracts of the RJs contained a high concentration of fatty acids (37.35%) followed by alcohols (13.21%), aromatic derivatives (10.34%), alkanes (5.45%), carbohydrates and sugary derivatives (4.02%), oligopeptides and amino acids (4.02%), alkenes (3.44%), quinolinic derivatives (2.01%), cholesterol derivatives (0.6%) and others (i.e. aldehydes, ketones, nitrilic derivatives; 19.54%).

The diethyl ether extract of the P1 RJ revealed the highest level of 10-HDA (7.41%) of the remaining ones. Moreover, only this sample presented the squalane (1.93%), an ester of the leucin (0.07%), a glycine dipeptide (0.22%) and the decanedioic (0.04%) and myristoleic (0.03%) acids. P1 methanol extract

Table I. Detailed GC-MS profiles of diethylether and methanol extracts of RJs.

RJ	Extract	Class	Molecules (%)
P1	diethyl ether	1	Eicosane (2.89); Dodecane, 2,6,11-trimethyl (3.58); Octacosyl trifluoroacetate (1.62); Phenantrenemetil-2 phenilcinnamato (1.25)
		2	Dodecane, 4,6-dimethyl (6.57); Nonane, 5-(2-methylpropyl) (0.17); 1-Heptanol, 2,4-diethyl (10.90); Undecane, 3,8-dimethyl (1.28);1-Decanol, 2-hexyl (0.14)
		3	Pentanedioic acid, 2,4-dimethyl-, dimethyl ester (0.14); Decane, 2,3,5,8-tetramethyl (0.53); Tetradecane (0.89); 1-Undecene, 7-methyl (9.68); 10-Methylnonadecane (0.91); Nonane, 2,6-dimethyl (0.43); trans-10-Hydroxy-2-decenoic acid (7.41); Octatriacontyl trifluoroacetate (5.43); 2-Methyl-1-undecanol (0.16); Dodecane, 4-methyl (3.13); Tetratriacontyl heptafluorobutyrate (4.20); Heneicosane (0.99); 12-Methyl-E,E-2,13-octadecadien-1-ol (0.06); 10-Undecenal (0.03); 9-Octadecenamide, (Z) (13.23); Triarachine (0.02); 7-Octenoic acid (0.39); Undecylenic Acid (0.04); 13-Tetradecenal (0.01); 11-Dodecenol (0.04); 8-Nonenoic acid (0.01); Oxalic acid, 6-ethyloct-3-yl heptyl ester (0.04)
		4	N-Cbz-glycylglycine p-nitrophenyl ester (0.22); dl-Leucine, N-[(phenylmethoxy)carbonyl] (0.07); 2-Undecene, 4,5-dimethyl-, [R*,R*-(E)] (0.16); Benzaldehyde, 2-methyl (0.61); 9-Decen-1-ol (1.08); 3-Tetradecene, (Z) (0.27); Nonane, 3,7-dimethyl (0.05);Tridecane (0.41); Decane, 3,7-dimethyl (0.24); Decane, 2,3,5,8-tetramethyl (0.53); Cyclooctane, methyl (0.11); 3-Ethyl-3-methylheptane (0.25); 1-Heptanol, 2-propyl (0.67); Octane, 2,3,6,7-tetramethyl (0.16); trans-2-undecenoic acid (0.55); 9-Decenoic acid (0.12); Tetracosane, 11-decyl (0.66); Butylated Hydroxytoluene (7.48); Tricosyl trifluoroacetate (2.72); trans-2-Decenoic acid (0.02); 5-Eicosene, (E) (0.04); 9-Hexadecenoic acid (0.07); Oleic Acid (0.04); 10-Hydroxydecanoic acid (0.14); Myristoleic acid (0.03); Decanedioic acid (0.04); Methyl 11,14-eicosadienoate (0.02); E-12-Tetradecen-1-ol (0.02); trans-2-Dodecenoic acid (0.02); l-(+)-Ascorbic acid 2,6-dihexadecanoate (0.02); Oleanitrile (0.42);1,2-Benzenedicarboxylic acid, diisooctyl ester (4.69); Squalane (1.93)
	methanol	2	2-Furancarboxaldehyde, 5-methyl (3.18); 2-Propanone, 1-(acetyloxy) (0.20); 2,5-Dimethyl-4-hydroxy-3(2H)-furanone (1.98); 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl (7.42); 1,6-Heptadiene, 2-methyl-6-phenyl (2.15); Decanoic acid, 2-hydroxy (1.63); 2-Furanmethanol (1.88); 2-Cyclopentene-1,4-dione (1.68); 2,4-Dihydroxy-2,5-dimethyl (4.09); Glutaconic anhydride (0.28); 2-Furancarboxaldehyde, 5-(hydroxymethyl (18.60)
		3	4-Hepten-3-one, 4-methyl (0.03); 2,5-Furandione, dihydro-3-methylene (0.04); Phenol, 3,5-bis(1,1-dimethylethyl) (0.47); 2(3H)-Furanone, 5-methyl-(0.98); trans-2-Decenoic acid (0.36); 9-Decenoic acid (0.33); 10-Undecenal (0.23); 17-Pentatriacontene (0.11); 8-Nonenoic acid (0.04); 9-Octadecenamide, (Z) (0.86); Octadecanamide (0.51); Dodecanoic acid, 1-methylethyl ester (0.46); Erucylamide (24.70); 1-Octacosanol (0.11)
		4	7-Hexadecenoic acid, methyl ester, (Z) (0.09); 2-Nonene, (E) (0.12); 9-Decen-1-ol (0.38); 1-(3H-Imidazol-4-yl)-ethanone (0.44); Butyrolactone (0.07); Cyclohexanone (5.39); Octanoic acid, 2-amino (0.09); cis-Pentenedioic acid (0.03); 2-Coumaranone (0.02); Acetic acid, pentyl ester (0.03); 6-Octen-2-one, (Z) (0.02); Propanoic acid, 2-methyl-, 2-propenyl ester (0.03); Malonic acid, di(2-methylpent-3-yl) ester (0.03); Undecanoic acid, 10-hydroxy-11-piperidin-1-yl-, propyl ester (0.03); Propan-2-ol, 1-(2-methyl-4-nitroimidazol-5-ylthio)-3-(1-piperidyl) (0.01); L-Glucose (0.15); D-Alanine, N-propargyloxycarbonyl-, isohexyl ester (0.03); l-Alanine, n-propargyloxycarbonyl-, pentyl ester (0.04); Propanoic acid, 3-hydroxy-, methyl ester (0.01); Malonic acid, ethyl 3-hexyl ester (0.01); Nonanal (0.17); Glutamine (0.01); Glycine, N-methoxycarbonyl-, isohexyl ester (0.01); L-Leucyl-L-alanine (0.01); Furan, 2,5-dihydro-2,5-dimethyl (0.16); beta.-Alanine, N-acryloyl-, isohexyl ester (0.40); beta.-Alanine, N-acryloyl-, isobutyl ester (0.16); Acetic acid, 2-propyltetrahydropyran-3-yl ester (0.10); Succinic acid, ethyl 4-heptyl ester (0.01); alpha.-D-Galactopyranose, 2-(acetylamino)-2-deoxy (0.06); Octanoic acid, cyclohexyl ester (0.01); Pentanoic acid, 2-acetyl-4-methyl-, ethyl ester (0.01); Pentanedioic acid, 2,4-dimethyl-, dimethyl ester (0.05); 2-Pentenoic acid, 4-methyl-, ethyl ester, (E) (0.01); Imidazole, 1-hydroxymethyl-2-nitro (2.06); Hexanoic acid, 3-hydroxy-5-methyl-, methyl ester (0.02); Propanedioic acid, dipropyl ester (0.19); 2-Furancarboxylic acid, pentadecyl ester (0.10); 1,3,5-Benzenetriol (0.90); Butanoic acid, 2-(1-oxobutyl)hydrazide (0.01); 9-Decen-2-one (0.01); N-Methyl-3-hydroxymethylpyrrolidin-2-one (2.90); Phenol, 2,6-bis(1,1-dimethylethyl)-4-methyl-, methylcarbamate (0.16); Dianhydromannito (l1.22); Phthalic acid, di-(1-hexen-5-yl) ester (0.21); 9-Octadecenoic acid (Z)-, phenylmethyl ester (0.03); Ribitol, 1,2,3,4,5-pentakis-O-(trimethylsilyl) (0.39); (4-Methyl-1-methylenepent-4-enyl)benzene (0.29); D-Fructose, 1,3,4,5,6-pentakis-O-(trimethylsilyl)-, O-methyloxime (0.36);d-Galactose, 2,3,4,5,6-pentakis-O-(trimethylsilyl)-, o-methyloxyme (0.18); Isoquinoline, 1-methyl (0.09); Benzene, (1,1-dimethyl-2-butynyl) (0.06); 5-Methyl-1-phenylbicyclo[3.2.0]heptane (0.65); 1-Tetradecanol (0.05); 16-Octadecenoic acid, methyl ester (0.05); 1,3-Cyclopentadiene, 5-(trans-2-ethyl-3-methylcyclopropylidene) (0.40); Benzene, 1-(1-buten-3-yl)-2-vinyl (0.03); 1,8-Nonadien-3-yne, 2,8-dimethyl-7-methylene (0.50); Nonane, 3-methyl-5-propyl (0.07); 1-Tetradecen-3-yne (0.06); Pentanoic acid, 2-methyl-, butyl ester (0.12); Palmitic acid .beta.-monoglyceride (3.37); Octadecanoic acid, octadecyl ester (0.06); 1,3,5-Cycloheptatriene, 7-ethyl (0.09); cis-13-Eicosenoic acid, methyl ester (0.20); Hexanoic acid, octyl ester (0.10); Indole-1-carboxylic acid, 4-amino-, methyl ester (0.12); Glycyl-L-tryptophylglycine (0.06); 1,2-Benzenedicarboxylic acid, diisooctyl ester (0.12); 1,2-Propanediol, 3-(hexadecyloxy)-, diacetate (0.07); 1-Heptadec-1-ynyl-cyclopentanol (0.07); D-Fructose, 3-O-[2,3,4,6-tetrakis-O-(trimethylsilyl)-.alpha.-D-glucopyranosyl] (0.48); Glycine, N-butoxycarbonyl-, propyl ester (0.43); Octadecanoic acid, 2,3-dihydroxypropyl ester (0.71); d-Ribose, 2-deoxy-bis(thioheptyl)-dithioacetal (0.24); Pentadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester (0.56); Cholest-8-en-3.beta.-ol, acetate (0.15); Cholesta-8,24-dien-3-ol, 4-methyl-, (3.beta.,4.alpha.) (0.15); Propanoic acid, 3,3'-thiobis-, didodecyl ester (1.26); Hexadecanoic acid, 15-methyl-, methyl ester (0.19)
P2	diethyl ether	1	Eicosane (10.27); Dodecane, 2,6,11-trimethyl (0.46); Octacosyl trifluoroacetate (7.39); Phenantrenemetil-2 phenilcinnamato (0.35)
		2	Dodecane, 4,6-dimethyl (3.98); 2-Isopropyl-5-methyl-1-heptanol (2.64); Nonane, 5-(2-methylpropyl) (0.55); 1-Heptanol, 2,4-diethyl (3.10); Undecane, 3,8-dimethyl (0.46); 1-Decanol, 2-hexyl (0.54); Nonane, 5-methyl-5-propyl (0.09); 2-Propenoic acid, tridecyl ester (0.98); 10-Undecen-1-ol (0.30); 1,6-Heptadiene, 2-methyl-6-phenyl (4.72); Diethyl Phthalate (2.30); Benzonitrile, m-phenethyl (29.41); Tetratetracontane (3.14)
		3	Octanoic Acid (1.17); Pentanedioic acid, 2,4-dimethyl-, dimethyl ester (0.10); Decane, 2,3,5,8-tetramethyl (0.08); 1-Decene, 3,3,4-trimethyl (0.08); 8-Nonen-2-one (3.02); Tetradecane (0.48); Heptadecane, 2,6,10,15-tetramethyl (3.95); Nonane, 3-methyl-5-propyl (0.14); Hexadecane (0.03); Cyclohexane, 1-ethyl-2-propyl (0.17); 10-Methylnonadecane (2.70); Pentadecane (0.94); 1,2-Diphenyl-1-isocyanoethane (2.88); Isotridecanol (0.83); Quinoline 2-(2-methylpropyl) (1.02); 5-(1-Phenyl-cyclopentyl)-[1,3,4]oxadiazole-2-thiol (0.48); Styrene (3.16); Heptane, 2,5,5-trimethyl (0.22); 3-Butyn-1-ol (0.14); 2,5-Hexanediol, 2,5-dimethyl (0.45); 1-Octanol, 2,7-dimethyl (0.21); Nonane, 2,6-dimethyl (0.10); 1-Tridecene (0.24); Benzene, 1,3-bis(1,1-dimethylethyl) (1.70); 3-Ethyl-3-phenyl-1-pentene (0.58); Oxalic acid, 6-ethyloct-3-yl heptyl ester (0.22)
		4	1-Hexanol, 3,5,5-trimethyl (0.06); 1-Trimethylsilyloxy-2-undecene (0.15); Acetamide, 2-(1-hydroxycyclohexyl (0.05); Decane, 2,3,5-trimethyl (0.12); 2-Hydroxy-8-methyl-nona-2,8-dien-4-one (0.07); 6-Methyl-bicyclo[4.2.0]octan-7-one (0.10); 2-Hydroxy-2,5-dimethyl-hept-6-en-3-one (0.07); Octadecyl trifluoroacetate (0.93); Cyclohexane, 1,4-dimethyl-2-octadecyl (0.03); 6-Methyl-bicyclo[4.2.0]octan-7-one (0.10); 2-Hydroxy-2,5-dimethyl-hept-6-en-3-one (0.07); Disulfide, di-tert-dodecyl (0.44); 13-Docosenamide, (Z) (0.98); Octane, 2,3,7-trimethyl (0.39); Benzene, (1-methylenepropyl) (0.27); N-dl-Alanylglycine (0.09); 1-Decene, 2,4-dimethyl (0.17); Propane, 1-(1,1-dimethylethoxy)-2,2-dimethyl (0.05); 5-Fluoro-3-trifluoromethylbenzoic acid, tridecyl ester (0.09)
	methanol	2	2-Furancarboxaldehyde, 5-methyl (4.61); 2-Propanone, 1-(acetyloxy) (1.01); 2,5-Dimethyl-4-hydroxy-3(2H)-furanone (4.75); 2,3-Dimethylfumaric acid (1.14); 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl (2.34); 1,6-Heptadiene, 2-methyl-6-phenyl (0.64); Decanoic acid, 2-hydroxy (2.62); Diethyl Phthalate (0.21);Benzonitrile, m-phenethyl (1.33); 2-Furanmethanol (3.34); 2-Cyclopentene-1,4-dione (2.62); 2,4-Dihydroxy-2,5-dimethyl (2.18); Glutaconic anhydride (1.42); 2-Furancarboxaldehyde, 5-(hydroxymethyl) (20.20); trans-10-decenoic acid (5.92)
		3	trans-2-undecenoic acid (0.01); 2-Cyclopenten-1-one, 2-hydroxy (3.61); Tetratetracontane (0.25); Oleic Acid (0.02); 7-Octenoic acid (1.79); 12-Methyl-E,E-2,13-octadecadien-1-ol (0.21); Heptadecane, 2,6,10,15-tetramethyl (0.04); Maltol (0.61); Pentadecane, 8-hexyl (0.03); 1,3-Benzodioxin-4-one,4a,5-dimethylperhydro-2-(1,1-dimethylethyl) (0.03); 10-Hydroxydecanoic acid (0.85); 12-Hydroxydodecanoic acid (0.42); 9-Decenoic acid (0.41); 10-Undecenal (0.03); Hexadecanoic acid, methyl ester (0.04); Octanoic Acid (2.38); 5-Acetoxymethyl-2-furaldehyde (1.48); Benzene, 1,1'-(2-pentene-1,5-diyl)bis (0.14); Quinoline, 2-propyl (0.04); Isoquinoline, 1-(2,2-dimethylpropyl) (0.06); Tridecanoic acid, 12-methyl-, methyl ester (0.02); 4,5-Diphenylocta-1,7-diene(meso) (0.07); Butyl citrate (0.18); 17-Pentatriacontene (0.05); 5-(1-Phenyl-cyclopentyl)-[1,3,4]oxadiazole-2-thiol (0.10); Heneicosane (0.12); 9-Octadecenamide, (Z) (0.14); Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-ethyl (0.04); 13-Octadecenal, (Z) (0.10); Octadecanamide (0.24); Dodecanoic acid, 1-methylethyl ester (0.04); 2-Propenoic acid, tridecyl ester (0.10); Erucylamide (4.64); n-Tetracosanol (0.02); 1-Octacosanol (0.03); Phenol, 2,4-bis(1,1-dimethylethyl) (0.26); Ethanone, 1-(2-furanyl) (0.38); 10-Hydroxydecanoic acid, methyl ester (0.50); 2(5H)-Furanone (1.62); 2(5H)-Furanone, 5-methyl (0.49); Propylene Carbonate (0.29); 1,2-Cyclopentanedione, 3-methyl (0.33); 8-Nonen-2-one (1.90); n-Decanoic acid (0.13); 1-Dodecanol (0.22); 10-Undecen-1-ol (1.91)

Table I. Continued.

		4	Butanoic acid, 2-methyl-3-oxo-, ethyl ester (9.55); Furane-2-carboxaldehyde, 5-(4-nitrophenoxymethyl) (0.46); Hydroperoxide, 1-ethylbutyl (0.89); 1-Carbmethoxy-3,3-dimethyldiaziridine (0.35); 2-Furanmethanol, 5-methyl (0.14); 2-Decenoic acid (1.01); Decanoic acid, 3-hydroxy-, methyl ester (0.14); 2H-Pyran-2-one, tetrahydro-6-pentyl (0.15); Oxime-, methoxy-phenyl (0.13); 3-Decenoic acid, (E) (0.09); 3-Hydroxydecanoic acid (1.07); Cyclopropanetetradecanoic acid, 2-octyl-, methyl ester (0.86); 15-Hydroxypentadecanoic acid (0.04); cis-13-Octadecenoic acid (0.01); Oxacyclododecan-2-one (0.01); Oxacyclotridecan-2-one (0.01); Cyclohexylmethyl formate (0.01); 1,11-Undecanediol (0.03); Heptadecanoic acid, heptadecyl ester (0.05); Oxacyclotetradecane-2,11-dione, 13-methyl (0.01); Undecylenic Acid (0.01); Z-8-Methyl-9-tetradecenoic acid (0.01); trans-Traumatic acid (0.01); 3-Decenoic acid (0.05); Azelaic Acid (0.01); Tridecanedioic acid (0.01); 2,4-Pentadien-1-ol, 3-propyl (0.01); Fumaric acid, dec-4-enyl heptadecyl ester (0.01); 13-Tetradecynoic acid, methyl ester (0.01); 1,2-15,16-Diepoxyhexadecane (0.01); Dodecanoic acid, 3-hydroxy (0.01); Phthalic acid, isobutyl cyclohexylmethyl ester (0.15); Benzene, (2-ethyl-4-methyl-1,3-pentadienyl) (0.01); 3,7,11,15-Tetramethyl-2-hexadecen-1-ol (0.02); 3-Octenoic acid, ethyl ester (0.01); 4,4,8-Trimethyl-non-5-enal (0.04); Decane, 5-ethyl-5-methyl (0.04); 10-Undecen-4-one, 2,2,6,6-tetramethyl (0.01); Benzene, 1,3,5-tris(3-methyl-3-butenyl) (0.06); 5-Methyl-Z-5-docosene (0.01); Pentatriacontane, 13-docosenylidene (0.02); d-Ribose, 2-deoxy-bis(thiononyl)-dithioacetal (0.02); Benzene, (2-decyldodecyl) (0.03); 10-Heneicosene, 11-phenyl (0.26); Isoquinoline, 1-isobutyl (0.08); 8,11,14-Docosatrienoic acid, methyl ester (0.02); 2-Propenoic acid, 5-methylene-6-heptenyl ester (0.02); (E)-13-Docosenoic acid (0.03); 1-Heneicosanol (0.02); 13-Docosenoic acid, methyl ester, (Z) (0.04); Z,E-3,13-Octadecadien-1-ol (0.05); 2-Myristynoyl-glycinamide (0.01); L-Ascorbic acid, 6-octadecanoate (0.01); 2-Hexen-1-ol (0.04); Benzeneacetaldehyde (0.59); 2,3-Butanedione, monooxime (1.78); Decanoic acid, 3-methyl (0.38); Butane, 1-propoxy (0.45)
P3	diethyl ether	1	Eicosane (3.18); Dodecane, 2,6,11-trimethyl (0.31); Octacosyl trifluoroacetate (1.92); Phenantrenemetil-2 phenilcinnamato (0.72)
		2	Dodecane, 4,6-dimethyl (0.32); 2-Isopropyl-5-methyl-1-heptanol (1.02); Nonane, 5-(2-methylpropyl) (0.17); 1-Heptanol, 2,4-diethyl (0.61); Nonane, 5-methyl-5-propyl (0.18); 2-Propenoic acid, tridecyl ester (3.33); 10-Undecen-1-ol (0.66); 1,6-Heptadiene, 2-methyl-6-phenyl (11.00); Diethyl Phthalate (5.37); Benzonitrile, m-phenethyl (23.64); Tetratetracontane (0.25)
		3	8-Nonen-2-one (2.93); 1,2-Diphenyl-1-isocyanoethane (2.39); Isotridecanol (0.11); Quinoline, 2-(2-methylpropyl) (0.80); 5-(1-Phenyl-cyclopentyl)-[1,3,4]oxadiazole-2-thiol (0.82); 3-Ethyl-3-phenyl-1-pentene (0.23); trans-10-Hydroxy-2-decenoic acid (1.64); Undecylenic Acid (5.62)
		4	1-Undecene (0.33); 2-(4,5-Dihydro-3-methyl-5-oxo-1-phenyl-4-pyrazolyl)-5-nitrobenzoic acid (0.10); 2,5-Furandione, dihydro-3-methylene (0.05); Butanal, 3-t-butyldimethylsilyloxy (13.46); 6-Methyloctahydrocoumarin (0.11); Octane, 2,4,6-trimethyl (0.17); Phenol, 2,4-bis(1,1-dimethylethyl) (2.05); Decanoic acid, trimethylsilyl ester (1.92); 1-Dodecanethiol (0.40); Benzene, 1,1'-(1,3-propanediyl)bis (0.86); Isophthalic acid, 2-methylprop-2-en-1-yl undecyl ester (0.58); Undec-10-ynoic acid (0.08); 4-Decenoic acid, 3-methyl-, (E) (0.05); 2-Butenedioic acid, 2-methyl-, (Z) (0.06); Carbonic acid, isobutyl octadecyl ester (0.11); 2-Heptenedioic acid. 4-cyclopropyl-, dimethyl ester, (E) (1.09); 1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester (0.53); 1-Hexanol, 5-methyl-2-(1-methylethyl) (0.22); Oxalic acid, bis(6-ethyloct-3-yl) ester (0.08); Heptacosanoic acid, methyl ester (0.52); 4,5-Diphenylocta-1,7-diene(meso) (2.47); Phthalic acid, 4-isopropylphenyl nonyl ester (1.97); Eicosanoic acid (0.51); Benzene, 1,1'-(2-pentene-1,5-diyl)bis (0.36); Propanoic acid, 3-mercapto-, dodecyl ester (0.17); Isoquinoline, 1-propyl (0.48); 1H-Indene, 3-butyl-1-methyl (0.22); Glycerol (1.07); Oxalic acid, 3,5-difluorophenyl tetradecyl ester (0.14); Benzene, 1,2-bis(1-buten-3-yl) (0.10); 4,6-Diphenylocta-1,7-diene(dl) (1.14); Fumaric acid, pent-4-en-2-yl tetradecyl ester (0.11); Butyl citrate (0.84); Octatriacontyl esafluoropropionate (0.18); Octadecanoic acid, 9,10-epoxy-, isopropyl ester (0.10); Benzene, (4-methyl-1-decenyl) (0.17)
	methanol	2	2-Furancarboxaldehyde, 5-methyl (3.34); 2-Propanone, 1-(acetyloxy) (1.17); 2,5-Dimethyl-4-hydroxy-3(2H)-furanone (4.17); 2,3-Dimethylfumaric acid (1.21); 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl (15.77); 1,6-Heptadiene, 2-methyl-6-phenyl (1.30); Decanoic acid, 2-hydroxy (3.36); Diethyl Phthalate (0.32); Benzonitrile, m-phenethyl (5.20); 2-Furanmethanol (2.67); 2-Cyclopentene-1,4-dione (2.16); 2,4-Dihydroxy-2,5-dimethyl (1.84); Glutaconic anhydride (1.51); 2-Furancarboxaldehyde, 5-(hydroxymethyl) (23.92); trans-10-decenoic acid (2.45)
		3	Tetratetracontane (0.12); 7-Octenoic acid (0.74); 2,5-Furandione, 3-methyl (0.47); Heptadecane, 2,6,10,15-tetramethyl (0.02); Maltol (0.73); 17-Octadecynoic acid (0.01); trans-2-Decenoic acid (0.51); 10-Hydroxydecanoic acid (0.36); Hexadecanoic acid, methyl ester (0.08); Octanoic Acid (0.36); 5-Acetoxymethyl-2-furaldehyde (0.38); Benzene, 1,1'-(2-pentene-1,5-diyl)bis (0.15); 1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester (0.23); Isoquinoline, 1-(2,2-dimethylpropyl) (0.09); 2-Propanol, 1-(2-methoxypropoxy) (0.19); 4,5-Diphenylocta-1,7-diene(meso) (0.10); Butyl citrate (1.54); 8-Nonenoic acid (0.01); 5-(1-Phenyl-cyclopentyl)-[1,3,4]oxadiazole-2-thiol (0.14); Isoquinoline, 1-butyl (0.07); Heneicosane (0.07); Propanoic acid, 3-mercapto-, dodecyl ester (0.04); 2-t-Butyl-6-methyl-5-(1-phenylbut-3-enyl)[1,3]dioxan-4-one (0.05); 2-Butanone, 1-(acetyloxy) (0.16); Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-ethyl (0.07); 13-Octadecenal, (Z) (0.04); Butanoic acid, 2,2-diethyl (0.26); Erucylamide (0.04); n-Tetracosanol (0.01); 1-Octacosanol (0.17); Phenol, 2,4-bis(1,1-dimethylethyl) (0.18); Ethanone, 1-(2-furanyl) (0.39); 10-Hydroxydecanoic acid, methyl ester (0.30); 2(5H)-Furanone (1.12); 2(5H)-Furanone, 5-methyl (0.44); Propylene Carbonate (0.40); 1,2-Cyclopentanedione, 3-methyl (7.33); 8-Nonen-2-one (0.44); n-Decanoic acid (0.09); 10-Undecen-1-ol (0.08)
		4	Phenol, 4-(methoxymethyl)-2,6-dimethyl (0.22); 1,4-Dioxane-2,3-diol, diacetate (1.60); Butanoic acid, 4-hydroxy (0.29); Pentanoic acid, 2,2-dimethyl-, ethenyl ester (0.14); Propanoic acid, 3-(acetylthio)-2-methyl (0.07); 4-Nonene (0.30); 2,5-Furandicarboxaldehyde (1.52); Quinoline, 8-hydrazino (1.28); Furan, 2-(2-furanylmethyl)-5-methyl (0.08); 6,7-Dihydro-5H-benzo[1,2,5]oxadiazol-4-one o-(2-fluoro-benzyl)-oxime (0.28); 2(3H)-Furanone, dihydro-4-hydroxy (0.01); 1,2,3-Propanetriol, 1-acetate (3.21); alpha.-D-Galactofuranoside, methyl 2,3,5,6-tetra-O-methyl (0.71); Benzene, 1,1'-(1,3-propanediyl)bis (0.14); Glycine, N-ethoxycarbonyl-, undec-10-enyl ester (0.01); trans-2-Dodecenoic acid (0.02); trans-13-Octadecenoic acid (1.29); 7-Dodecenol (0.01); Undecane, 5-methyl (0.01); Spiro[2.3]hexan-4-one, 5-chloro-6-methyl (0.01); Methyl 7,12-octadecadienoate (0.01); Spirohexan-4-one, 5-chloro-6,6-dimethyl (0.01); 1,4-Dioxaspiro[5.5]undecan-2-one (0.01); Decane, 1,1'-oxybis (0.01); Indole 2-carboxylic acid, 3-[methyl(2-phenylethyl)aminomethyl]-, methyl ester (0.01); l-(+)-Ascorbic acid 2,6-dihexadecanoate (0.05); 3-Dodecanol (0.03); n-Nonadecanol-1 (0.01); 11,14,17-Eicosatrienoic acid, methyl ester (0.02); Octadecanoic acid, methyl ester Stearic acid, methyl ester n-Octadecanoic acid (0.04); 1-(3,4-Dihydro-2-naphthyl)-pyrrolidine (0.01); 1-Heptanol, 2,4-diethyl (0.03); Isotridecanol (0.02); 3-Hexadecanol (0.01); Benzene, (1-propyl-1-nonenyl) (0.01); 9-Hexadecenoic acid, phenylmethyl ester (0.02); Tetracosane (0.10)
G1	diethyl ether	1	Eicosane (16.58); Dodecane, 2,6,11-trimethyl (0.21); Octacosyl trifluoroacetate (13.27); Phenantrenemetil-2 phenilcinnamato (0.54)
		2	Dodecane, 4,6-dimethyl (3.20); 2-Isopropyl-5-methyl-1-heptanol (2.04); 1-Heptanol, 2,4-diethyl (2.10); Undecane, 3,8-dimethyl (1.67); 1-Decanol, 2-hexyl (0.45); 1,6-Heptadiene, 2-methyl-6-phenyl (1.10); Tetratetracontane (4.17)
		3	Pentanedioic acid, 2,4-dimethyl-, dimethyl ester (0.32); 1-Decene, 3,3,4-trimethyl (0.18); Heptadecane, 2,6,10,15-tetramethyl (3.15); Hexadecane (0.46); 1-Undecene, 7-methyl (0.04); trans-10-Hydroxy-2-decenoic acid (0.18); Hexadecane, 2,6,11,15-tetramethyl (0.22); Octatriacontyl trifluoroacetate (1.74); 3-metil-undencenolo (3.64); 1-Dodecanol, 3,7,11-trimethyl (0.22); 2-Methyl-1-undecanol (0.51); Dodecane, 4-methyl (0.06); Tetratriacontyl heptafluorobutyrate (3.75); Heneicosane (2.38); 12-Methyl-E,E-2,13-octadecadien-1-ol (0.02); 10-Undecenal (0.04); 9-Octadecenamide, (Z) (4.07); Triarachine (0.01); 7-Octenoic acid (0.01); Undecylenic Acid (0.01); 13-Tetradecenal (0.02); 11-Dodecenol (0.01); 8-Nonenoic acid (0.01)
		4	Methyl-cinnamene (0.71); 2H-Pyranmethanol, tetrahydro-2,5-dimethyl (0.54); Octane, 3,5-dimethyl (0.31); Octane, 2,3,7-trimethyl (0.40); Octane, 1-ethoxy (0.11); Styrene, .alpha.-ethyl (0.13); 2,5-Hexanediol, 2,5-dimethyl (0.21); p-Octyloxybenzonitrile (0.50); Decane, 3,7-dimethyl (0.08); 2-Nonanone (0.11); Nonanal (0.04); Butanoic acid, 3,3-dimethyl (0.04); 2-Phenyl-1-pentene (0.07); 10-Undecen-1-ol (0.19); Hexane, 3-phenyl (0.19); 2-Undecanone, 6,10-dimethyl (0.04); 1-Tridecene (0.31); Dodecane, 2,7,10-trimethyl (0.08); Dodecane (0.28); Undecane, 4,6-dimethyl (0.10); 1,3-Cyclopentanedicarboxylic acid, dimethyl ester (0.06); 1-Decene, 3,3,4-trimethyl (0.18); Benzene, m-di-tert-butyl (2.41); Tetradecane, 5-methyl (0.04); 1-Octanol, 2-butyl (0.07); Benzeneacetic acid, 2-tridecyl ester (0.06); Nonane, 5-methyl-5-propyl (2.30); Nonane, 2,6,11-trimethyl (0.13); Oxalic acid, 2-ethylhexyl tetradecyl ester (0.12); Decane, 2,3,7-trimethyl (0.03); Decane, 3,8-dimethyl (0.04); Isobutyric acid, tridecyl ester (0.10); 1-Hexadecanol, 2-methyl (0.07); Tetradecanal e C-14 (0.04); 2-Acetyl-2,3-dimethylbutyl acetate (0.08); Dodecane, 2,6,10-trimethyl (0.13); Methyl 2-methylhexanoate (0.33); Heptadecane (2.63); Cyclohexane, 1-ethyl-2-propyl (0.22); Pentadecane (0.57); Tetratriacontyl pentafluoropropionate (1.45); Octatriacontyl pentafluoropropionate (1.97); 10-Dodecenol (0.12); Tetratriacontane (0.30); Hexatriacontane (0.33); Nonahexacontanoic acid (0.10); 1,2-Propanediol, -1,2-diacetyle (3.43); Hexatriacontyl pentafluoropropionate (0.11); Hexatriacontyl trifluoroacetate (7.43); 5-(1-Phenyl-cyclopentyl)-[1,3,4]oxadiazole-2-thiol (0.71); Malonic acid, 2,4-dimethylpent-3-yl isobutyl ester (0.13); 1,5-Hexadiene, 2,5-bis(4-methylphenyl) (1.59); Nonane, 2-methyl-5-propyl (0.07); 1-Tetradecene (0.04); Benzene, (1-methylenepentyl) (0.25); 2-Propenoic acid, oxybis(methyl-2,1-ethanediyl) ester (1.32); 13-Octadecenal, (Z) (0.02); 17-Octadecynoic acid (0.01); 1,5-Hexadiene, 3-methyl (0.02); Cyclopentadecanone, 2-methyl (0.01); 2-Pentene, 1-ethoxy-4,4-dimethyl (0.02); Capric ether (0.10)

Table I. Continued.

	methanol	2	2-Propanone, 1-(acetyloxy) (1.65); 2,3-Dimethylfumaric acid (1.48); 1,6-Heptadiene, 2-methyl-6-phenyl (2.19); Decanoic acid, 2-hydroxy (4.74); Diethyl Phthalate (0.36); Benzonitrile, m-phenethyl (4.13); 2-Furanmethanol (4.18); 2-Cyclopentene-1,4-dione (3.48); trans-10-decenoic acid (4.76)
		3	7-Octenoic acid (2.14); 2,5-Furandione, 3-methyl (0.57); 12-Methyl-E,E-2,13-octadecadien-1-ol (0.44); Benzene, 3-pentenyl-, (E) (0.28); 6-Hepten-1-ol (0.05); Octanoic acid, 7-oxo (0.94); trans-2-Decenoic acid (0.58); 10-Hydroxydecanoic acid (0.34); 12-Hydroxydodecanoic acid (0.07); 9-Decenoic acid (0.58); 10-Undecenal (1.10); Benzene, 1,1'-(2-pentene-1,5-diyl)bis (0.41); 3-Eicosene, (E) (0.05); Quinoline, 2-propyl (0.08); 1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester (0.46); 2-Propanol, 1-(2-methoxypropoxy) (0.35); Tridecanoic acid, 12-methyl-, methyl ester (0.04); Octadecanoic acid (0.54); Butyl citrate (1.50); 5-(1-Phenylcyclopentyl)-[1,3,4]oxadiazole-2-thiol (0.18); Isoquinoline, 1-butyl (0.14); Propanoic acid, 3-mercapto-, dodecyl ester (0.05); 2-t-Butyl-6-methyl-5-(1-phenylbut-3-enyl)[1,3]dioxan-4-one (0.10); 2-Butanone, 1-(acetyloxy) (0.24); Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-ethyl (0.12); 13-Octadecenal, (Z) (0.07); Butanoic acid, 2,2-diethyl (0.47); 2-Propenoic acid, tridecyl ester (1.60); Phenol, 2,4-bis(1,1-dimethylethyl)(1.03); Ethanone, 1-(2-furanyl) (0.35); 10-Hydroxydecanoic acid, methyl ester (0.31); 2(5H)-Furanone (2.13); 2(5H)-Furanone, 5-methyl (0.57); 1,2-Cyclopentanedione, 3-methyl (5.83); 1-Dodecanol (0.07)
		4	Propanoic acid, 2-oxo-, methyl ester (11.81); 4-Ethylbenzoic acid, cyclopentyl ester (0.76); Hydroquinone (4.18); Pentanoic acid, 2,4-dioxo-, methyl ester (0.45); Succinic acid diisopropyl ester (2.82); Butanoic acid, 4-(1,1-dimethylethoxy)-3-hydroxy-, methyl ester (0.34); 2-Butenedioic acid, 2-methyl-, (E) (1.97); dl-Alanyl-l-leucine (3.49); Pentanoic acid, 4-oxo-, ethyl ester (12.52); 5-Hydroxymaltol (3.28); oleic acid hexyl ester (1.48); Hydrocinnamic acid (0.16); 4-Pentenoic acid, 3-hydroxy-, ethyl ester (0.56); Isopropylphosphonic acid, dicylcopentyl ester (1.30); 1-Tetradecene (0.06); 1H-2-Indenone,2,4,5,6,7,7a-hexahydro-3-(1-methylethyl)-7a-methyl (0.03); Phenol, 2-(1,1-dimethylethyl)-4-(1-methyl-1-phenylethyl) (0.32); 2-Myristynoic acid (0.25); Tetradecanoic acid, 12-methyl-, methyl ester (0.10); Pentadecanoic acid (1.08); Quinoline, 2-(2-methylpropyl) (0.19); Nonanoic acid, 9-(3-hexenylidenecyclopropylidene) (0.02); 1-Phenanthrenol, 1,2,3,4,4a,9,10,10a-octahydro-4a-methyl-, (1.alpha.,4a.alpha.,10a.alpha.) (0.02); 9,12,15-Octadecatrienoic acid, (Z,Z,Z) (0.82); Benzene, 1,1'-(2-butene-1,4-diyl)bis (0.04); Propane-1,1-diol diacetate (1.19)
G2	diethyl ether	1	Eicosane (16.08); Dodecane, 2,6,11-trimethyl (0.89); Octacosyl trifluoroacetate (13.68); Phenantrenemetil-2 phenilcinnamato (0.33)
		2	Dodecane, 4,6-dimethyl (2.85); 2-Isopropyl-5-methyl-1-heptanol (0.81); Nonane, 5-(2-methylpropyl) (0.17); 1-Heptanol, 2,4-diethyl (0.57); Undecane, 3,8-dimethyl (0.29); 1-Decanol, 2-hexyl (2.14); Nonane, 5-methyl-5-propyl (0.90); 2-Propenoic acid, tridecyl ester (1.52); 10-Undecen-1-ol (0.15); 1,6-Heptadiene, 2-methyl-6-phenyl (5.86); Diethyl Phthalate (0.62); Benzonitrile, m-phenethyl (13.13); Tetratetracontane (1.09)
		3	1-Decene, 3,3,4-trimethyl (0.12); Heptadecane, 2,6,10,15-tetramethyl (5.21); Nonane, 3-methyl-5-propyl (0.42); Hexadecane (0.35); Cyclohexane, 1-ethyl-2-propyl (0.60); Pentadecane (0.77); Isotridecanol (6.63); Styrene (1.70); Heptane, 2,5,5-trimethyl (2.41); 2,5-Hexanediol, 2,5-dimethyl (0.37); 1-Octanol, 2,7-dimethyl (0.61); 1-Tridecene (0.26); Benzene, 1,3-bis(1,1-dimethylethyl) (2.94);1-Hexanol, 5-methyl-2-(1-methylethyl) (0.15); Benzene, 1,1'-(1,3-propanediyl)bis (0.49); Hexadecane, 2,6,11,15-tetramethyl (0.44); 4,5-Diphenylocta-1,7-diene (0.82); Octatriacontyl trifluoroacetate (2.33); 2-Methyl-4-phenylthiolane, 1-oxide (0.51)
		4	1-Pentanol, 2-ethyl (0.22); 3-Ethyl-3-methylheptane (0.22); 2-Undecene, 4,5-dimethyl-, [R*,S*-(Z)] (0.71); Dodecane (0.24); Hexadecane, 1,1-bis(dodecyloxy) (0.30); Sulfurous acid, hexyl octyl ester (0.14); Nonane, 5-butyl (0.20); Glycerin triacetate (0.53); 8-Nonen-2-one (0.44); Butanoic acid, 2,3-dimethyl-, methyl ester (0.23); Cyclohexanepropanoic acid (0.17); 2,5,5-Trimethyl-3-phenyl-cyclohexanone (0.26); Phenol, 3,5-bis(1,1-dimethylethyl) (2.38); Cyclopentane, 1-pentyl-2-propyl (0.66); Benzene, 3-butynyl (1.23);2-Heptenedioic acid, 4-cyclopropyl-, dimethyl ester (0.50); Phthalic acid, diisobutyl ester (0.29); Bicyclo[4.1.0]heptan-2-ol, 1-phenyl-, endo (0.26); Hexacosyl trifluoroacetate (0.37); Quinoline, 2-propyl (0.28); Quinoline, 2-(2-methylpropyl) (1.03); Heptadecane, 2-methyl (0.24); Cyclotetradecane, 1,7,11-trimethyl-4-(1-methylethyl) (0.20); 3-Hexadecanol (0.68)
	methanol	2	2-Furancarboxaldehyde, 5-methyl (0.27); 2,5-Dimethyl-4-hydroxy-3(2H)-furanone (5.90); 2,3-Dimethylfumaric acid (2.15); 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl (38.22); Benzonitrile, m-phenethyl (10.20)
		3	2-Cyclopenten-1-one, 2-hydroxy (19.64); Tetratetracontane (0.89); 2,5-Furandione, dihydro-3-methylene (0.94); Phenol, 3,5-bis(1,1-dimethylethyl) (0.34); Benzene, 3-pentenyl-, (E) (0.78); Heptadecane, 2,6,10,15-tetramethyl (0.35); Maltol (9.87); Pentadecane, 8-hexyl (0.58); 1,3-Benzodioxin-4-one,4a,5-dimethylperhydro-2-(1,1-dimethylethyl) (0.58)
		4	3-Butyn-1-ol (2.97); Propanoic acid, 2-oxo-, ethyl ester (3.13); 2-Butenethioic acid, 3-(ethylthio)-, S-(1-methylethyl) ester (0.58); N-dl-Alanylglycine (0.15); Hexanoic acid, dodec-9-ynyl ester (0.01); Tritetracontane (0.17); d-Mannitol, 1,1'-O-1,16-hexadecanediylbis (0.10); i-Propyl 9-tetradecenoate (0.07); Octadecanoic acid, ethenyl ester (0.01); 10-Undecen-1-al, 2-methyl (0.01); Propionitrile, 2-(3-fluorophenylhydrazono)-3-imino-3-(4-morpholyl) (0.01); 1,3-Pentadiene, 2,4-di-t-butyl (0.04); 4-Ethyl-trans-3-oxabicyclo[4,4,0]decane (0.01); 10-Methyl-3,4,5,8,9,10-hexahydrooxecin-2-one (0.03); Benzoic acid, 3-methyl-2-trimethylsilyloxy- trimethylsilyl ester (0.02); Stearic acid, 3-(octadecyloxy)propyl ester (0.05); 5-Ethoxy-3,4-dihydro-2H-pyrrole-2-carboxylic acid, ethyl ester (0.29); 4H-Pyran-4-one, 3,5-dihydroxy-2-methyl (1.49); 1-Undecanol (0.13); 1,3-Benzenediol, o,o'-dihexanoyl (0.01)
G3	diethyl ether	1	Eicosane (1.66); Dodecane, 2,6,11-trimethyl (1.14); Octacosyl trifluoroacetate (1.11); Phenantrenemetil-2 phenilcinnamato (0.19)
		2	2-Isopropyl-5-methyl-1-heptanol (0.67); Nonane, 5-(2-methylpropyl) (0.41); 1-Decanol, 2-hexyl (0.30); Nonane, 5-methyl-5-propyl (0.12); 10-Undecen-1-ol (0.41); 1,6-Heptadiene, 2-methyl-6-phenyl (2.64); Diethyl Phthalate (3.87); Benzonitrile, m-phenethyl (43.70)
		3	Octanoic Acid (0.75); Decane, 2,3,5,8-tetramethyl (1.11); 3-Butyn-1-ol (1.47); 3-Ethyl-3-phenyl-1-pentene (0.12); 1-Hexanol, 5-methyl-2-(1-methylethyl) (0.25); Benzene, 1,1'-(1,3-propanediyl)bis (1.40); 4,5-Diphenylocta-1,7-diene (0.46); 2-Methyl-4-phenylthiolane, 1-oxide (0.31); 3-metil-undencenolo (1.55); 1-Dodecanol, 3,7,11-trimethyl (0.15); Oxalic acid, 6-ethyloct-3-yl heptyl ester (0.42)
		4	2-Cyclopenten-1-one, 2-hydroxy (9.22); p-Dioxane, methylene (0.22); 2,5-Furandione, dihydro-3-methylene (0.41); 2,5-Dimethyl-4-hydroxy-3(2H)-furanone (0.07); 2,3-Dimethylfumaric acid (1.26); 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl (12.58); Benzoic acid, o-(trimethylsiloxy) (0.21); Methyl 3-O-acetyl-2,4,6-tri-O-ethyl-.alpha.-d-mannopyranoside (0.65); 1-Undecanol (0.12); 1-Propen-2-ol, acetate (0.77); 1,2-Propadiene-1,3-dione (0.16); 3-Eicosene (0.10); Methyl 2,4-di-O-acetyl-3,6-di-O-methyl-.alpha.-D-glucopyranoside (0.75); Imidazole-1-carboxylic acid, methyl ester (0.16); Phenol, 2,6-bis(1,1-dimethylethyl) (0.94); 1-Dodecanethiol (0.21); 1,2-Diphenyl-1-isocyanoethane (3.21); 1,2-Diphenylcyclopropane (0.42); Cyclopropanecarboxylic acid, 3-(2-carboxy-1-propenyl)-2,2-dimethyl-, [1.alpha.,2.beta.(E)] (0.08); 4-(7-Allyl-7-hydroxy-2,2-dimethyltetrahydro[1,3]dioxolo[4,5-c]pyran-4-yl)-3-methylbut-2-enoic acid (0.15); Decane, 5-ethyl-5-methyl (0.13); Tetradecanoic acid, 12-methyl-, methyl ester (0.25); Benzene, (2-decyldodecyl) (0.42); Phenol, 2-methyl-4-(1,1,3,3-tetramethylbutyl) (0.88); Docosanoic acid, docosyl ester (0.08); 1-Pentacontanol (0.15); 7-Phenyl-6-tridecene (1.08); Acetic acid, 3,7,11,15-tetramethyl-hexadecyl ester (0.07); Benzene, (1,1-dimethyl-2-butynyl) (0.66); 9-Undecenal, 2,6,10-trimethyl (0.05); Malonic acid, 2-heptyl pentadecyl ester (0.15); Benzenebutanoic acid, .alpha.-oxo-, ethyl ester (0.12); Tetracosane, 11-decyl (0.09)
	methanol	2	2-Furancarboxaldehyde, 5-methyl (2.30); 2,5-Dimethyl-4-hydroxy-3(2H)-furanone (1.45); 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl (0.03); Diethyl Phthalate (1.33); Benzonitrile, m-phenethyl (27.67); 2-Furanmethanol (0.06); 2-Cyclopentene-1,4-dione (1.84); 2,4-Dihydroxy-2,5-dimethyl (4.77); Glutaconic anhydride (2.35); 2-Furancarboxaldehyde, 5-(hydroxymethyl) (32.14); trans-10-decenoic acid (0.70)
		3	4-Hepten-3-one, 4-methyl (0.14); trans-2-undecenoic acid (0.01); Oleic Acid (0.29); 2(3H)-Furanone, 5-methyl (1.90); 2,5-Furandione, 3-methyl- (0.85); 6-Hepten-1-ol (0.20); Octanoic acid, 7-oxo (0.09); 17-Octadecynoic acid (0.01)
		4	Pentanoic acid, 2-hydroxy-, methyl ester (0.14); 2-Nonanone, 9-hydroxy (2.52); 2-Propynoic acid, methyl ester (0.03); 2-Hepten-1-ol (0.23); Cyclopentaneundecanoic acid (1.46); DL-Proline, 5-oxo (0.02); Benzene, 3-pentenyl (2.69); Phenol, p-(benzyloxy)-, benzoate (0.43); 1H-Pyrazole-4-carboxylic acid (0.04); Pentanenitrile, 4-methyl (0.01); 3-Cyclohexen-1-ol (0.04); 7-Octen-3-ol, 2,6-dimethyl (0.05); Oxalic acid, 2-methylphenyl pentadecyl ester (0.08); 3-Pyrrolidinecarboxylic acid, 5-oxo-1-(2-pyridinylmethyl) (0.02); 2-Butenoic acid, 4-hydroxy-, methyl ester (0.02); 7-Octene-1,2-diol (0.07); Cyclohexanol, 3,3,5-trimethyl-, trans (0.07); Pentanal, 3-hydroxy-2-methyl (0.01); Nonanal dimethyl acetal (0.02); E-10-Dodecen-1-ol propionate (0.02); 3-Methyl-5-(8-nonenyl)pyrrolizidine (0.05); Pentanoic acid, 2-propyl-, 8-methyl-8-azabicyclo[3.2.1]oct-3-yl ester, endo (0.05); 1,7-Octanediol, 3,7-dimethyl (0.04); Naphtho[1,2-b]furane-2,8-dione-octahydro-9-methyl-3-(3,3-dimethyl-1-piperidylmethyl) (0.03); Pyrimidine-2,4(1H,3H)-dione, 5-amino-6-nitroso (2.41); alpha.-D-Glucose (0.45); 9-Oxabicyclo[4.2.1]nonan-2-ol, acetate (0.05); Undecanal, 2-methyl (0.03); 1,8-Octanediol (0.01); Valeric acid, 3-methylphenyl ester (0.02); Hydrogen azide (0.66); 6-Oxa-bicyclo[3.1.0]hexan-3-one (10.05); l-Proline (0.06)

Gas-chromatographic profiles. For each diethyl ether and methanol extract of the RJs (P1, P2, P3, G1, G2 and G3), the list of molecules identified by GC-MS analysis was shown. For each fraction, all compounds were grouped in 4 classes, according to their detection in the RJ samples (class 1, including compounds identified in all the RJs; class 2, containing molecules present, at least, in 4 RJs; class 3, presenting rare compounds detectable in 2 or 3 RJs; class 4, clustering all the substances which could be found only in one RJ). The percentage of relative abundance of each molecule, with respect to the amount of total compounds revealed in the same sample (100%), was also reported in brackets after each compound. All results represented the mean of three independent measurements. For each compound the maximum s.d. was always lower than 5% of the value corresponding to the relative chromatographic peak area.

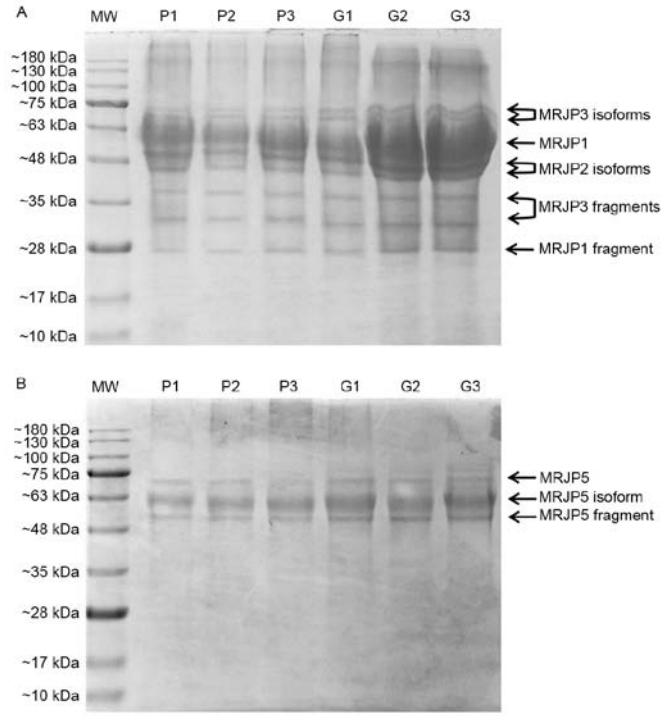

Figure 2. Protein detection. RJ hydrophilic (A) and lipophilic (B) proteins were separated on one-dimensional SDS PAGE and revealed by Coomassie blue staining. Representative gels of three independent replicates, with similar results, are shown. Molecular ladders (MW) were loaded into the gel, together with the RJ samples (P1, P2, P3, G1, G2 and G3), to determine the weight of each protein spot. On the right, the identity of each band was reported.

was characterized by high doses of 2-Furancarboxaldehyde, 5-(hydroxymethyl) (18.60%) and Erucylamide (24.70%). It was also the richest of the RJs in typical molecules (class 4), including 80 different peculiar compounds (see Table I).

P2 sample showed the greatest amounts of 2-Isopropyl-5-methyl-1-heptanol (2.64%), 8-Nonen-2-one (3.02%) and Octanoic acid (1.17%), with respect to all the other diethyl ether extracts. On the other hand, it was the least characteristic of the RJs, according to the low number (n. 19) of specific compounds (class 4) which typified it. The methanol extract of the P2 RJ was rich in 2-furancarboxaldehyde, 5-(hydroxymethyl) (20.20%), 10-HDA (5.92%), 2,5-dimethyl-4-hydroxy-3(2H)-furanone (4.75%), erucylamide (4.64%) and 2-furancarboxaldehyde, 5-methyl (4.61%). Among the substances unique in this sample we found an ester of the butanoic acid (9.55%), the 2-decenoic acid (1.01%), the 3-Hydroxydecanoic acid (1.07%) and traces of ribose and ascorbic acid.

P3 showed a diethyl ether extract which presented the highest concentration of 1,6-heptadiene, 2-methyl-6-phenyl (10.99%) among all the other RJs. Moreover, it typically contained phthalic and butanal derivatives. The methanol preparation of this RJ was rich in 2-furancarboxaldehyde, 5-(hydroxymethyl) (23.92%), 4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl (15.77%) and 1,2-cyclopentanedione, 3-methyl (7.33%). The same extract singularly included trans-13-octadecenoic acid (1.29%), quinoline, 8-hydrazino (1.28%), ascorbic acid (0.05%) a galactofuranoside derivative (0.71%), a pentanoic acid ester (0.14%) and a glycin (0.01%) ester.

Table II. Protein quantitation.

RJ	Hydrosoluble protein content (μg/mg)	Liposoluble protein content (μg/mg)
P1	0.854±0.017	0.262±0.005
P2	0.831±0.024	0.396±0.007
P3	0.967±0.048	0.207±0.006
G1	1.072±0.053	0.313±0.009
G2	1.021±0.020	0.545±0.010
G3	1.174±0.035	0.525±0.026

Hydrosoluble and liposoluble protein content of each RJ (P1, P2, P3, G1, G2 and G3) was reported as μg per mg of sample. Data represent the mean ± s.d. of the results obtained in three different measurements.

The GC profile of the G1 diethyl ether extract revealed the highest level of eicosane (16.58%) and tetratetracontane (4.17%). Sixty-two different molecules, such as methyl-cinnamene and capric ether, typically characterized this sample (class 4, Table I). The methanol extract of the G1 RJ presented the most elevated doses of decanoic acid, 2-hydroxy- (4.74%), 2-furanmethanol (4.18%) and 2(5H)-furanone (2.13%) with respect to the other RJs. Moreover, propanoic acid (11.81%) and pentanoic acid (12.52%) esters represented the most abundant specific markers of this same sample.

The diethyl ether extracts of the G2 and G3 RJs showed the higher concentration of octacosyl trifluoroacetate (13.68%) and benzonitrile, m-phenethyl (43.70%), respectively. In particular, G2 sample was typified by the presence of 3-butynyl-benzene, 3,5-bis(1,1-dimethylethyl)-phenol and 2-(2-methylpropyl)-quinoline, while G3 preparation was characterized by high doses of 2-Cyclopenten-1-one, 2-hydroxy- (9.22%) and 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl- (12.58%). The G2 methanol extract revealed elevated amounts of 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl- (38.22%), benzonitrile, m-phenethyl (10.20%) and 2-cyclopenten-1-one, 2-hydroxy (19.64%). The same extract singularly included 3-butyn-1-ol (2.97%), N-dl-alanylglycine (0.15%), Stearic acid ester (0.05%) and a mannitol derivative (0.1%). On the other hand, the methanol extract of the G3 RJ was rich of benzonitrile, m-phenethyl (27.67%), 2-furancarboxaldehyde, 5-(hydroxymethyl) (32.14%) and glutaconic anhydride (2.35%). Finally, 6-oxa-bicyclo-hexan-3-one, 5-amino-6-nitroso-pyrimidine-2,4(1H,3H)-dione, 3-pentenyl-benzene, 9-hydroxy-2-nonanone and cyclopentaneundecanoic acid were the compounds that could be identified only in G3 sample.

Protein quantitation and analysis. Hydrosoluble and liposoluble protein portions of the RJs were purified as reported in Materials and methods and then quantified by Bradford method (30). As shown in Table II, all the RJs contained a greater amount of hydrosoluble proteins than liposoluble ones. In particular, the highest and the lowest contents of hydrophilic proteins were detected in G3 (1.174 μg/mg) and

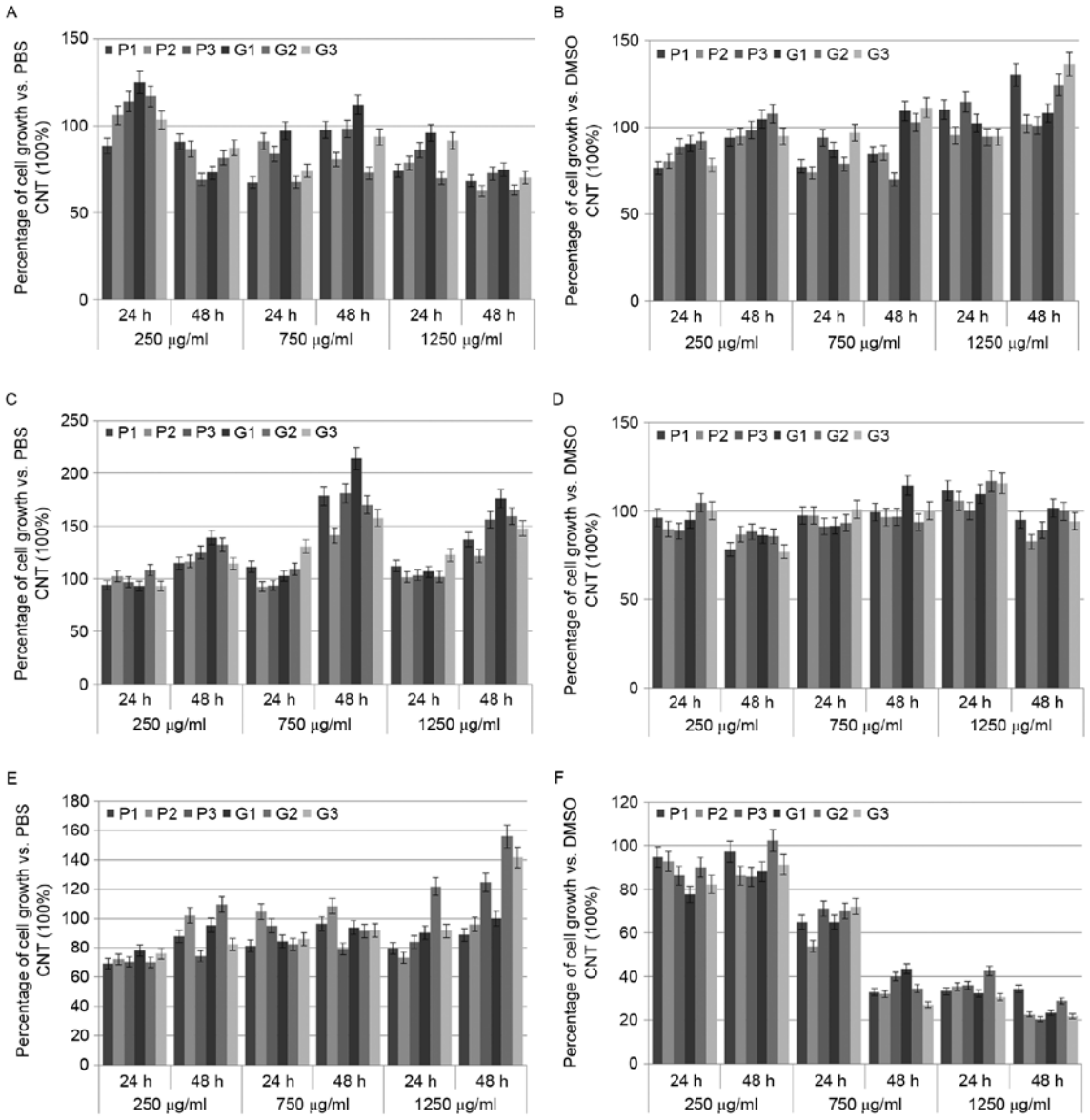

Figure 3. RJ bioactivity on mammalian cells. The graphs represent the results obtained by MMT assay performed on C2C12 (A and B), PC3 (C and D) and SH-SY5Y (E and F) cells after treatment, for 24 and 48 h, with hydrophilic (A, C and E) or lipophilic (B, D and F) extract of RJs (P1, P2, P3, G1, G2 and G3) at different concentrations (250, 750 and 1250 μg per ml of culture medium). Results are reported as percentage of cell growth compared to the respective control (PBS or DMSO). Data are expressed as mean of three independent measurements ± s.d. p-values vs. control: $p<0.05$ for (A, B, D and E); $p<0.03$ for (C); $p<0.01$ for (F).

P2 (0.831 μg/mg) samples, respectively. On the other hand, G2 was the richest sample in lipophilic proteins (0.545 μg/mg) among all the RJs, while P3 (0.207 μg/mg) the poorest one.

Protein extracts were then subjected to one dimensional denaturing gel electrophoresis and Coomassie Blue staining (Fig. 2). No qualitative difference could be observed among the various samples. In general, the hydrophilic protein profile of all the RJs was characterized by 8 principal bands. According to literature data (5,32) and thanks to a standard of molecular weights (MW), we were able to recognize each one of the protein spots. In particular, MRJP1, MRJP2, MRJP3, their isoforms and their fragments were identified (Fig. 2A). In the same manner, we also identified the three protein bands detected in the gel lanes where the lipophilic portions of the RJs were fractionated. They corresponded to MRJP5 and to its isoform and fragment (Fig. 2B).

Biological activity of the RJs on mammalian cells. The lipophilic and hydrophilic fractions of the RJs were purified as described in Materials and methods and used to treat, for 24 and 48 h, three mammalian cell lines: C2C12, PC3 and SH-SY5Y. In particular, all the treatments were performed at three different concentrations, that is the equivalent of the hydrosoluble or liposoluble molecules contained in 250, 750 and 1250 μg of RJ, respectively, per ml of culture medium. Cell growth was measured by MTT assay and the data are reported in Fig. 3A-F.

In C2C12, the hydrophilic extracts of the RJs, except that P1, slightly induced an increase of cell proliferation after 24 h of incubation with the lowest dose (maximum value +24.95% for G1). On the other hand, all the other treatments, at 24 and 48 h with the remaining concentrations, showed a weak reduction of cell growth, reaching the best antiproliferative effect (-37.53%) using the P2 hydrophilic fraction at 1250 μg/ml for

48 h. Singularly, G1 sample determined an increase of C2C12 cells after 48 h of exposure to 750 μg/ml (Fig. 3A).

All lipophilic portions of the RJs caused a slight decrease of C2C12 proliferation after 24 h of exposure with 250 and 750 μg of extract per ml of culture medium (maximum value -26.01% for P2 at 750 μg/ml). The same treatments, after 48 h, showed a rescue of the cell growth (maximum value +36.51% for G3 at 750 μg/ml), except the P3 sample. At the highest concentration, the RJ fractions did not exercise a significant variation of the cell reproduction at 24 h, while, at 48 h, P1, G2 and G3 samples induced hyperproliferative phenomena (maximum value +36.51% for G3) (Fig. 3B).

PC3 cells did not show any substantial modification of their cell growth after exposure, for 24 h, to all doses of the hydrophilic fractions, except G3 (+30.46% at 750 μg/ml; +22.41% at 1250 μg/ml). In contrast, after 48 h, all treatments caused an increase of the cell count. In particular, G1 was the most proliferative extract, amounting to +75.95% and +114.14% at 750 and 1250 μg/ml, respectively (Fig. 3C).

In PC3 cells, all lipophilic extracts of the RJs did not induce significant changes in the proliferation rate. In general, results of all treatments at 250 μg/ml, for 24 h, at 750 μg/ml, for 24 and 48 h, and at 1250 μg/ml, for 48 h were very similar to the control, expect that G1 (+14.20%) and P2 (-17.41%) at 750 and 1250 μg/ml, in that order, after 48 h. On the other hand, we observed that the lowest dose of treatment with all RJ fractions caused, after 48 h, a weak antiproliferative effect (maximum value -24.00% for G3), while the highest one determined, at 24 h, a feeble pro-proliferative effect (maximum value +16.63 for G2) (Fig. 3D).

SH-SY5Y cells reduced their proliferation after exposure, for 24 h, to the lowest dose of hydrophilic extracts (maximum value -30.83% for P1). All the other treatments at 250 μg/ml, for 48 h, at 750 μg/ml, for 24 and 48 h, and at 1250 μg/ml, for 24 h, determined a variable decrease of the cell growth (maximum value -26.83% for P2 at 1250 μg/ml), except that P2 and G2 extracts in some cases. After 48 h, the highest doses of P1, P2 and G1 samples did not produce any significant alteration compared to the control, while P3, G2 and G3 ones induced an increase of the cell proliferation of +24.74%, +55.97% and +41.72%, respectively (Fig. 3E).

Surprisingly, all lipophilic fractions of the RJs caused a strong reduction of SH-SY5Y cell growth, in a dose- and time-dependent manner. In particular, after treatment at 1250 μg/ml for 48 h, the cell proliferation was reduced by RJ extracts in a range which varied between -65.62% (P1 sample) and -79.53% (P3 sample) (Fig. 3D).

Finally, to facilitate the comprehension of the biological effect exerted by all RJs on the mammalian cell lines and individuate the general trend of their treatments, the mean of the results previously obtained after exposure with the different RJ fractions, in each condition, was calculated (Fig. 4).

Discussion

We studied the biochemical composition of six different RJs, with the purpose of understanding if these matrixes were characterized by homogeneous and similar chemical profiles or by specific and typical spectra. To do it, a high-throughput GC-MS analysis was carried out both on methanol and on diethyl ether extracts of the RJs (Fig. 1), detecting a total of 348 and 276 different compounds, respectively (Table I). Literature data usually report a substantially lower number of molecules associated to RJ (11,26), indicating that the present research can be considered a very in-depth and original work which does not limit itself to the identification of the main chemical markers of the RJ but reveals all its components. In general, the amount of substances that were peculiar of only one sample (class 4 for each type of extraction, Table I) was always greater than those commonly detectable in two or more RJs (classes 3, 2 and 1), suggesting that these bee products are almost impossible to standardize. In fact, RJ composition is strongly dependent on several factors, such as environment conditions, genetics and physiology of the bee colony, plant biodiversity distributed around the hive (33).

The chromatographic study revealed that the acids, including free fatty acids, were the most abundant compounds in all RJs, as widely reported in literature (34,35). In particular, 10-HDA and its saturated form, considered as authenticity and freshness markers of RJs (36), were detected in all matrixes, except in G2 and G3, suggesting that these molecules cannot be used as absolute parameters for RJ certification and/or that G2 and G3 samples were partially spoiled (deriving from large distribution network, see Materials and methods). In the RJs, we also detected high concentrations of plant phenolic derivatives (i.e. m-phenethyl-benzonitrile) and alcans (i.e. eicosane) with antimicrobial and antioxidant properties, in coherence with other works (37,38). On the other hand, low doses of sugars and peptides could be revealed only in methanol extracts.

Another innovative aspect of the current work was the fractionation of the RJ proteins according to their lipophilicity. All RJs showed an higher content of hydrosoluble proteins than liposoluble ones (Table II). Thanks to one-dimensional SDS-PAGEs (Fig. 2), we were also able to separate and distinguish these proteins in all samples. In particular, MPRJ1 appeared as the most abundant of the hydrophilic proteins, followed by MPRJ2 and 3. On the contrary, MPRJ5 was the only one which could be found in the lipophilic protein extracts. Finally, MPRJ4 was not detectable in any fractions, as expected due to the low expression level of its mRNA in nurse bees (39). The multiple isoforms and fragments of the RJ proteins could be easily explained by the existence of several repeated regions into their amino acidic sequences and by degradation processes, respectively (32). In general, our results did not reveal qualitative differences in the protein profiles of the six samples, as happened in GC-MS analysis, possibly because the protein composition of the RJ is influenced by fewer factors than its chemical assortment.

Since only a few scientific works describe the antineoplastic properties of the RJ, we focused our research on *in vitro* analysis of the biological effect of RJs on cell growth of three different mammalian cell lines (C2C12, PC3 and SH-SY5Y). The choice of these specific cell models was encouraged by the lack of studies on RJ bioactivity on them. Moreover, for the first time in literature, the cell treatments were carried out using, separately, both hydrophilic and lipophilic fraction of the RJs, at various concentrations and times. With respect to the control, each fraction of RJ exercised a typical effect on the growth rate of the treated cells (Fig. 3). However, in most cases, we observed that extracts of different RJs surprisingly induced

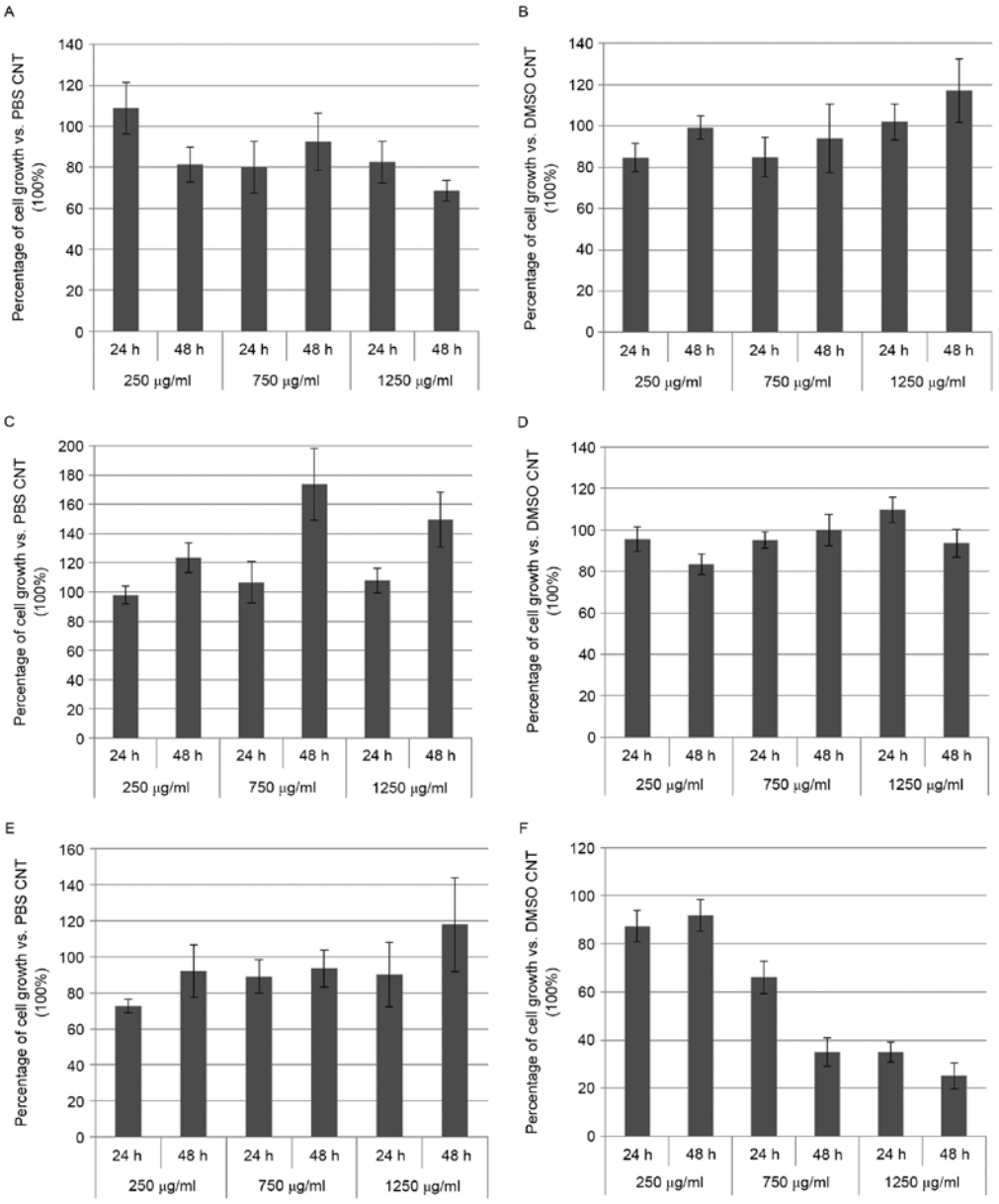

Figure 4. Resuming diagrams of the MTT results reported in Figure 3. Each graph represent the mean value ± s.d. of the six biological effects induced by the hydrophilic (A, C and E) or lipophilic (B, D and F) extracts of the RJs (P1, P2, P3, G1, G2 and G3) on C2C12 (A and B), PC3 (C and D) and SH-SY5Y (E and F) cells, after treatment (for 24 and 48 h) at different concentrations (250, 750 and 1250 μg per ml of culture medium). Results were reported as percentage of cell growth compared to the respective control (PBS or DMSO); p-values vs. control: $p<0.05$ for (A, B, D and E); $p<0.02$ for (C); $p<0.01$ for (F).

a similar proliferative trend on the same cells; although each RJ had a peculiar biochemical profile (Table I) and protein content (Table II).

In general, the hydrophilic fraction of the RJs caused a small decrease of C2C12 proliferation, while the respective lipophilic portion determined just a slight increase of the cell growth, both in a time- and dose-dependent manner (Fig. 4A and B). On PC3, the hydrophilic extracts caused a time-dependent over-proliferation, while the lipophilic ones did not show any significant effect (Fig. 4C and D). Finally, the hydrophilic samples did not effectively influence SH-SY5Y growth rate, whereas antiproliferative effects were extraordinarily revealed by the lipophilic fractions, according to concentration and exposure of the treatments (Fig. 4E and F). This last outcome could suggest a potential medical application of the lipophilic portion of the RJ as antineoplastic treatment for human neuroblastoma.

Similar results were already described in Tamura *et al* (25), where the authors reported that lipid components of the RJ presented antineoplastic property against slow-growing cancers (i.e. P388 and L1210 murine lymphocytic leukemias, 180 murine sarcoma), such as SH-SY5Y neuroblastoma we studied. Obviously, other *in vitro* and *in vivo* studies should be carried out to confirm this hypothesis. Finally, the use of RJ to prevent the onset and slow down the growth of the neuroblastoma could also be supported by the evidence, documented in Hashimoto *et al* (40) and Hattori *et al* (41), which proved that RJ fatty acids (in particular 10-HAD) showed neurotrophic and differentiative activity on murine neuronal cells.

It is quite difficult to establish which RJ compound exerts antiproliferative effects, since RJs usually possess very different compositions, as demonstrated in the present study. However, we hypothesize that this specific bioactivity is strongly linked to lipophilic molecules, such as those detected in all our samples (eicosane; dodecane, 2,6,11-trimethyl; octacosyl trifluoroacetate; phenantrenemetil-2 phenilcinnamato; class 1, Table I). The predominant biological role that literature assigned to 10-HAD (and its saturated form) in the RJ was unexpectedly not confirmed in the current research. In fact, all RJs induced SH-SY5Y growth inhibition, although some of them were lacking this fatty acid. Previous evidence let us to believe that all RJ compounds work in synergy to reach the final observed effect.

In conclusion, all lipophilic fractions extracted from chemically different RJs surprisingly shared a common antitumoral feature against human neuroblastoma.

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