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ORIGINAL ARTICLE

The key phytochemistry of rosemary (*Salvia rosmarinus*) contributing to hair protection against UV

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Abstract

Extracts from rosemary (*Salvia Rosmarinus)* are analysed for their phytochemistry using LC–MS and the phytochemistry identified. The same extracts were tested for their efficacy to act as antioxidants by both hydrogen-atom transfer (ORAC) and single electron transfer (FRAP). A correlation analysis was performed to identify the key phytochemistry responsible for antioxidant efficacy. The top performing extracts were then tested in a peptide model and in hair with the presence of UV to measure ability to protect against UV-induced peptide and protein damage. Polyphenols (e.g. rosmarinic acid, glycosides of selgin) and abietane diterpenes (e.g. carnosic acid) in rosemary were identified as the principal compounds which enables the extracts to protect hair from UV.

Objective: The objective of this work was to correlate the phytochemistry of rosemary (*Salvia rosmarinus)*, a botanical with known antioxidant properties, to a UV protection benefit in hair. These data will give insights into mechanisms of UV damage, the ROS formed and their reactivity.

Methods: LC–MS was used to compare the compounds in 10 commercial extracts of rosemary. ORAC (oxygen radical antioxidant capacity) and FRAP (ferric reducing antioxidant power) were used to measure the antioxidant capacity of the rosemary extracts. The ORAC assay measures ability of an antioxidant to react with a peroxyl radical via hydrogen atom extraction and FRAP measures electron transfer through reduction of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) by antioxidants present in the samples. Correlation of extract composition with antioxidant measures was performed using principal component analysis. Selected extracts were assessed for their ability to protect hair from UV damage in a model peptide system and on hair. In addition, the same methods were used to test rosmarinic acid and carnosic acid, key phytochemistries in the rosemary extracts. The model system was a peptide and its decomposition on exposure to UV was monitored by LC–MS in the absence and presence of the rosemary extracts. Hair degradation in the presence of UV was measured by exposure of UV in an Atlas weatherometer followed by extraction of degraded protein in water. A fragment

of the S100A3 protein was used as a marker of UV damage ($m/z=1278$) and quantified via LC–MS.

Results: Ten rosemary extracts were assessed for antioxidant performance and correlated with their compositions. The phytochemistry in each extract varied widely with a total of 33 individual compounds identified. The differences were most likely driven by the solvent and extraction method used by the supplier with extracts varying in the proportion of polar or non-polar compounds. This did influence their reactivity in the ORAC and FRAP assays and their efficacy in preventing protein damage. Two of the key compounds identified were rosmarinic acid and carnosic acid, with rosmarinic acid dominating in extracts with mainly polar compounds and carnosic acid dominating in extracts with mainly nonpolar compounds. Extracts with higher rosmarinic acid correlated with ORAC and FRAP scores, with UV protection on hair and in the peptide model system. The extracts chosen for hair experiments showed hair protection. UV protection was also measured for rosmarinic and carnosic acid.

Conclusions: Despite the variation in the profile of phytochemistries in the 10 rosemary extracts, likely driven by the chosen extraction method, all rosemary extracts had antioxidant activity measured. This study suggests that the polyphenols (e.g. rosmarinic acid, glycosides of selgin) and abietane diterpenes (e.g. carnosic acid) are the principal compounds which enables the extracts to protect hair from UV.

KEYWORDS

carnosic acid, chemical analysis, formulation/stability rosemary, hair treatment, rosmarinic acid

Résumé

Introduction: Les extraits de romarin (*Salvia Rosmarinus)* sont analysés par LC-MS pour établir et identifier leur profil phytochimique. Les mêmes extraits ont été testés pour leur efficacité à agir comme antioxydants à la fois par transfert d'atome d'hydrogène (ORAC) et par transfert d'électrons uniques (FRAP). Une analyse de corrélation a été réalisée pour identifier les propriétés phytochimiques clés responsables de l'efficacité antioxydante. Les extraits les plus performants ont ensuite été testés dans un modèle peptidique et sur les cheveux en présences d'UV pour mesurer la capacité à protéger contre les dommages induits par les UV su les peptides et protéines. Les polyphénols (par ex. acide rosmarinique, glycosides de selgin) et les diterpènes d'abiétine (par ex. acide carnosique) dans le romarin ont été identifiés comme les principaux composés permettant aux extraits de protéger les cheveux des UV.

Objectif: L'objectif de ce travail était de mettre en corrélation la phytochimie du romarin (*Salvia rosmarinus)*, une plante aux propriétés antioxydantes connues, et les bénéfices d'une protection contre les UV dans les cheveux. Ces données fourniront des informations sur les mécanismes des dommages causés par les UV, la formation du ROS et leur réactivité.

Méthodes: La LC-MS a été utilisée pour comparer les composés de 10 extraits commerciaux de romarin. L'ORAC (Oxygen Radical Antioxidant Capacity/

Capacité d'absorption des radicaux d'oxygène) et la FRAP (Ferric Reduction Antioxidant Power/Pouvoir antioxydant de réduction ferrique) ont été utilisés pour mesurer la capacité antioxydante des extraits de romarin. Le dosage ORAC mesure la capacité d'un antioxydant à réagir avec un radical peroxyl par extraction d'atome d'hydrogène et la FRAP mesure le transfert d'électrons par réduction du fer ferrique (Fe^{3+}) en fer ferreux (Fe^{2+}) par les antioxydants présents dans les échantillons. La corrélation entre la composition de l'extrait et les mesures des antioxydants a été effectuée en analysant les composants principaux. Les extraits sélectionnés ont été évalués pour leur capacité à protéger les cheveux des dommages causés par les UV dans un modèle de système peptidique et sur les cheveux. En outre, les mêmes méthodes ont été utilisées pour tester l'acide rosmarinique et l'acide carnosique, principales caractéristiques phytochimiques dans les extraits de romarin. Le système modèle était un peptide et sa décomposition à l'exposition aux UV a été suivie par LC-MS en l'absence et en présence des extraits de romarin. La dégradation des cheveux en présence d'UV a été mesurée par l'exposition aux UV dans un indicateur de désagrégation Atlas suivi de l'extraction de protéines dégradées dans l'eau. Un fragment de la protéine S100A3 a été utilisé comme marqueur de dommage UV (m/z = 1278) et quantifié par LC-MS.

Résultats: Dix extraits de romarin ont été évalués en termes de performance antioxydante et mis en corrélation avec leurs compositions. La phytochimie de chaque extrait variait considérablement, avec un total de 33 composés individuels identifiés. Les différences étaient très probablement dues à la méthode du solvant et de l'extraction utilisée par le fournisseur avec des extraits variant dans la proportion de composés polaires ou non polaires. Cela a effectivement influencé leur réactivité dans les dosages ORAC et FRAP et leur efficacité dans la prévention des dommages protéiques. Deux des composés clés identifiés étaient l'acide rosmarinique et l'acide carnosique, l'acide rosmarinique dominant dans les extraits contenant principalement des composés polaires et l'acide carnosique dominant dans les extraits contenant principalement des composés non polaires. Les extraits avec un taux d'acide rosmarinique plus élevé étaient mis en corrélation avec les scores ORAC et FRAP, avec une protection UV sur les cheveux et dans le système de modèle peptidique. Les extraits choisis pour les expériences sur les cheveux ont montré une protection des cheveux. La protection contre les UV a également été mesurée pour l'acide rosmarinique et l'acide carnosique.

Conclusions: Malgré la variation des profils phytochimiques dans les dix extraits de romarin, probablement induite par la méthode d'extraction choisie, l'activité antioxydante de tous les extraits de romarin a été mesurée. Les polyphénols (par ex. acide rosmarinique, glycosides de selgin) et les diterpènes d'abiétane (par ex. acide carnosique) dans le romarin ont été identifiés comme les principaux composés permettant aux extraits de protéger les cheveux contre les UV.

INTRODUCTION

Damage to hair from sunlight has been well documented and has been shown to change cuticle and cortex protein structures [1], cell membrane lipids [2] and melanin [3]. The consequences of this damage are noticed by women, especially those with a lighter colour, as lightened hair tips, lack of tip alignment, breakage, and split ends [4]. Absorption of UVB light (280–315 nm) in hair by amino acids tyrosine and tryptophan and to a lesser extent

histidine and phenylalanine is thought to be the first step in this damage. The amino acids are photoionized and produce aromatic free radicals. The subsequent chemistry is complex but oxidation of the tyrosine [5] and tryptophan residues [6] has been measured with tryptophan producing the well-studied, yellow-coloured kynurenines. Singlet oxygen and superoxide radical anion are formed by photosensitization of both aromatic amino acid residues and melanin pigments [7] and these reactive oxygen species (ROS) can propagate further reactivity in the proteins and lipid structures [8]. Redox metals such as copper have been shown to accelerate these radical reactions by reacting with any formed hydrogen peroxide or alkoxyl radicals leading to additional protein damage [9].

Reduction of UV damage can be achieved by various strategies including reducing UV absorption by addition of UV absorbers [10], removal of exogenous copper via chelation [11] or addition of antioxidants that terminate radical reactions by quenching free radicals. Polyphenols can act as antioxidants via these mechanisms and are important as they are commonly found in many botanical extracts such as tea (*Camellia sinensis* L.) [12], grape (*Vitis vinifera* L.) [13] and pomegranate (*Punica granatum* L.) [14]. The polyphenols scavenge ROS via three main mechanisms: hydrogen atom transfer, single electron transfer and metal chelation.

This study focused on the antioxidant efficacy of rosemary extracts. Rosemary (*Salvia rosmarinus L.*) is an aromatic plant of the Lamiaceae family (with thyme, mint, sage, basil). It originates from the Mediterranean and is a perennial that grows in many environments. Rosemary oil obtained from steam distillation of the twigs/fresh leaves is often used in aromatherapy to reduce stress levels. However, in this work, we used extracts from rosemary-dried leaves where the compounds are removed by solvent extraction, typically water or ethanol. Rosemary's clinical efficacy has been studied and extracts have been shown to have anti-inflammatory, antioxidant and antimicrobial properties [15]. It has been shown to deliver photoprotection and antiageing in skin models [16] but there is only limited data relating to hair. The phytochemistry of rosemary extracts has also been well studied with three key compounds identified, rosmarinic acid, carnosic acid and carnosol (Figure 1). However, it also known that extracts of botanicals include many different chemistries, and these could all be contributing to the efficacy and there may also be synergy between chemistries.

The objective of this study was to measure if rosemary extracts can deliver UV protection to hair, propose a mechanism and identify which compounds in the extracts are responsible for the protection.

METHODS

Hair source

Two gram, six inch Caucasian-source light brown-untreated hair (i.e. no chemical treatment) was purchased from International Hair Importers & Products Inc. (Glendale, NY).

Material information

Rosemary samples were sourced from 10 different suppliers. These were coded with a unique number identified from 01 to 10. Eight of the samples were supplied as powders and two as liquids. The liquids were prepared in either sunflower seed oil (Extract 09) or glycerin (Extract 10). Rosmarinic acid and carnosic acid were purchased from 1PlusChem (San Diego, CA). The peptide N-acetylalanine (N-Ac-Ala-OH) was purchased from Fluorchem (Hadfield, UK).

LC–MS sample preparation and analysis

The dry powder of eight rosemary samples were weighed into 5 mL glass vial followed by addition of methanol

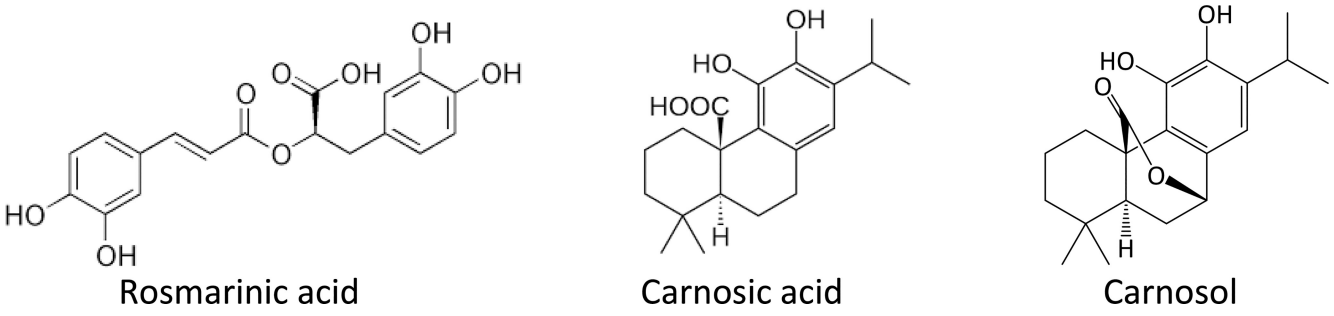


FIGURE 1 Structures of rosmarinic acid, carnosic acid and carnosol key compounds in rosemary extracts.

to a concentration of 10 mg/mL. They are considered as 1% dilution. The two liquid samples (Extracts 9 and 10) were further diluted with methanol to 1/10 before analysis by LC–MS. The samples were vortex mixed for 60 s and sonicated (Fisherbrand FB15050, Elma Schmidbauer GmbH, Germany) for 5 min. To remove insoluble excipient, sample solutions were centrifuged at 18000 x *g* (centrifuge 5420, Eppendorf AG, Germany) and the supernatants were transferred to 1.5 mL LC–MS glass vials.

The profile of the compounds in the commercial samples of rosemary and their identity were obtained using an ultra-high performance liquid chromatography coupled to high-resolution electrospray ionization mass spectrometry (UHPLC-UV-HR-ESI-MS) platform. UV spectra and tandem MS/MS data were recorded from a Vanquish UHPLC system coupled to a 100 Hz photodiode array detector (PDA) and an Orbitrap Fusion Tri-hybrid high-resolution tandem mass spectrometer (Thermo Scientific, Waltham, MA, USA). Chromatographic separation was performed with 5 µL injection volume using a Luna C18 column (150 mm × 3 mm i.d., 3 µm, Phenomenex) at 30°C and a mobile phase consisting of methanol: water: acetonitrile (acidified with 1% formic acid) and linear gradient elution (0:90:10 to 90:0:10) for 20 min then held for further 5 min before re-equilibrated to initial conditions for 5 min (flow rate: 400 µL/min). The PDA was set to record from wavelengths 210–550 nm, with a 1 nm resolution. Mass spectrometry detection was performed in both positive and negative ionization modes using the full scan and data-dependent MS2 and MS3 acquisition modes. The mass spectrometer was set to acquire the range of 125–1800 *m/z*; The spray voltages for positive and negative ionization modes were set to +3.5 and −2.5 kV respectively; full scan resolution of 60 000 FWHM; capillary temperature of 350°C; ion transfer tube temperature of 325°C; 50% RF lens; 4.0×10^5 (full scan) and 1.0×10^4 (MS^n) as automatic gain control targets; intensity threshold of 1.0×10^4; the collision-induced dissociation energy was set to 35 eV; activation Q of 0.25; and an isolation window of 4 *m/z*. Nitrogen was used as the drying, nebulizer and fragmentation gas. Compound identification achieved by comparison of accurate mass measurements, UV spectra and MS^n spectra data with standard compounds and those in literature and database resources including SciFinder and the Dictionary of Natural Products (https://dnp.chemnetbase.com).

ORAC and FRAP measurements

OxiSelect™ Oxygen Radical Absorbance Capacity (ORAC) activity assay kit from Cell Biolabs (San Diego, CA) was used for ORAC measurements and performed as described in the product manual.

Arbor Assays (AA) K043-H1, FRAP™ (ferric reducing antioxidant power) Colorimetric Detection Kit was used for FRAP measurements and performed as described in the product manual by Abor Assays (Ann Arbor, Michigan, US).

Peptide irradiation

Stock solutions for all antioxidants were prepared in MeOH as they are insoluble in H_2O. All reactions were carried out in D_2O (with a minimal amount of MeOH as the antioxidants were prepared in MeOH). Irradiations were carried out by exposing solutions of the protected amino acids (1 mM) in the presence of aqueous H_2O_2 (100 mM), and the antioxidant (0.1 mM) (3 mL final reaction volume) under UV light using a Philips HPK 125 W high pressure Hg lamp with a H_2O filter (5 cm) which provides broad-band UV light. The light output from this HP lamp provides maximum energy at 365 nm with substantial radiation also at 435, 404, 313 and 253 nm. The reactions were carried out in a quartz cuvette (open to air), and the cuvette was placed 5 cm in front of the UV lamp (the distance from the lamp was chosen to achieve a ca. 10% decomposition of the amino acids in the absence of any antioxidant). Irradiations were stopped after 2 min. All reactions were analysed prior to and at the end of the exposure. All reactions were run in triplicates.

Substrate decomposition

Liquid chromatography–mass spectrometry (LC–MS) calibration curves were constructed by preparing the most concentrated sample of the substrates (1 mM for both substrates), and then preparing serial dilutions on these (0.2–0.8 mM). The method afforded excellent calibration curves ($R^2 > 0.997$ in all cases). For *N*-Ac-Phe-OH, the UV detector was used, and the calibration curves were constructed by integrating the peak area of the peptide. For *N*-Ac-Ala-OH, the total ion count (TIC) of the $[M+H]^+$ extracted ion chromatogram (EIC) was used. Daily and weekly variations of the calibration curves were assessed by repeating LC–MS injections with fresh solutions for all diluted samples.

Hair treatment

Hair was treated with rosemary extract solutions before each cycle. A solution of the extract in ethanol: water

(50:50) was made and 2 mL of solution was added to each 2 g tress (five tresses per treatment) and then dried in a hot box. Ethanol: water was used for the control. Each cycle consists of the following—treatment with solution, leave for 15 min, dry in hot box, in UV box for 10 h at 85% RH in Atlas weatherometer. Wash with clarifying shampoo in between each cycle for a total of six cycles. The no UV exposure gets the wash cycle each time but no UV exposure.

For the leave-on product testing, the extract was added to a cream gel network leave-on treatment and speed mixed until the extract was completely dissolved and mixed homogeneously. The product was applied to hair after washing with a clarifying shampoo at a dose of 0.05 g/g hair.

Hair exposure to artificial irradiation

Sun exposure was simulated by irradiation with an Atlas Ci3000+ weather-o-meter (Atlas, Chicago, Illinois, US). An internal and outer quartz filter was used to simulate broad-spectrum, outdoor daylight with a specific irradiance of 1.48 W/m^2 at 420 nm. During the irradiation process, temperature and relative humidity (RH) were kept constant at 35°C and 80% RH respectively.

Biomarker analysis

Samples (0.5 g) of hair from treated tresses were cut and placed into 50 mL tubes with 5 mL of water added. Tubes with hair and water were mixed on a multi-tube vortex shaker for 60 min at 2500 rpm. Water portion was then transferred from the tubes by pipette into glass scintillation vials. A 10 mg/mL solution of MALDI matrix (alpha-cyano-4-hydroxy cinnamic acid) was mixed with the hair extract samples in a 1:1 volume ratio. A 1 µL of this solution was used to spot onto the MALDI plate and MALDI MS spectra was acquired (1000 shots). Intensity of the UV damage marker peptide at m/z 1278 was measured. This biomarker was identified as a fragment of the S100A3 protein that is involved in cuticle cell adhesion [11] and is directly related to level of UV exposure of untreated hair.

RESULTS AND DISCUSSION

A total of 33 peaks were identified in most of the 10 rosemary extracts, although their abundance varied among the extracts. Of these 33 peaks, 31 could be assigned to known compounds (Table 1) and two were unassigned. The main groups of compounds are hydroxycinnamic acid derivatives (caffeic acid, rosmarinic acid), abietane diterpenes (carnosic acid, carnosol, rosmanol), various methoxyflavones (hispidulin, cirsimaritin, luteolin and selgin) and their glycosides as listed in Table 1.

A heat map based on intensity of representative ions (ESI+/−) for the different peaks was created to illustrate the difference in abundance of the peaks in the 10 extracts (Figure 2). The darker colour indicates a higher level of each compound in the extracts. The compounds detected from extracts are ordered in retention time from the mass spectrometer chromatograms, that is, hydrophilic materials which are eluted earlier from the column than hydrophobic materials. This enables an overview of the extract compositions in terms of what types of compounds are abundant in each extract. Extracts 01, 02, 03 and 10 contain mainly hydrophilic compounds as compared to extracts 05 and 09 which have mainly hydrophobic compounds. Extracts 04, 05, 06 and 07 have a mix of both hydrophobic and hydrophilic compounds. Extract 08 has low levels of both hydrophilic and hydrophobic compounds. Differences between extracts containing either high hydrophilic or high hydrophobic compounds will be influenced by the composition of the starting leaf material, also by the type of solvents and/or method used by the supplier to extract the rosemary leaves. Aqueous solvents will extract more hydrophilic materials and ethanolic solvents will extract more hydrophobic materials.

This basic heat map with raw ion intensities (from either negative or positive ESI-Mass) data provides a quick overview of the phytochemistry of the 10 extracts, the darkness of green colour in each cell represents the relative intensity of that compound in the row. However, another way to illustrate the similarity in the chemical profile of extracts is to produce a heat map based on Z-Score as used in Fang et al. [17], which is to scale each compound from highest to lowest across 10 extracts and the colour shows relative abundance on this scale (Figure 3). Red colour indicates high concentration, blue indicates low concentration. Extracts are clustered according to similarity in their chemical traits.

As seen in Figure 3, extracts from the 10 samples can be broadly separated into two groups based on similarity in their chemistry. Extracts 05 and 09 are clustered together due to their higher levels of abietane diterpenes (e.g. rosmanol isomer III (R-24), carnosol isomer (R-25), rosmanol methyl ether (R-28), carnosic acid (R-32) and 12-methoxy-carnosic acid (R-33)) and some 7-methyl flavones (luteolin-7,4′-dimethyl ether (R-20), genkwanin (R-23), salvigenin (R-26) and apigenin-7,4′-dimethyl ether (R-30)). Extracts 06, 03 and 08 are clustered due to similar levels of rosmarinic acid (R-7) and some flavonoid glycosides (selgin-3-glucoside (R-3), hesperidin (R-4),

TABLE 1 Compounds identified in one or more of the rosemary extracts.

[a]	Compound names	MW	[a]	Compound names	MW
R-1	Quinic acid	192	*R-18*	Cirsimaritin	314
R-2	Caffeic acid	180	*R-19*	Rosmanol Isomer I	346
R-3	Selgin-3-glucoside	478	*R-20*	Luteolin-7,4′-dimethyl ether (or isomer)	314
R-4	Hesperidin	610	*R-21*	Rosmanol	346
R-5	Hispidulin-7-O-glucoside	462	*R-22*	Rosmanol Isomer II	346
R-6	Luteolin-7-O-glucuronide	462	*R-23*	Genkwanin	284
R-7	Rosmarinic acid	360	*R-24*	Rosmanol isomer III	346
R-8	Selgin-glucosyl-glucuronide	654	*R-25*	Carnosol isomer	330
R-9	Selgin-rutinoside	624	*R-26*	Salvigenin	328
R-10	Cirsimaritin glycoside	476	*R-27*	Rosmadial isomer I	344
R-11	Luteolin-3′-acetyl-O-glucuronide	504	*R-28*	Rosmanol methyl ether	360
R-12	Luteolin	286	*R-29*	Carnosol	330
R-13	Selgin (3'-O-methyltricetin)	316	*R-30*	Apigenin-7,4′-dimethyl ether	298
R-14	Hispidulin	300	*R-31*	Rosmadial isomer II	344
R-15	Unknown-A	N/A	*R-32*	Carnosic acid	332
R-16	Unknown-B	N/A	*R-33*	12-methoxy-carnosic acid	346
R-17	Hispidulin isomer	300			

Abbreviation: MW, molecular weight.

[a]They are listed in the order they eluted, and each compound had been given an R number between 1 and 33.

luteolin-7-*O*- glucuronide (R-6) and luteolin-3′-acetyl-*O*-glucuronide (R-11)). Extract 10 is particularly high in flavonoid glycosides (selgin-3-glucoside (R-3), hesperidin (R-4), hispidulin-7-O-glucoside (R-5), luteolin-7-*O*-glucuronide (R-6), selgin-glucosyl-glucuronide (R-8), selgin-rutinoside (R-9) and cirsimaritin glycoside (R-10) which could be due to an extraction procedure that is targeting water-soluble components. Extracts 01, 03, 06, 07 and 10 have higher levels of rosmarinic acid (R-7), whereas extracts 04, 05 and 09 have higher levels of carnosic acid (R-32).

The ORAC assay measures the ability of an antioxidant to undergo a hydrogen atom transfer (HAT) to a peroxyl radical and the FRAP assay measures the ability of an antioxidant to undergo an electron transfer. The chemistry of extracts were correlated with the results of testing the 10 rosemary extracts in the ORAC and FRAP antioxidant assays (Table 2). For instance, Ext. 07 showed highest ORAC score, and it also contain high proportion of rosmarinic acid (R7) than other extracts. A complete correlation analysis is presented in Table 3.

All the extracts score highly in both assays and there is a high correlation between both measures ($R^2 = 0.88$; Pearson correlation coefficient = 0.94) indicating all extracts can act as antioxidants via either hydrogen atom transfer or electron transfer. This ability to react via different mechanisms has been reported for polyphenols such as quercetin, catechin, apigenin and naringenin [18]. For comparison, a benchmark comparison for antioxidancy is an ORAC score for Vitamin E, a well-known antioxidant in Beauty Care products. This is reported to have an ORAC score of 129 000 mM/100 g [19]. Extracts 01, 02 and 07, have the highest values in both assays up to 6 975 542 mM/100 g (ORAC Score) and 74 673 621 μM/100 g (FRAP Score), whereas Extracts 05 and 09 have the lowest scores.

A pairwise correlation analysis was run between the extracts' phytochemistries and ORAC and FRAP scores, and the pairs with significant correlations are listed in Table 3 and Table S1 (in supplementary material). Selgin-3-glucoside (R-3), hispidulin-7-O-glucoside (R-5), rosmarinic acid (R-7), Selgin-glucosyl-glucuronide (R-8) and Selgin-rutinoside (R-9) have the highest correlation to antioxidant activities (FRAP and ORAC). These are all hydrophilic natural metabolites as seen in Table 1.

These data show that the rosemary extracts contain a complex mixture of compounds that differ in abundance among extracts from the various suppliers. These data suggest that it is not only one key compound, but a combination of polyphenols that are responsible for antioxidant activity. The correlation analysis shows that levels of five (selgin-3-glucoside (R-3), selgin-glucosyl-glucuronide (R-8), selgin-rutinoside (R-9), hispidulin-7-O-glucoside (R-5) and rosmarinic acid (R-7)) of the 33 compounds correlate with the antioxidant levels in both antioxidant assays. It is of interest to note that all these compounds

Hydrophilic

Hydrophobic

PPT#	01	02	03	04	05	06	07	08	09	10
R-1	93552472	224057724	115395098	50973447	3535227	192718114	81565476	106891028	0	9869039
R-2	99513765	81274250	55646482	163441360	1837904	60502264	168910220	20020013	659487	13966849
R-3	116753383	72653499	108701126	65132409	1888294	178288737	233647481	95978601	4389101	412383808
R-4	6835188	1714748	43783902	33078834	356598	69660548	92831524	20810676	600491	79990253
R-5	107184039	40754851	88140706	77163258	1175729	128687193	287194538	72464496	5462203	509199908
R-6	229275456	419805420	328644827	114968672	19799917	494361764	319888671	287169649	1383135	775258163
R-7	426540963	174086787	362218414	311422191	5290639	466045691	887955118	415856889	3059339	873085205
R-8	29265142	55818705	61361871	101141573	2355445	90287412	367845173	33597303	3002931	146275769
R-9	22564020	17732825	29575316	58097788	703682	51608992	173945727	12289926	923686	60925417
R-10	362925670	5510764	87466470	82084337	1159957	115635777	284013842	87526802	7173637	423518518
R-11	4001914	116445582	124665655	146052421	839801	209686518	327552074	55289715	542050	85640903
R-12	60768190	190609684	89229988	118010885	157275462	10887794	220628600	66910452	19603174	19248185
R-13	25210677	140063914	22155467	139780020	56123942	38095405	149311542	11352279	14788032	136484593
R-14	9118938	13594086	10114231	84562952	35808731	23684638	115231208	10058288	26709179	139689349
R-15	9460096	17453394	3035944	368628391	582927	27988187	32086640	1322814	267550	2642027
R-16	6085450	24486624	9722975	350694153	401607	22650916	51087166	2322657	357768	6283820
R-17	80045352	141834114	50811737	79938969	112423302	104463607	118396848	40017885	171766836	101092020
R-18	341900709	159966561	175941152	275327702	366773714	438153596	635854026	202284342	455794858	240375038
R-19	164082124	55727167	119541978	734648899	303432632	235249838	474704719	89323875	344019646	35605951
R-20	34006885	49456701	37488013	288877681	277783534	142702937	698789030	24147499	557667978	29723711
R-21	141364368	63205742	73327828	398345333	385116712	142615929	115388977	130843750	1170773036	29778350
R-22	67172235	18269429	65270804	182433716	282447811	85689482	144307466	133245782	513021256	33310654
R-23	68147580	32639676	68416286	592940052	1329697061	449367223	1133982700	108601592	1314938888	94029929
R-24	14685747	3818328	16456541	134203015	159968770	31651901	56961174	4578773	94878556	12209281
R-25	0	1978424	3150656	7120455	65054799	0	0	602805	252883378	742737
R-26	179171554	34945625	58854317	1624969039	606339260	195212187	672158262	30690571	967316072	15398415
R-27	22325344	62336684	16154851	163754230	279792126	36952433	44498597	64459359	283145100	47996165
R-28	38145371	3765758	17640799	255495882	451198048	73954465	77569941	25556383	321436934	1964770
R-29	374786662	68795827	155913137	225563251	429035231	286697644	256909454	80625515	954733903	36803796
R-30	23371013	7392540	25145332	757168661	975466296	228292905	836889391	28746439	2065231353	29201602
R-31	29657945	13404365	21852275	583610769	183278015	55838962	112206192	17008836	400616660	56703478
R-32	80085141	9437318	12823344	471976880	561855009	60684143	132577118	3482528	1011367665	65860
R-33	4304291	3270832	7691072	172005102	236517148	16089200	16735242	14036892	287980161	5874920

FIGURE 2 Heat map based on intensity of representative ions (ESI+/−) of the compounds in the rosemary extracts. Columns represent the 10 rosemary samples and rows R1–R33 the compounds (see Table 1 for identification of the compounds R1-R33).

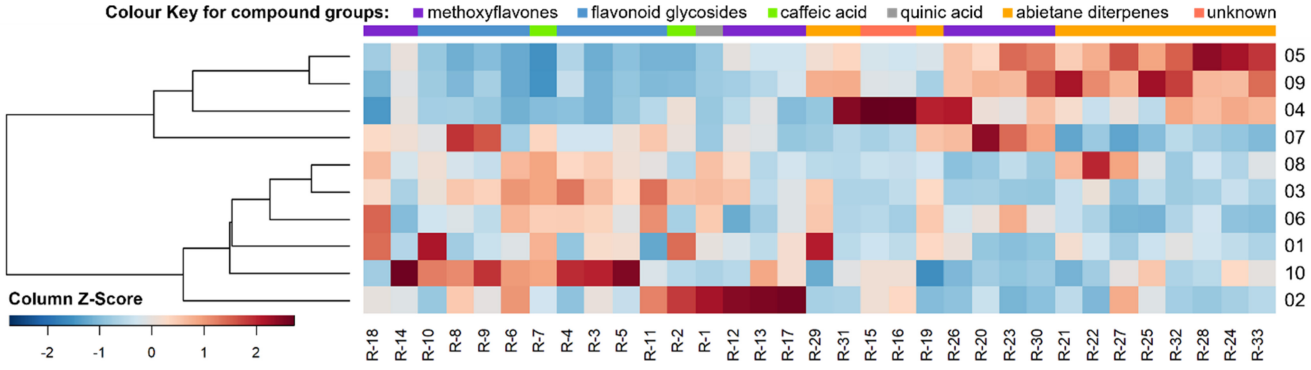


FIGURE 3 Heat map showing scaled relative intensity of each compound across 10 samples. Cluster analysis performed based on similarity of chemical profiles. Similar compounds are colour coded as one group; see Table 1 for identification of the compounds R1-R33.

are hydrophilic (Figure 1). This might be due to the fact that the antioxidant potency is measured by assays with aqueous buffers.

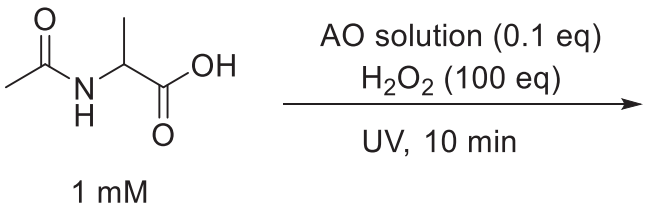
The ability of these extracts to protect protein oxidation from UV oxidation was first tested in a model peptide system before moving to hair which is a lot more complex. A small peptide chosen, N-acetylalanine (N-Ac-Ala-OH) and the effect of added antioxidant was monitored by exposing aq. solutions of *N*-Ac-Ala-OH (1 mM), in presence of 100 eq H_2O_2 and 0.1 eq of the antioxidant to UV light (Scheme 1). In the first set of experiments, the pure chemistries, rosmarinic acid and carnosic acid were tested and in the second set of experiments, a select group of the rosemary extracts were tested.

TABLE 2 ORAC and FRAP scores for rosemary extracts.

Rosemary unique identifier	ORAC score Trolox Equiv. (mM/100 g)	% RSD	FRAP score Fe2+ Equiv (nMFeCl2/mg sample)	% RSD
Ext. 01	4 243 156	6.5	4894	1.9
Ext. 02	3 648 007	5.6	4451	2.4
Ext. 03	2 659 565	15.0	4836	3.3
Ext. 04	1 890 475	12.0	2358	2.6
Ext. 05	1 117 194	6.6	1502	4.6
Ext. 06	2 912 767	14.0	3800	1.1
Ext. 07	6 975 542	6.0	9845	0.8
Ext. 08	2 764 056	3.3	2592	3.6
Ext. 09	404 622	3.7	950	0.5
Ext. 10	2 762 822	5.4	3930	1.0
Rosmarinic Acid	9 043 167	8.0	22 990	1.8
Carnosic Acid	3 248 400	28.0	8712	2.5

TABLE 3 The Pearson correlation coefficients with *p*-values are reported for each pair of assay and compound. This table only shows correlations with $p < 0.01$; the full table of pairwise correlation analysis is provided as (Table S1).

Assays	Codes	Compounds	Coeff.	*p*-Value
ORAC	FRAP		0.96	0.0000
ORAC	R-3	Selgin-3-glucoside	0.85	0.0019
ORAC	R-5	Hispidulin-7-O-glucoside	0.89	0.0005
ORAC	R-7	Rosmarinic acid	0.86	0.0015
ORAC	R-8	Selgin-glucosyl-glucuronide	0.81	0.0047
ORAC	R-9	Selgin-rutinoside	0.79	0.0064
FRAP	R-3	Selgin-3-glucoside	0.83	0.0028
FRAP	R-5	Hispidulin-7-O-glucoside	0.90	0.0004
FRAP	R-7	Rosmarinic acid	0.82	0.0033
FRAP	R-8	Selgin-glucosyl-glucuronide	0.85	0.0019
FRAP	R-9	Selgin-rutinoside	0.83	0.0031



SCHEME 1 Experimental conditions for the UV exposure of N-Ac-Ala in the presence of H_2O_2 and antioxidant solutions.

The efficiency of the AO was evaluated by monitoring the decomposition of the parent amino acid via LC–MS and comparing with a control reaction where no AO was added (Figure 4a,b). Both rosmarinic acid and carnosic acid prevent a loss of the peptide and are statistically different versus no antioxidant control (99% significance in student *t*-test) with rosmarinic acid statistically equal to carnosic acid. All the rosemary extracts tested also showed a protection benefit giving significantly lower levels of peptide lost (99% significance in student *t*-test) versus the control. The two extracts with the highest carnosic acid levels (Extract 05 and Extract 09) gave the lowest level of protection, consistent with data from testing the pure compounds.

These data are also consistent with measured ORAC and FRAP scores which showed a higher correlation with hydrophilic chemistries including rosmarinic acid and with published studies where ORAC and FRAP scores were compared for rosemary extracts standardized to the same amount of rosmarinic acid and carnosic acid [20].

UV protection of hair protein with these chemistries and extract was measured by extracting created protein fragments after UV exposure and quantifying a specific UV hair biomarker at $m/z = 1278$. This biomarker is a fragment of the S100A3 protein showed in previous work to correlate with UV oxidation [11]. It was not possible to measure total protein loss as a measure of UV oxidation as the extracts all contain an amount of protein. In all experiments, two controls were added, hair that had no UV exposure (low control) and hair that was exposed to UV but with no treatment (high control).

In the first experiment, we measured the activity of pure phytocompounds—carnosic acid and rosmarinic acid (Figure 5). Both showed high activity when tested at 0.5% solution active dosed from a 50:50 ethanol: water solution at 1 mL per gram of hair, an equivalent of 0.005 g of extract per gram of hair. Carnosic acid was more efficient than

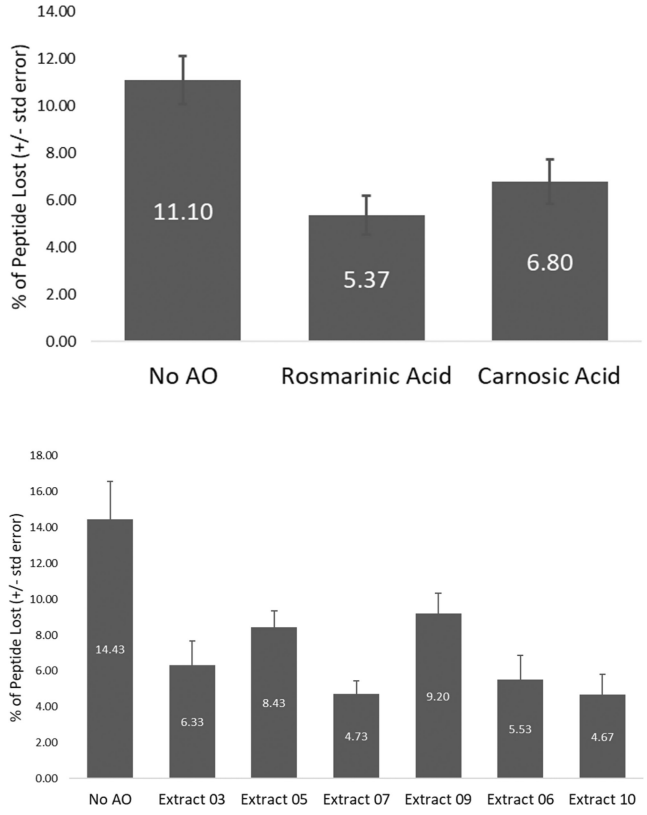


FIGURE 4 Decomposition of the parent amino acid upon exposure of aq. solutions to UV light. Aliquots were analysed by LC–MS ($N=3$). (a) comparison to rosmarinic acid and carnosic acid, (b) comparison to rosemary extracts.

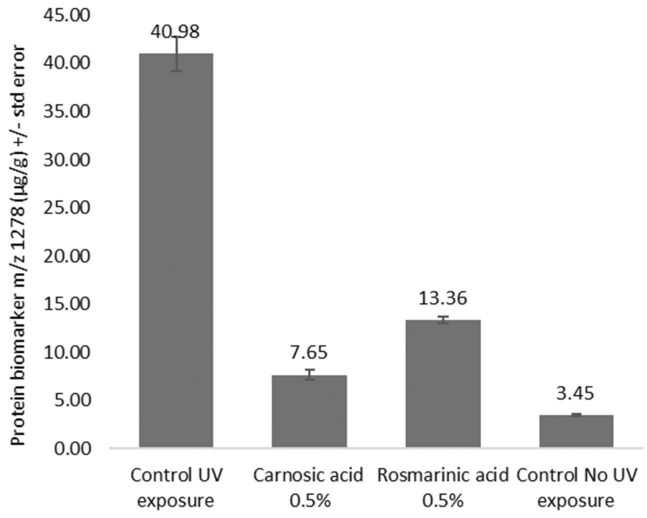


FIGURE 5 Protein biomarker formation on sunlight/UV exposure of hair treated with 0.5% carnosic acid and 0.5% rosmarinic acid ($N=9$). Controls were hair with no treatment and hair with no UV exposure. All test legs are statistically different from each other (99% confidence in a student *t*-test).

rosmarinic acid, which is the opposite to what was shown in the peptide data where rosmarinic acid was more efficient. This inconsistency is likely due to the added

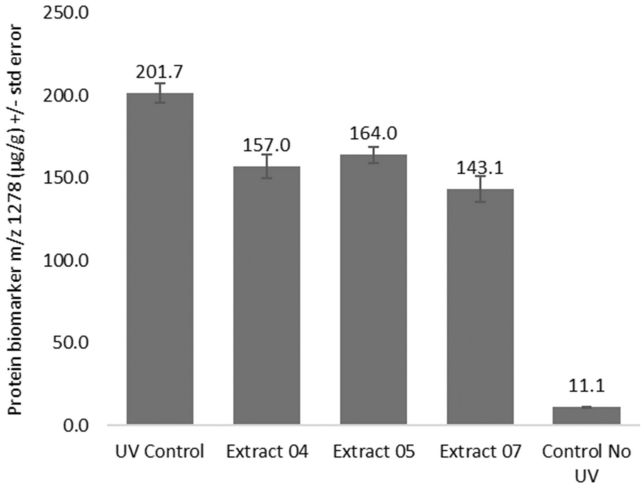


FIGURE 6 Protein biomarker formation on sunlight/UV exposure of hair treated with 0.1% rosemary extracts ($N=9$). Controls were hair with no treatment and hair with no UV exposure.

complexity of the hair which is a heterogeneous system in these experiments. This means other factors could be important for extract efficacy including penetration into hair and where the antioxidant is located in hair. Carnosic acid is more hydrophobic than rosmarinic acid and this may make it easier to penetrate into the lipid-rich parts of hair including the cell membrane complex.

In the second experiment, three extracts (extracts 04, 05 and 07) were chosen from the set of 10 to test on hair at 0.1% active in ethanol: water (50:50) giving a concentration of 0.001 g/hair. Extract 05 has the highest level of carnosic acid, Extract 07 has the highest level of rosmarinic acid and Extract 04 has a mix of both hydrophilic chemistries (including rosmarinic acid) and hydrophobic chemistries (including carnosic acid). The data show that all three extracts provide UV protection to hair with similar efficacy values (Figure 6). All three extracts have statistically lower protein biomarker than the UV control with no extract (significant to 99% confidence in a student *t*-test). All three extracts have similar protection to each other. These data show again there is not a high correlation of the peptide and ORAC/FRAP data with hair UV protection likely driven by the more complex heterogeneous hair.

Even though there is no strong correlation with ORAC/FRAP or peptide data, the initial hair experiments did show the rosemary extracts can deliver UV protection. The third experiment was to demonstrate if this benefit could be delivered by a leave-on treatment. Extract 06 was chosen as it was a medium performing extract based on the ORAC and FRAP data and was added to a cream leave-on treatment at 0.5%, 1.5% and 3.0% concentrations and applied on hair at 0.05 g/g. This

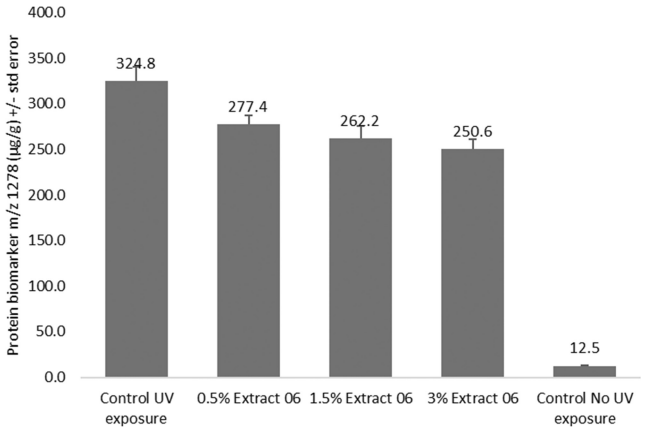


FIGURE 7 Protein biomarker formation on sunlight/UV exposure of hair treated with leave-on treatment containing Extract 6 at 0.5%. 1.50% and 3.0% ($N=9$). Controls were hair with no treatment and hair with no UV exposure.

is equivalent to 0.00025, 0.00075 and 0.0015 g of extract per gram of hair. All the three products showed a benefit versus no treatment and there was a dose-dependent increase in protection as level of botanical extract was increased (Figure 7).

CONCLUSION

A selection of rosemary extracts has been shown to protect hair against UV oxidative damage, either added as a solution or from a leave-on treatment. Analysis of the extracts showed they have a range of phytochemistries, and all have good antioxidant properties as demonstrated in ORAC and FRAP assays and in a peptide model system.

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CONFLICT OF INTEREST STATEMENT

Authors RF, MSJS, NV and VC have no conflict of interest to declare.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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