https://doi.org/10.31925/farmacia.2022.3.5 **ORIGINAL ARTICLE**

PHYSICO-CHEMICAL CHARACTERIZATION OF THE ANTIOXIDANT MIXTURE RESVERATROL-FERULIC ACID FOR APPLICATIONS IN DERMATO-COSMETIC PRODUCTS

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Abstract

The aim of the paper was to identify the best combination of resveratrol - ferulic acid with the purpose of increasing the antioxidant activity and maintaining the qualities of resveratrol in the management of skin disorders. Aqueous solutions of resveratrol (1%, 3% and 5% concentrations) and hydro alcoholic solution (30%) of ferulic acid were used. The following resveratrol - ferulic acid mixtures (v/v) were considered: 1:1; 1:2; 1:3 and 1:4. The antioxidant activity of the mixtures was determined and compared with that of resveratrol, and also the physico-chemical were performed by thermogravimetric analyses and UV-VIS spectrophotometry. The results underline that the mixtures of 1% and 3% resveratrol with 0.5% ferulic acid in a ratio of 1:1 and 1:2 ensure the improvement of the antioxidant activity of resveratrol.

Rezumat

Scopul lucrării a fost de a identifica cea mai bună combinație resveratrol - acid ferulic cu scopul de a potența activitatea antioxidantă, menținând calitățile resveratrolului, în gestionarea unor patologii cutanate. S-au folosit soluții apoase de resveratrol (având concentrația 1%, 3% și 5%) și o soluție hidroalcoolică (30%) de acid ferulic. Au fost luate în considerare următoarele amestecuri de resveratrol - acid ferulic (v/v): 1:1; 1:2; 1:3 și 1:4. S-a determinat activitatea antioxidantă a acestor amestecuri și a fost comparată cu cea a resveratrolului. Amestecurile au fost, de asemenea, caracterizate fizico-chimic prin analize termogravimetrice și spectrofotometrice UV-VIS. Rezultatele subliniază că amestecurile de resveratrol 1% și 3% cu acid ferulic 0,5%, în raport de 1:1 și 1:2 asigură îmbunătățirea activității antioxidante a resveratrolului.

Keywords: antioxidant activity, ferulic acid, resveratrol, dermato-cosmetic product

Introduction

Resveratrol (RV), like many other natural compounds with antioxidant activity, is among the most studied and recommended ingredients to combat oxidative stress in human body [1-6]. The significant amount of the developed studies concerning RV is focused on its internal use and specific mechanism of action on various levels in human body [7-9]. In the recent years resveratrol also gained special consideration as an antioxidant for skin disorders and dermato-cosmetic formulas [10-13].

From a dermatological perspective, the use of an antioxidant is currently a must, as the implication of oxidative stress in many commonly found and high-impact diseases is well-documented [1, 14]. Also, for the clinical and dermatological purposes, an association of antioxidant ingredients can be a powerful method to protect the skin and contribute to therapeutic success. The sensitive point and the concerns here are represented by the dose and the general amount of the administered antioxidant in order to prevent harmful effects.

From a chemical perspective, the focus is on the physical and chemical characteristics which can interfere in exerting an expected, high-performant antioxidant activity. Not in the least, as much experimental research has already proven, in certain associations, natural compounds had an improved antioxidant activity than individually [4, 5]. The challenge here is to establish the most efficient and safe concentration and association of natural antioxidants for a dermato-cosmetic formula.

On a first overview, it could seem an intriguing mismatch between, on a hand, the huge amount of literature about the benefits of resveratrol, the importance of dosage and the significant ground gained by anti-oxidants in the already impressively dynamic dermato-cosmetic industry, and on the other hand, the small number of studies on resveratrol for external use and

dermato-cosmetic products. If we put together the studies on all natural antioxidants for dermato-cosmetic products, the proportions remain the same. While for internal administration, RV concentration is considered to be safe at up to 5 g [8], for external use we can find products with 1%, 3% or maximum 5% RV formulation, but without scientific articles talking about specific resveratrol concentrations. Moreover, the RV-based or RV including cosmetics manufacture has a serious advance toward studies which could certify specific concentrations or associations.

On what basis are the current RV concentrations chosen? What stands in the way of new associations between well-known and cost-effective natural anti-oxidants? Are there any other question marks concerning RV in dermato-cosmetic products [15]? We know about poor solubility and bioavailability issues, but yet, proven and numerous health benefits exceed concerns already analysed or partially answered [16, 17].

Another widely spread natural phenolic compound is ferulic acid (FA), [18-20], a phenolic hydroxy-cinnamic acid used as therapeutic antioxidant in various health conditions including skin diseases. There is also a wide range of natural resources for FA, such as sugar beet, parsley, spinach, grapes, rice, oat, wheat, peanut, artichoke [18-20] and is considered to increase cellular antioxidant activity (free radical scavenger, inhibitor of free radical catalysis, enhancer of scavenger enzyme activity) [21]. Further the internal administration, FA is reported to exert anti-inflammatory effect, to regulate the expression of markers in autophagy, to have anti-carcinogenic and antidiabetic effect and to prevent or ameliorate cardiovascular diseases. In recent years, ferulic acid is used as an antioxidant in cosmetic industry, both as a therapeutic ingredient, but also as an auxiliary agent with antioxidant, anti-microbial and cross-linking agent. As an active ingredient in cosmetic products, FA has an immense potential, as a protector against UV rays, anti-photo aging and brightening agent. It is considered to inhibit melano-genesis, to enhance angiogenesis and to promote healing processes. It has also an extra advantage of low toxicity, but there is a limitation in its use, being rapidly oxidized. It is used alone or associated with vitamin C and E.

The aim of this article is to provide new insights into experimental research and understanding the RV behaviour dose-depending and the association with another antioxidant. The general objective is to establish and characterize the best RV + FA association in terms of RV concentration and optimum proportion of the compounds, in order to enhance the antioxidant activity, preserving RV qualities, so as to enable the best contribution in skin disorders management. The evaluation of the efficiency of the analysed mixtures was made based on the measured antioxidant activity compared to that of the individual RV. Also, the mixtures were physical-chemical characterized using UV-Vis spectrophotometry and thermogravimetric analyses.

Materials and Methods

Materials

Reference substances were RV and FA with analytical purity degree. Also, standard reagents for radical scavenging assays – DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2'-azino-bis-(3-ethyl-benzo-thiazoline-6-sulfonic acid)) of analytical purity and purchased from Sigma Aldrich were used.

Methods

Determination of Antioxidant Activity

The mixtures under study consisted of RV aqueous solution in concentrations of 1%, 3% and 5% and hydro-alcoholic (30%) solution of FA 0.5% in a ratio (v/v) of 1:1, 1:2; 1:3 and 1:4, respectively.

DPPH method

The DPPH assay was prepared according to Brand-Williams recommendations, with slight modifications [22, 23]. The stock solution was used at a 0.1 mM final concentration: 2 mg of DPPH reagent were suspended in 50 mL of ethanol 96%.

From the tested samples, 50 µL was taken and then a volume of 200 µL of DPPH ethanolic solution was added on 96 well microplate. The mixture was incubated for 30 min and kept at room temperature in the dark. Experimental data were acquired on a multimode microplate reader, Tecan 5082 (Austria GmbH), set at 517 nm using ethanol as blank sample. α-tocopherol ethanolic solution (1 mg/mL) was used as a positive control.

The DPPH radical scavenging ability of the samples was calculated as the inhibition percentage (I %) using the equation 1:

$$I\% = ((A_0 - A_t)/A_0) \times 100, \quad (1)$$

where: A_0 = the absorbance value of the DPPH radical and ethanolic solution of 0.1 mM (without extracts); A_t = the absorbance value of DPPH radical and the tested samples.

ABTS method

ABTS assay was carried out according to a previously published method [24, 25]. The $ABTS^{\bullet+}$ free radical was generated by treating the aqueous solution of 2,2'-azino-bis-(3-ethyl-benzo-thiazoline-6-sulfonic acid) ABTS (7 mM) with ammonium persulfate (2.46 mM). This stock solution was kept during 12 to 16 hours in the dark and at room temperature in order to allow chemical equilibrium. The $ABTS^{\bullet+}$ solution was then diluted with ethanol (approximately 1:90, v/v) in order to obtain an absorbance of 0.7 ± 0.02 at 734 nm. Absorbance measurements were performed on a multi-mode microplate reader, Tecan 5082 (Austria GmbH). The reaction mixture was prepared by mixing 50 µL of each sample and 250 µL $ABTS^{\bullet+}$ working solution. The mixture was left to stand for 6 min, after which the absorbance at 734 nm was measured using ethanol as

blank sample [26-29]. α-tocopherol ethanolic solution (1 mg/mL) was used as a positive control. The $ABTS^{\bullet+}$ radical scavenging ability of the samples was calculated as the inhibition percentage (I%) using the equation 2:

$$I\% = ((A_0 - A_t)/A_0) \times 100, \quad (2)$$

where: A_0 = the absorbance value of the ethanolic solution of $ABTS^{\bullet+}$, A_t = the absorbance value of the analysed samples.

Thermogravimetric analysis

A Mettler Toledo TGA-SDTA851^e device was used to analyse thermogravimetric behaviour. Thermogravimetric (TG), derivative thermogravimetric (DTG) and differential thermal analysis (DTA) curves were recorded in inert atmosphere (N_2) at a 20 mL/min flow rate and 10°C/ min heating rate, within the 25 - 700°C temperature range. The samples subject to thermal analysis weighed 1.9 to 3.5 mg. The reproducibility of data acquisition was checked by repeating the determinations in identical working conditions. Mettler Toledo's STAR software was used to process the curves in order to determine the thermal characteristics.

UV-VIS spectroscopy method

The UV-VIS spectra for the RV, FA and mixture of RV-FA were drawn with a Jasco UV 550 VIS spectrophotometer.

Results and Discussion

Evaluation the antioxidant activity

For the evaluation of the antioxidant activity, *in vitro*, the scientific literature presents numerous methods, the difference between them consisting mainly in the mechanism of action (CUPRAC), $ABTS^{\bullet+}$ or TEAC, FRAP, DPPH, ORAC, TRAP [4, 5, 30]. Even if it is based on different mechanisms, we have chosen for the evaluation of antioxidant capacity the $ABTS^{\bullet+}$ or TEAC method (Trolox equivalent antioxidant capacity) and DPPH (free radical scavenging properties by diphenyl-1-picrylhydrazyl radical) methods to avoid biased results that can be obtained with only one method, so to rely on solid information on tested antioxidant mixtures, useful information in further studies. The DPPH method is known to provide lower Trolox-related values than the $ABTS^{\bullet+}$ method due to the higher stability (and thus lower reactivity) of the DPPH radical. The results obtained with the two methods are dependent on the reduction potentials involved (-1.2 V for DPPH and -0.67 V for $ABTS^{\bullet+}$) [31]. Therefore, the antioxidant spectra caused by DPPH and $ABTS^{\bullet+}$ are partially different [31].

The studied mixture was evaluated in terms of antioxidant capacity by the selected two methods. The values obtained were compared with those of the pure RV component. The results obtained from both test methods are presented in Table I. All the determinations were performed in a triplicate and the results were expressed as arithmetic average ± standard deviation (SD, σ).

The values presented in Table I indicate an obvious increase in the antioxidant capacity of RV by mixing with FA. The comparative analysis of the inhibition percentage values, characteristic of the two used methods, which are directly correlated with the antioxidant capacity, provides indications about which mixture is most feasible in terms of practical efficiency in dermatological products correlated with the cost-profit principle.

Table I

Antioxidant capacity values for mixtures RV-FA using the DDPH and $ABTS^{\bullet+}$ methods

Mixture RV + FA	Ratio (v/v)	**$ABTS^{\bullet+}$**	**DPPH**
		I% ± σ	**I% ± σ**
RV 1%:FA 0.5%	R1 1:1	94.602 ± 0.117	87.977 ± 0.3241
	R2 1:2	94.856 ± 0.059	87.929 ± 0.140
	R3 1:3	95.194 ± 0.059	87.743 ± 0.324
	R4 1:4	94.450 ± 0.155	85.871 ± 0,.292
	RV1%	93.909 ± 1.0599	72.444 ± 1.210
RV 3%:FA 0.5%	R5 1:1	95.279 ± 0.406	87.929 ± 0.140
	R6 1:2	95.025 ± 0.406	86.947 ± 0.421
	R7 1:3	94.416 ± 0.442	87.228 ± 0.506
	R8 1:4	94.145 ± 0.7619	79.275 ± 4.801
	RV3%	94.788 ± 0.422	78.8070 ± 1.725
RV 5%:FA 0.5%	R9 1:1	95.076 ± 0.2686	86.807 ± 0.643
	R10 1:2	94.924 ± 0.465	87.041 ± 0.214
	R11 1:3	94.619 ± 0.203	86.199 ± 0.531
	R12 1:4	94.958 ± 0.211	83.813 ± 1.627
	RV5%	94.518 ± 0.352	66.316 ± 1.2406

In this context, the mixtures that attract attention for further studies are those consisting of 1% and 3% RV - 0.5% FA in a ratio of (v/v) 1:1. Their performances in terms of "antioxidant activity" are almost similar, the criterion that will make the difference being the therapeutic properties of the final dermato-cosmetic preparation and the ratio "final product-benefit".

Physico – chemical characterization of the studied RV + FA mixture

The mixture of RV + FA with the best antioxidant activity was characterized by physico-chemical methods in order to identify potential chemical interactions between the two components. Thus, it was used UV-VIS spectroscopy and thermogravimetric analysis.

Thermogravimetric analysis

The TG and DTG thermogravimetric curves represented in Figure 1 enabled us to centralize the main thermogravimetric characteristics in Table II: T_{onset} – temperature of the thermal degradation onset in every stage, T_{peak} – temperature at which the degradation rate reaches its maximum, T_{endset} – temperature of the thermal degradation endset in every stage, W% – percentage mass loss in each stage and the amount of residue remaining at 700°C.

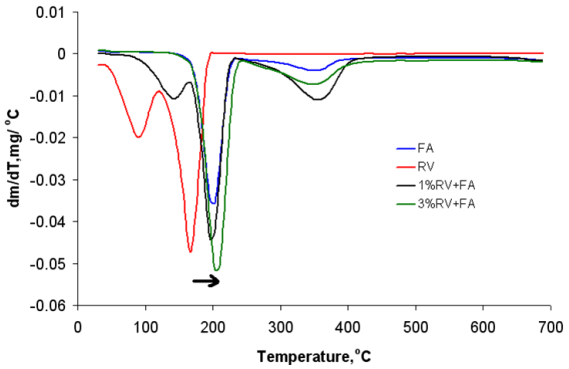

Figure 1.
Comparative DTG curves

Table II
Main thermogravimetric characteristics

Sample	Stages	T_{onset} (°C)	T_{peak} (°C)	T_{endset} (°C)	W (%)	Residue
FA	I	182	200	215	61.32	3.75
	II	315	355	377	34.93	
RV	I	59	91	107	35.86	3.39
	II	140	167	178	60.75	
1% RV + FA (1:1)	I	117	143	160	17.73	10.21
	II	184	198	212	38.86	
	III	308	357	386	33.20	
3% RV + FA (1:1)	I	184	207	220	56.14	6.48
	II	281	351	382	37.38	

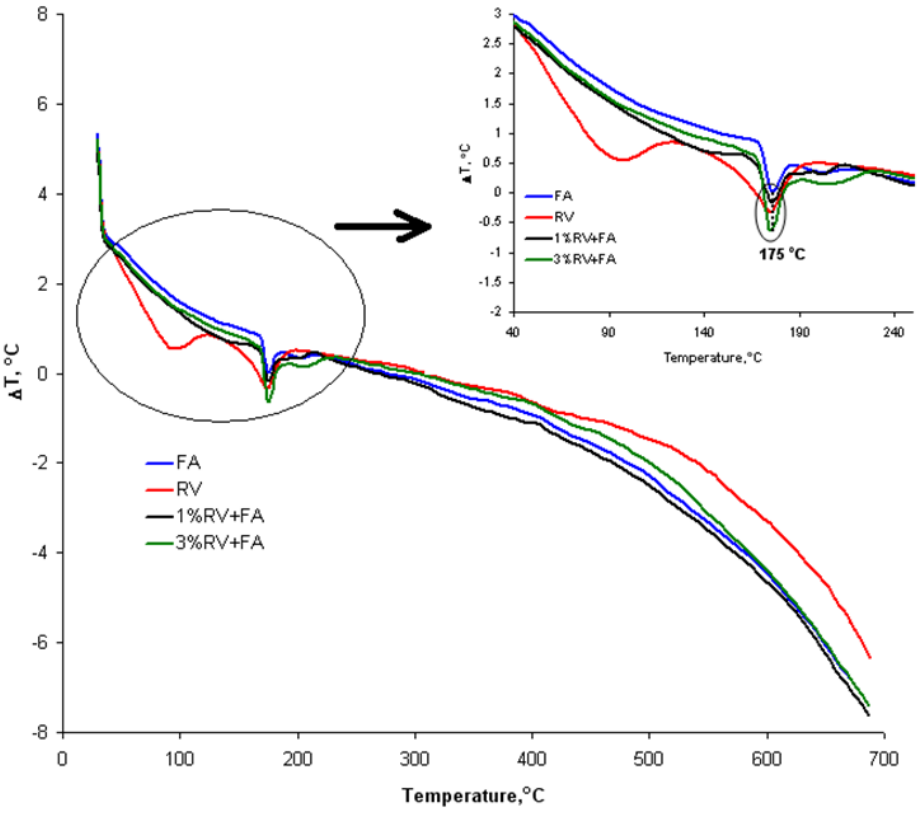

Figure 2.
Comparative DTA curves

As shown in literature, the thermal decomposition of ferulic acid occurs in an inert atmosphere in two stages [32, 33]. The decarboxylation process takes place in the first stage, the peak degradation rate temperature being 200°C and the mass loss being 61%. 4-hydroxy-3-methoxystyrene results in these stages [32, 33], the decomposition onset temperature of which is 315°C. The T_{peak} temperature for this stage is 355°C and the mass loss is approximately 35%. These two degradation stages that have close thermal characteristics are also identified in the case of the samples marked "1% RV + FA" and "3% RV + FA", highlighting very clearly the presence of FA. Figure 2, which shows the DTA curves for the recorded samples, also shows the melting temperature of FA, which is around 175°C [34].

The thermogravimetric curves recorded for the RV-containing sample marked "RV" indicate two endothermic processes, the first within the 59 - 107°C temperature range, which corresponds to water evaporation, and the second within the 140 - 178°C range with a mass loss of about 61%, which corresponds to butylene glycol evaporation. According to the literature data, the thermal decomposition of RV in inert atmosphere (nitrogen) takes place mainly within the 240 - 420°C temperature range, with a mass loss of about 50% [35]. The thermogravimetric curves recorded for sample "RV" do not show significant mass losses within this temperature range, due to the presence of an extremely small amount of RV in the solution.

Thermal analysis reveals three stages in the case of sample "1% RV + FA" containing 1% RV in a 1:1 mixture with FA. As already mentioned, the last stages correspond to the thermal degradation of FA. The first stage within the 117 - 160°C temperature range may correspond to the removal of solvent traces.

The thermal decomposition in inert atmosphere of sample "3% RV + FA" containing 3% RV in a 1:1 mixture with FA takes place in two stages, which have thermal characteristics close to those of FA. However, we found that the peak degradation rate temperature in the first stage in which the process of decarboxylation occurs is shifted to higher temperatures (T_{peak} = 207°C), suggesting possible interactions between RV and FA. We also found larger residue quantities by a few percent in the samples of the two mixtures, compared to those obtained for FA and RV.

This analysis led to the conclusion that shifting the T_{peak} temperature in the first decomposition stage to temperatures 7°C higher in sample "3% RV + FA" compared to FA may suggest low interactions between FA and RV.

UV-VIS spectroscopy

To obtain the UV-VIS spectra, aqueous solutions of RV and hydro-alcoholic (30%) FA were used, the components having the same concentration: 3.33 x 10^{-4} %.

The analysis of the spectra does not show significant changes in the spectrum of the mixture of compounds, but only attenuations of the maximums of the characteristic bands of individual compounds, which suggests that there are no chemical reactions between the components of the mixture but only physical associations.

This observation is in agreement with the conclusion of the thermogravimetric analysis and responds to some aspects pointed out in the introduction of this article about of the opportunity of this mixture of antioxidants. Based on the scientific published works supporting topical application as a more convenient way to benefit from antioxidant activity of RV and AF [36], many associations between antioxidants have emerged: RV (1 % to 5%) is associated with vitamin C, E, baicalin, catechine, bisabolol etc. and ferulic acid (0.5 to 1%) [21] with vitamin C or E, in order to increase the antioxidant potential and stability of the bioactive compound.

However, despite the evidence and references to the advantages of the combinations, there are no available data on the superiority of certain concentrations and the ratio of the associated ingredients. At the same time, the data on the concentrations chosen in the products available on the market have a weak scientific basis, with limited investigations of formulas.

Conclusions

Therefore, this paper systematically studies mixtures, different concentrations, ratios, as well as their physico-chemical stability, to create a scientific basis for the recommendations related to the association of RV:AF in a dermatological-cosmetic formula that we will develop and further analysis with anti-inflammatory, antioxidant and protective activity.

In this direction, experimental studies aimed at improving the antioxidant capacity of RV while maintaining its recognized biologically active properties have led to the hypothesis that mixing with FA is extremely beneficial. Thus mixtures of 1% and 3% RV with 0.5% FA in a ratio of 1:1 and 1:2 proved to be effective in this direction. The criteria that will influence the choice of a mixture in practical use will also take into account the therapeutic properties of the final dermato-cosmetic preparation and the "final product-benefit cost" ratio.

Based on the experimental results, we can consider dermato-cosmetic formulation a way forward for the biological effects valorisation of RV - FA mixture. With a better insight on ingredients concentration and mixture ratio for the best antioxidant activity, we gained higher perspective of meeting criteria related to superior therapeutic efficacy, but also good patient compliance and stability.

Conflict of interest

The authors declare no conflict of interest.

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