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ARGUMENTS FOR VITILIGO ANTI-OXIDATIVE STRESS PROTECTIVE/RESTORATIVE THERAPEUTIC MANAGEMENT WITH SELECTED PLANT EXTRACTS FROM ROMANIAN NATIVE FLORA

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Vitiligo is a disease characterized by localized melanocyte extinction leading to restricted epidermal depigmented macular lesions.
Etiopatogenetically, this multifactorial polygenic disorder compounds genetic and epigenetic determining factors, set off by several incompletely recognized environmental stressors that induce REDOX imbalances activating several mechanisms of melanocyte destruction.
Albeit vitiligo equally affects both sexes in cca 1% of the world population, its therapeutic management is still flawed by uncertain efficacy and persistent negative side-effects; demanding identification of new, better therapeutic agents.
The aim of our study was the activity-based, data-driven identification of plant species candidates for extracts usable in anti-oxidative protective and restorative therapeutic management of vitiligo.
We performed a targeted data survey examining the known phytochemical profiles of a comprehensive group of previously not used Romanian native plants with specific ecological and agronomical characteristics, rich in flavonoids and their derivatives we considered as a melanocyte-protective best-suited class of antioxidant compounds.
The advanced selection of the plant species was performed based on the following criteria:
(i) the phytochemical profiles,
(ii) well-balanced activity-matching antioxidant, vulnerary and antimicrobial properties,
(iii) multiple, accessory, ecological, horticultural/agrotechnical and other collateral pragmatic criteria.
Based on these criteria, we argument herein the selection for further bio-molecular assessment of 2 Romanian Crassulaceae native species.

Keywords: Vitiligo therapeutic management, oxidative stressors,Flavonoids, *Crassulaceae*, phytotherapy.

INTRODUCTION

Vitiligo is a disease characterized by localized melanocyte extinction leading to restricted epidermal depigmented macular lesions. Etiopathogenetically, this multifactorial polygenic disorder compounds genetic and epigenetic determining factors, set off by several incompletely recognized environmental stressors that induce REDOX imbalances activating several mechanisms of melanocyte destruction.

Melanocytes from vitiligo patients appear to be unable to efficiently regulate the oxidative imbalances caused by the environmental and physiological physical factors, and so bear physiological, biochemical and morphological damage incompatible with normal cellular life, ultimately leading to discontinuous irregular lesional patches of discolored tegument[1]

Albeit vitiligo equally affects both sexes in cca 1% of the world population[2], its therapeutic management is still flawed by uncertain efficacy and persistent negative side-effects; demanding identification of new, better therapeutic agents.

The aim of our study was the activity-based, data-driven identification of plant species candidates for extracts usable in anti-oxidative protective and restorative therapeutic management of vitiligo.

Melanocytes' death is caused by an imbalance in the REDOX status of the cell.

Already since 1997, one in-vitro study[3] demonstrated the presence of an imbalance in the anti-oxidant system in cultured melanocytes from

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vitiligo patients and showed that the degree of free-radical toxicity correlated strictly with the anti-oxidant status in the cellular environment, suggesting that the alteration in the antioxidants was the basis for sensitivity to the external oxidative stress, thus providing support for the hypothesis that the initial pathogenic event in melanocyte degeneration in vitiligo was a free radical-mediated damage.

As the primary pathogenetic mechanism in vitiligo involves the oxidative REDOX imbalance ultimately responsible for melanocyte destruction, the most logical way of counteracting it in the protective approach in the therapeutic management of vitiligo would be the restoration of the local and systemic cellular REDOX balance by the introduction of antioxidant and radical scavenger compounds in the local environment, by either topic or systemic input –as was already proposed as early as 1995 by Passi *et al.*[4]

FLAVONOIDSHAVE PREVIOUSLY DOCUMENTED USES IN VITILIGO THERAPEUTIC MANAGEMENT

Flavonoids are polyphenolic compounds, in which a C6-C3-C6 “aglycone” core (Figure1) often glycosylated to the most frequently natural form in which flavonols occur in plants, a glycoside bound form.

Flavonoids have many interesting biological properties, but the ones that suscitate the greatest interest for vitiligo management are their antioxidant, free-radical scavenging, anti-inflammatory and antimicrobial properties. Many flavonoidic compounds are present ubiquitously in plants and in many dietary products –*e.g.* natural beverages like wine, beer, tea; the pigmentary layers of pungent onions, colored fruits – cranberries, blueberries; all Citrus fruits – especially in the peels; cocoa and dark chocolate among others.

Based on their antioxidant activity, flavonoids have previously been proposed as agents in the treatment of vitiligo patients[5], so we have chosen this rich class of antioxidant compounds, ubiquitous in many plant families, for our screening.

One of the most and best studied flavonoid is quercetin, for which both in vitro and in vivo evaluatory tests have been performed for the treatment of vitiligo[6]. Various studies documented the protective effect of quercetin against oxidative damage in keratinocytes and melanocytes, some also suggesting its utility in oral therapy of vitiliginous patients[7,8], and also that topical application of quercetin has protective effect against cellular damage by UV radiation[9].

Flavonoids effect their antioxidant activity via multiple intertwined mechanisms[10,11],of which we hereby emphasize 2 very importantones:

(i) suppression of ROS formation by chelating trace metals involved in free radical generation;

(ii) scavenging ROS; and RNS.

The antioxidant capacity of flavonoids is strongly influenced by the number and position of functional groups substituents.

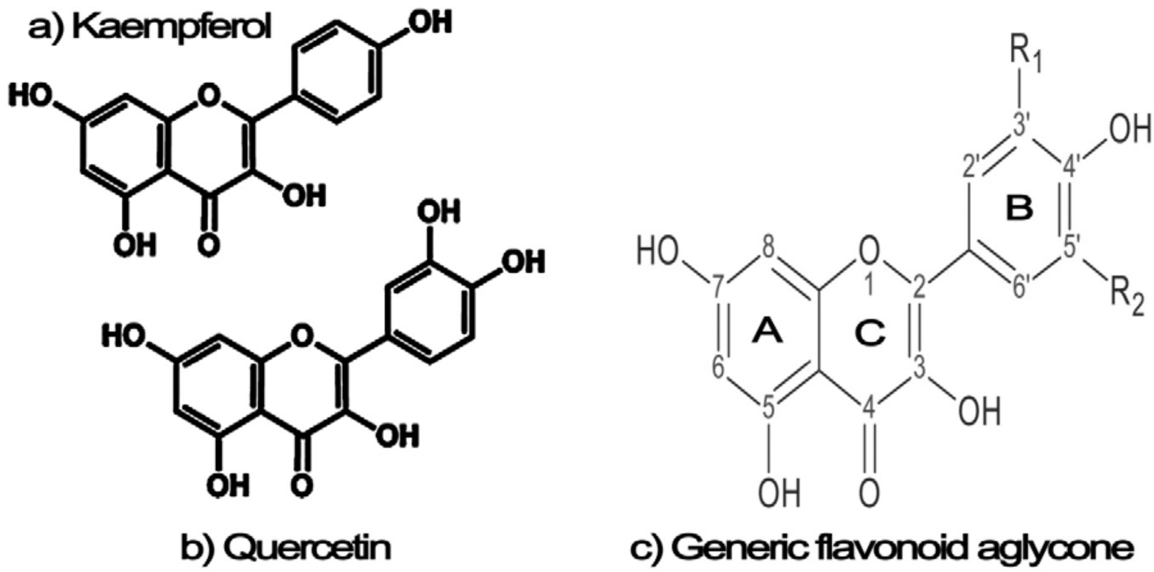

Figure 1. Flavonoid structures; a) Kaempferol; b) Quercetin; c) Generic flavonoid aglycone.

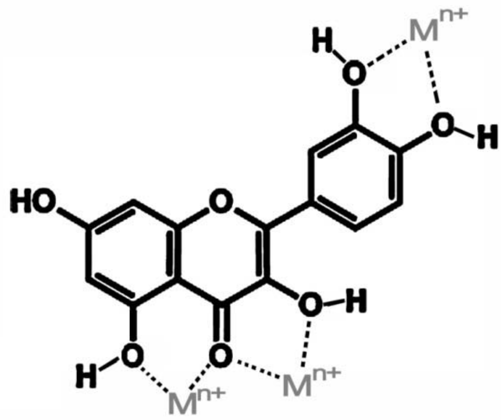

Figure 2. Metal chelating capacity of quercetin.

It is especially their radical scavenging and metal ion chelation ability is significantly modulated by the configuration, substitution, and total number of hydroxyl groups[12,11].

The presence of – hydroxyl substituents in the 5 of ring A and 3 of ring C – as in quercetin and kaempferol – significantly increases the chelating capacity of flavonoids as depicted in Figure 2

By presenting this particularly favorable structure, both quercetin and kaempferol show potential metal-chelating properties, and actually quercetin show significant iron-chelating and iron-stabilizingproperties[11]. Trace metals bind at specific positions of different rings of flavonoid structures[13].

Quercetin has over kaempferol (Figure1) an additional advantage conferred by the 3',4'-catechol structure in the B-ring which enhances the chelating capacity adding an additional site, as depicted in Figure 2.

The B ring acts as hydrogen and electron donor to hydroxyl, peroxyl, and peroxynitrite radicals, thus stabilizing them and converting themselves in the process into relatively stable flavonoid radicals. Hence their hydroxyl configuration of the B ring influences dramatically their ROS and RNS scavenging capacity[14].

Conversely, by having lower redox potentials, the flavonoids having a 3',4'-catechol structure in the B ring – like quercetin –, have considerable reductive capacity, reducing by hydrogen atom donation the highly oxidizing free radicals (redox potentials in the range 2.13–1.0V) such as superoxide, peroxyl,alkoxyl, and hydroxyl radicals[11] via the reaction in (Figure 3).

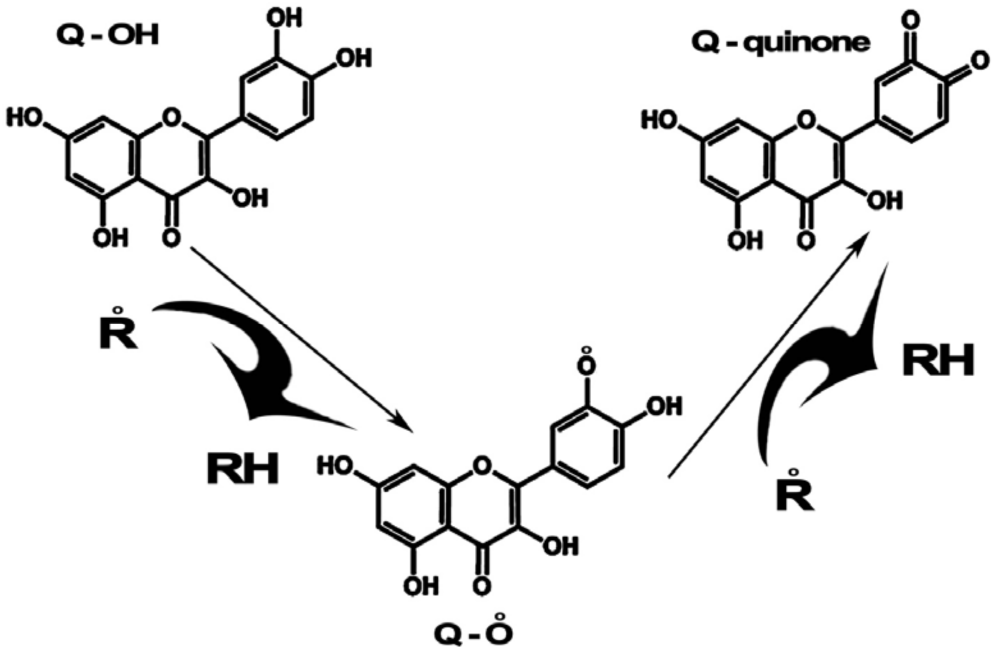

Figure 3. Free radical scavenging steps of quercetin.

Another interesting aspect is the antagonistic effect of glycosylation of flavonoids. It is generally accepted that aglycones are usually stronger antioxidants than their glycosides, but the bioavailability of flavonoids glycosylated with glucose rests is generally thought to be higher, and it is specifically the glycosidic forms of flavonoids that constitute the highest proportion of flavonoids, with glycosidicmoieties most frequentlyoccurring at the 3- or 7-position.[11]

The relationships structure-antioxidant capacity exposed above warrants a privileged position for quercetin and kaempferol as main flavonols, preferably with minimal glycosylation for topical use.

GENUS SEMPERVIVUM AND ITS PHYTOCHEMISTRY

The genus *Sempervivum*has a wide Eurasian range, from the mountains south of the Caspic Sea in Iran and Turkmenistan to Spain, France and Ireland in the West, and from Norway's Svalbard islands (cultivated) to High Atlas Mts of Morocco in the South. In Romania it is represented by several species, of which S.*marmoreum* Griseb occurs along with other medicinally-important Crassulacean species studied by[15,16,17,18,19,20,21,22,23,24,25,26,27], over the entire country.

It is important to emphasize that the Romanian populations show a great ecological amplitude, occurring in diverse various habitats from the Danube banks to the highest altitudes in the Romanian Carpathians, (between 50–2550 m a.s.l.).

Romanian populations show declining trends amplified by indiscriminate collection from natural stands by "traditional healers" and small-scale providers of "natural remedia"[15]. Despite this, the species is not protected and could benefit from industrial cultivation as it would decrease the pressure on natural populations.

Romanian ethnoiatric tradition recommends the use of the fresh juice squeezed extemporaneously in the ear from leaves of *S. marmoreum* Griseb (and of other members of the genus Sempervivum s.l.) to alleviate pain and inflammations caused by the external and medial otitis. Even the vernacular Romanian names for the plant mentioned by Pantu Z.[28], as "urechelnița" or "iarba de ureche" (meaning "ear herb" or "herb for the ear") illustrate this widely known usage, Stanciu A. et Niculae M.[27] mentioned other interesting domestic uses for one of its close relatives Jovibarbaheuffelli.

The phytochemistry of *Sempervivum sp*., is mostly known from research done on *S. tectorum* L., as summarized in SašaS. *et al.*[29] who attributes the benefits of juice and tea prepared from the leaves of *S. tectorum* among othersto the presence of polyphenols, phenol carboxylic acids and flavonoidic compounds.

For both *S. tectorum* and *Jovibarba heuffelli*, the presence in the leaf juice of a host of potentially useful bioactive compounds was documented[30,31,32]...

Already long ago Stevens J. *et al.* in 1996[33] and Abram V. *et al.*[34] reported isolation of glycosides of kaempferol, quercetin, isorhamnetin, scutellarein, myricetin, herbacetin, proanthocyanidins (prodelphinidin and procyanidin) and delphinidol from *Sempervivum* sp. extracts.

Steven *et al.* in 1996[33] found that the only flavonols in Sempervivum are kaempferol and quercetin.

Blázovics *et al.*[30] report in 2000 that the main characteristic monosaccharides in *Sempervivum* sp. are rhamnose, arabinose, xylose, mannose, and galactose.

Kery *et al.*[35] found that the polyphenols and flavonoids contents of *Sempervivum* sp. leaf extracts reach as much as 0.70% and 4.2%, respectively.

Superoxide scavenger activity of *S. tectorum* extract in vitro was reported as early as 1993 by Blázovics *et al.*[32], and i*n vivo* and *in vitro* antioxidant activity was documented by the same group[32], and also more recently by Kekesi *et al.* in 2003.[36]

Previous studies on *S.marmoreum* L. (sic!) documented that whole extracts from leaves from southern Serbian populations have anti-oxidant properties[29].

MATERIALS AND METHODS

We performed a targeted data survey of the pertinent literature in the available databases PubMed,

The evaluatory process followed a multistep algorithm consisting of the following phases:

1) evaluation of the known etiopathogenic molecular pathways involving radiative stressors; -it indicated that REDOX imbalance of the dermal and adjacent tissues as a primary targetable etiopathogenic mechanism.

2) evaluation of available data to identify the best-suited class of antioxidant compounds usable to counteract the REDOX imbalance; -it suggested that the flavonoids and their derivatives are the best suited chemical agents to protect and/or restore the melanocytes.

3) preliminary evaluatory screening of a comprehensive group of plant species candidates previously not used in specific procedures of clinical therapeutic management of vitiligo, based on composition and content of flavonoids.

4) an advanced selection of final candidate plant species, based on complex criteria as follows.

Advanced complex criteria for selection of finalplant species usable as sources of flavonoids:

a) **Phytochemical profile**ensuring adequate active substances content, bioavailability and ease of extraction, formulation, and conditioning for topical application and/or systemic administration. Priority was given to flavonoids with 3',4'-catechol in ring B; to flavonols hydroxylated in positions 5 of ring A and 3 of ring C, and with minimal glycosylation of the aglycone moieties.

b) **Previous clinical experience** or indications of use in academic, medical or traditional/ ethnoiatric settings

c) **Availability in Romanian or European/ Eurasian flora** assures that these species are relatively safe to cultivate industrially

d) **Invasive potential and biodiversity concern** they should have none/minimal invasive propensity and pose no potential threat against local native biodiversity

e) **Ecological horticultural/agronomic characteristics**, assuring relatively easier (industrial) cultivation and higher yields as medicinal crops with minimal costs and effort,

f) **Collateral aspects** involving the spp of interest, e.g.:

– known or potential ***ornamental function***,

– known or potential ***human habitat-improving*** plants,

– known or potential ***conservation importance*** of the plants themselves and/or of other species of conservationimportance – e.g. food plants, habitat constituents or providers etc.

RESULTS AND DISCUSSION

The first two steps of the study resulted in the following main conclusions:

1) the analysis of the known etiopathogenic molecular pathways involving radiative stressors, indicated that the REDOX imbalance of the dermal and adjacent tissues was the primary targetable etiopathogenic mechanism;

2) the evaluation of available data antioxidant compounds usable; -it suggested the flavonoids and their derivatives as the best-suited class of chemical agents to counteract the local tissular REDOX imbalance to protect and/or restore the melanocytes for the following reasons:

– relative good water solubility

– good bioavailability,

– well-known metabolic pathways,

– inexistent or low acute or chronic toxicity in humans at the applicable dosage,

– good proven biometabolic and excretory clearance in case of overdose,

– compatible with both topical and systemic use

In the following steps, according to the complex set of criteria, and based on our previous experience the advanced selection process leads us to the initial proposal of several species from the native genera Sempervivum, Jovibarba, and Hyllotelephium (Crassulaceae) which satisfy these criteria and moreover present some additional advantages as enumerated below.

The final selected species of our study are *Sempervivum tectorum* L (cultivated) and *S. marmoreum* Griseb, for which the existing data are most comprehensive and suggest both high efficacy and absent or minor safety issues for the doses normally used.

The phytochemical profile is qualitatively and quantitatively adequate in terms of flavonoid composition and content.

Detailed chemical surveys' findings summarized in Stevens *et al.*[33] show that Sempervivum (and the closely related genus Jovibarba) have quite a uniform array of flavonoids with kaempferol as the principal flavonol in all studied species, which simplifies the burden of side-effect assessment making these genera very attractive for selection of plants as flavonoids sources.

By contrast, the same authors found a much higher variation of flavonols in Eurasian *Sedum*s pecies, derived mostly via 8-hydroxylation and 8-*O*-methylation, posing harder questions about their potential side-effects. Supplementary, the phytochemical profile is interesting as it provides additional compounds that have been demonstrated to have beneficial action in the therapeutic management of dermal lesions as further described.

Besides the good yield of polyphenols and flavonoids in Sempervivum sp. leaf extracts**,** Kéry *et al.*[35] found some 11.2% polysaccharides also potentially further increasing the antioxidative and reductive capacity of leaf extracts of Sempervivum plants.

Previous documented "antimicrobial activities against *Aspergillus niger* and *Candida albicans* only but not against the tested bacteria" by[29] for leaf whole extracts from southern Serbian *S. marmoreum* L. (sic!) populations suggest that these extracts could be safely used in topical applications where besides their anti-oxidant properties would be complemented by antiseptic effects.

The elemental analysis provided in Blazovics A. *et al.*[30] showing as main cations are Ca (76.2 mg/g), K (40.47 mg/g) and Mg (817.85 mg/g), and no toxic elements in *S. tectorum* extracts[37] recommends also the related species S. marmoreum as a candidate drug source.

Blazovics group also documented in a rat model system a lipid-lowering, free radical scavenger, membrane-stabilizingeffects of *S. tectorum* extracts suggesting liver-protecting effects after oral intake[38].

The modulation of the clastogenic effect of cytostatic CCNU on rat nucleated blood cells documented by Barca[16] for whole leaf extracts from *S. marmoreum* Griseb., together with a slight intrinsic clastogenic effect, indicates additional areas of interest for this plant, but also raises some concerns. The quinone methides of flavonols are apt to react by nucleophilic attack with DNA forming potentially pro-mutagenic adducts[39] and this might explain the slight intrinsic clastogenic activity observed[16]. The propensity for this behaviour is pH-dependent though[40], making these processes far from simple, but thus suggesting ways to manipulate this behaviour and minimize the negative side-effects

These concerns warrant further investigation, but, since fresh *S. marmoreum* leaves are used in the human diet, prepared fresh as salads since immemorial times without any reported issues, the toxicity issues at usual doses should be absent.

In terms of ecological constraints, *Sempervivum* species are able to thrive inhabitats with diverse climate and pedology[19,16] but have a preference for mostly dry habitats in rocky landscapes, which ensures successful cultivation in dry and warm climates and in urban settings, on brownfields and green roofs, thus providing consistent and inexpensive ground cover in anthropic degraded habitats, in the eve of climatic changes.

Several studies provide consistent evidence to support their good ecological, horticultural, and agro-technical fit-for-the-purpose, as follows.

Also, in terms of **invasiveness and biodiversity concern**, all selected species are native to Romanian local flora[19] and are noninvasive under normal circumstances.

In terms of conservation importance, some of the selected species are themselves rare, localized, and/or somewhat threatened in Romania and/or Europe[15], so their cultivation would reduce the sozological pressures involving them.

Moreover, most of the selected species are themselves unique or alternate food-plants for insect species that are rare/extremely rare and threatened and protected in many countries in EU and worldwide like *Parnassius apollo*, or *Scolitantides orion* Pallas (see also[22]) so their cultivation could benefit also the insect species depending on them.

Besides these very rare and localized Lepidoptera, insect herbivory is very low on Sempervivum, and the only natural herbivore specialized in Sempervivum with significant populations is a weevil – Aizobius sedi Germ. (Apionidae, Curculionoidaea). Like in Hyllotelephium[21], this herbivore is rare, and the crop losses it could sensibly produce are insignificant.

CONCLUSIONS

We hereby report the selection of 2 Crassulacean candidate species for further investigation of their bioactivity as phytotherapeutic antioxidant remedies usable in the protective and restorative therapeutic managment of vitiligo. The final selected species of our study are *Sempervivum tectorum* L (cultivated) and *S. marmoreum* , for which the existing data are most comprehensive and suggest both high efficacy and absent or minor safety issues for the doses normally used, necessitating only minor processing for topical application, with possibilities of ample industrial cultivation in both intensive and extensive green-roof settings and also on reclaimed brownfields. These species' intensive cultivation could also benefit 2 Lepidopteran threatened species and also reduce the pressure on the natural populations.

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