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FELLOWSHIP FINAL REPORT

Strategies to enhance cosmeceuticals in *in vitro* cultures of herbal plants

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ABSTRACT

Herbal plants accumulate large amounts of phenolics and pentacyclic triterpenes. The present research project deals with the *in vitro* culture induction from stem and leaf explants of several medicinal plant species of Centre-Val de Loire under various plant growth regulators (PGRs) for the production of antioxidant and anti-ageing compounds. Among all the tested PGRs, auxins and cytokinins used alone or in combination induced callogenesis in stem/leaf-derived explants. Callus culture displayed feasible total phenolic content and antioxidant activity under optimum hormonal combination. HPLC analysis revealed the presence of plectranthoic acid, oleanolic acid, betulinic acid, caffeic acid and rosmarinic acid. Complete antioxidant and anti-aging potential of extracts with very contrasting phytochemical profiles were investigated. Correlation analyses revealed rosmarinic acid as the main contributor for antioxidant activity and anti-aging hyaluronidase, advance glycation end-products inhibition and SIRT1 activation, whereas, pentacyclic triterpenoids were correlated with elastase, collagenase and tyrosinase inhibition. Altogether, these results clearly evidenced the great valorization potential of herbal plants from CVL for the production of antioxidant and anti-aging bioactive extracts for cosmetic applications.

1- Introduction

Cosmeceuticals are plant derived products intended to improve the health and beauty of the skin by providing a specific result. Most of cosmeceuticals are defined by their biologically active ingredients (BAIs). Phenolic compounds are well established BAIs of these cosmeceuticals. Caffeic acid, quercetin, flavonoids, gallic acid, etc. are common phenolic compounds used in cosmeceuticals. These compounds are extracted from different plant species like *Echinacea, Silybum, Linum, Citrus*, etc. However, plant derived phenolic compounds are privileged in cosmetic industry due to benefits they offer. Therefore, whole cosmetic industry is looking for efficient and feasible production platforms for cost efficient production of these cosmeceuticals by herbal plants.

Use of plant cell culture technology offers an attractive alternative for the production of plant-based cosmeceuticals. Compared to field grown plants, cell cultures can be cultivated in a controlled and contained environment. Production in cell culture also offers possibilities for process optimization,

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independent of environmental effects, etc. Although plant cell culture technology is very widespread in phytopharmaceutical industry, but it is not yet very common in the cosmetic industry.

The problem, usually encountered by plant cell culture is the low production rate and yield of BAIs. Therefore, several strategies have been devised to enhance concentration of these compounds in cell cultures of herbal plants. Elicitation of BAIs is considered as most reliable and feasible strategy. Elicitation not only enhances biosynthesis of BAIs but also influencing their production by stimulating biomass accumulation. Elicitors are the chemical compounds from abiotic and biotic sources that can stimulate stress responses in plants, leading to enhanced biosynthesis of BAIs. The number of parameters, such as elicitor type, dose, treatment schedule, duration of exposure are major factors influencing biosynthesis of BAIs in plant cell.

2- Experimental details

Chemicals and reagents :

All the extraction solvents employed in this study were of analytical grade, provided by Thermo Scientific. Standards and reagents were obtained from Sigma-Aldrich.

Plant material :

The seeds of herbal plants were provided by Christophe Hano. Seeds were surface sterilized prior to culturing in order to escape contamination. The air-dried seeds were then germinated on Murashige and Skoog (MS) (1962) medium comprising 0.8% agar and 30 g/l sucrose [63]. The pH of all the media was maintained at 5.8 before autoclaving at 121°C for 20 minutes. For the establishment of callogenesis, stem and leaf explants were cut out from 4 weeks old *in vitro* grown plantlets and cultured at various concentrations of PGRs, either alone or in combination.

Callogenic frequency :

Three different PGRs (NAA, TDZ and BAP) at varied concentrations (1.0-5.0 mg/l), used either alone or in combination with 1.0 mg/l TDZ, were employed for callus induction in the present study. The explants were maintained in a growth room for 16/8 h (light/dark cycle) at 25 ± 1°C. Observation of callogenic frequency and callus morphology was done on weekly basis with visual eye. Respective calli were then subcultured on fresh medium supplemented with the same PGRs concentrations after every 4 weeks of the culture. Fresh weight and dry weight were also determined for subsequent examinations.

Quantification and identification by HPLC :

The contents of caffeic acid, oleanolic acid, rosmarinic acid, plectranthoic acid and betulinic acid were determined by HPLC. Following the protocol documented by Bourgeois et al. (2016), a reversed-phase HPLC equipped with autosampler, Varian Prostar 230 pump and a Varian Prostar 335 photodiode array detector was used and controlled with Galaxie software (Varian v1.9.3.2) [15]. Briefly, the separation was achieved on an RP-18 column (5 µm, 250 × 4.0 mm id (Purospher Merck)) at 35 °C. The mobile phase was comprised of acetonitrile (C_2H_3N) (as solvent A) and 0.1% (v/v) formic acid acidified ultrapure water (as solvent B). The composition of the mobile phase varied during a 60-min run according to a linear gradient ranging from a 5:95 (v/v) to 100:0 (v/v) mixture of solvents A and B, respectively, at a flow rate of 0.6 ml/min. Detection was accomplished at 210 nm and 254 nm. Quantification and identification of each compound was done by coupling with retention times, UV spectra to those of authentic reference standards and by standard additions. Calibration curve (6 points) was used to quantify each standard with the range of 5 µg/mL-1mg/mL and correlation coefficient of at least 0.9994.

Antioxidant DPPH assay :

For this assay, 20 µl of each sample extract was combined with 180 µl of DPPH reagent and OD was recorded at 517 nm with the help of a microplate reader. The following equation was then used to calculate DPPH activity: % scavenging = 100 × (Abc-Abs/Abc)

Where, Abc denotes absorbance of the control while Abs is absorbance of the sample or expressed as TAEC (Trolox C equivalent antioxidant capacity).

Antioxidant ORAC assay :

Oxygen radical absorbance capacity (ORAC) assay was carried out as suggested by Prior et al. (1998). In brief, 10 µl of the extracted sample was mixed with 190 µl of 0.96 µM fluorescein in 75 mM phosphate buffer (pH 7.4) and incubated for about 20 minutes at 37 °C. Then, 20 µl of 119.4 mM 2,2'-azobis-amidinopropane (ABAP) was added and the fluorescence intensity was recorded every 5 minutes during 2.5 h at 37 °C with the help of a fluorescence spectrophotometer (BioRad) set with an excitation at 485 nm and emission at 535 nm. Assays were made in triplicate and antioxidant capacity determined using ORAC assay was expressed as TAEC.

Antioxidant ABTS assay :

ABTS assay was accomplished with the method of Mohd et al. (2018) [65]. Briefly, 2,2-azinobis (3-ethylbenzthiazoline-6-sulphonic acid (ABTS) solution was made by mixing equal proportion of ABTS salt (7 mM) with potassium persulphate (2.45 mM) and the mixture was kept in the dark for 16 h. The absorbance of the solution was measured at 734 nm and adjusted to 0.7 and then mixed with the extracts. The mixture was again kept in the dark for 15 minutes at 25 ± 1 °C and the absorbance was recorded at 734 nm using a BioTek ELX800 Absorbance Microplate Reader (BioTek Instruments). Assays were made in triplicate and antioxidant capacity was expressed as TAEC.

Antioxidant FRAP assay :

Modified method of Benzie and Strain (1996) was used for the determination of ferric reducing antioxidant power (FRAP) assay [66]. In brief, 10 µl of the extracted samples were mixed with 190 µl of FRAP solution (composed of 20 mM $FeCl_3$, 10 mM TPTZ, $6H_2O$ and 300 mM acetate buffer (pH 3.6) in a ratio 1:1:10 (v/v/v)). Reaction mixtures were then incubated at 25 ± 1 °C for 15 minutes. Absorbance of the reaction mixture was noted at 630 nm using a BioTek ELX800 Absorbance Microplate Reader (BioTek Instruments). Assays were made in triplicate and the antioxidant capacity determined using this assay was expressed as TAEC.

Antioxidant CUPRAC assay :

Cupric ion reducing antioxidant capacity (CUPRAC) assay was evaluated by some modifications in the method of Apak et al. (2004) [67]. Briefly, 10 µl of samples and 190 µl of CUPRAC reaction solution (containing 7.5 mM neocuproine, 10 mM Cu(II) and 1 M acetate buffer (pH 7) in a ratio 1:1:1 (v/v/v)) were mixed. Reaction mixtures were then incubated at 25 ± 1°C for 15 minutes and absorbance was recorded at 450 nm using a BioTek ELX800 Absorbance Microplate Reader (BioTek Instruments). Assays were made in triplicate and antioxidant capacity determined using this assay was expressed as TAEC.

Metal Chelating Activity Assay :

The ferrous ion chelating activity of herbal plants extracts was evaluated following a slightly modified method of Srivastava et al. (2012) [68]. In brief, 10 µl of extracts were mixed with ferrous iron at a final concentration of 50 µM in HEPES (pH 6.8) buffers and 50 µl ferrozine (5 mM aqueous solution). All experiments were performed in a 96-well microplates at room temperature (25 ± 1 °C). Each sample was measured with and without (blank) the addition of ferrozine. Absorbance was noted at 550 nm instantaneously after addition of ferrozine and 5 min later with a BioTek ELX800 Absorbance Microplate Reader. Chelating activity values were expressed in µM of fixed Fe^{3+}.

Collagenase assay :

Collagenase clostridium histolyticum (Sigma Aldrich) was employed for this assay and its activity was determined with the aid of a spectrophotometer by making use of N-[3-(2-furyl)acryloyl]-Leu-Gly-Pro-Ala (FALGPA; Sigma Aldrich) as a substrate in accordance to the protocol of Wittenauer et al. (2015) [69].

The absorbance decrease of FALGPA was followed at 335 nm for 20 min using a microplate reader (BioTek ELX800; BioTek Instruments). Triplicated measurements were used and the anti-collagenase activity was revealed as % inhibition relative to corresponding control (adding same volume of extraction solvent) for every extract.

Elastase assay :

Elastase assay was performed by using porcine pancreatic elastase (Sigma Aldrich) and its activity was determined with spectrophotometer by making use of N-Succ-Ala-Ala-Ala-p-nitroanilide (AAAVPN; Sigma Aldrich) as a substrate and following p-nitroaniline release at 410 nm using a microplate reader (BioTek ELX800; BioTek Instruments) by adopting the method of Wittenauer et al. (2015) [69]. Triplicated measurements were used and the anti-elastase activity was expressed as % inhibition relative to the corresponding control (adding same volume of extraction solvent) for every extract.

Hyaluronidase assay :

Hyaluronidase inhibitory assay was carried out as suggested by Kolakul et al. (2017) using 1.5 units of hyaluronidase (Sigma Aldrich) and hyaluronic acid solution (0.03% (w/v)) as substrate [70]. The precipitation of undigested form of hyaluronic acid occurred with acid albumin solution (0.1% (w/v) BSA). The absorbance was measured at 600 nm using a microplate reader (BioTek ELX800; BioTek Instruments). The hyaluronidase inhibitory effect was expressed as % inhibition relative to the corresponding control (adding same volume of extraction solvent) for every extract.

Tyrosinase assay :

Method of Chai et al. (2018) was used for the determination of tyrosinase assay [71]. In brief, L-DOPA (5 mM; Sigma Aldrich) was used as diphenolase substrate and mixed in sodium phosphate buffer (50 mM, pH 6.8) with 10 µl of plant extract. Finally, 0.2 mg/mL of mushroom tyrosinase solution (Sigma Aldrich) was added to this mixture to make a final volume of 200 µl. Control, with equal amount of extraction solvent replacing the extract, was routinely carried out. The reaction processes were traced by using a microplate reader (BioTek ELX800; BioTek Instruments) at a wavelength of 475 nm. The tyrosinase inhibitory effect was expressed as % inhibition relative to the corresponding control for each extract.

Anti-AGE formation activity :

The inhibitory capacity of AGE formation was determined as described by Kaewseejan and Siriamornpun (2015) [72]. Herbal plants extracts were mixed with 20 mg/ml BSA (Sigma Aldrich) solution prepared in 0.1 M phosphate buffer (pH 7.4), 0.5 M glucose (Sigma Aldrich) solution prepared in phosphate buffer and 1 ml of 0.1 M phosphate buffer at pH 7.4 containing 0.02 % (w/v) sodium azide. This mixture was incubated at 37°C for 5 days in the dark and then the amount of fluorescent AGE formed was determined using a fluorescence (VersaFluor fluorometer; Bio-Rad) set with 330 nm excitation wavelength and 410 nm emission wavelength. The percentage of anti-AGEs formation was revealed as % inhibition relative to the corresponding control (adding same volume of extraction solvent) for every extract.

SIRT1 assay :

SIRT1 activity was determined using the SIRT1 Assay Kit (Sigma Aldrich) following manufacturer instructions and fluorescent spectrometer (Biorad VersaFluor) set with 340 nm excitation and 430 nm emission wavelengths. The relative SIRT1 activity was revealed as percentage relative to the corresponding control (adding same volume of extraction solvent) for every extract.

Statistical analysis :

Each experiment was carried out in triplicate. OriginPro (Windows, 2017) was used for statistical analysis. A Duncan's multiple range test (DMRT) ($P < 0.05$) was used for measuring significant differences.

3- Results and discussion

Optimization of Callogenesis from different initial explants

For the determination of callus induction frequency, stem and leaf explants from several species were cultured on MS medium encompassing different concentrations (1.0-5.0 mg/ml) of several PGRs (TDZ, NAA and BAP) used either alone or in conjunction with TDZ. Callus was initiated after 10-12 days of culturing explants. In case of leaf explants, TDZ (1.0 mg/ml, 2.0 mg/ml and 3.0 mg/l) and 1.0 mg/ml TDZ + NAA (1.0 mg/ml, 2.0 mg/ml and 3.0 mg/l) led to highest callus induction (95-100%) as compared to BAP alone or combination of BAP with TDZ. NAA alone resulted in around 80% callus induction but the value greatly increased (up to 90%) when TDZ was used alone. Similarly, the induction frequency for stem explants was close to 100% when TDZ was employed either alone or combined with NAA. However, higher concentration of all the tested PGRs restricted callus induction in both stem and leaf explants, possibly due to repression of some endogenic PGRs retarding callus formation. Indeed, changes in callus response formation have already been ascribed to diverse endogenous hormonal responses pointing the variable sensitivity of tissues toward these PGRs. Sreedevi et al. (2013) and Anjum et al. (2017) reported similar observations. No callogenesis was observed on MS medium lacking these PGRs which has been observed for various other plant species such as *Stevia rebaudiana*.

Visual morphological variations were also detected in calli. Generally, stem-derived calli were more friable, while leaf-derived calli were compact in texture. Similar results have previously been reported for several other medicinal plant species. We also observed that the callogenic response and morphological changes were markedly influenced by the exogenously applied PGRs. Physiological response of calli also radically varied in accordance to the type of initial explant. The potential growth rate was higher in stem-derived calli as compared to the calli derived from leaf as starting explants.

Evaluation of secondary metabolites production

Total polyphenols accumulation in stem-derived calli of herbal plants on all the tested PGRs ranged from 49.99 to 90.06 mg/g DW. Calli cultured on media supplemented with TDZ (1.0 mg/l) and NAA (3.0 mg/l) biosynthesized optimum levels (90.06 mg/g DW) of phenolic compounds, while lowest accumulation (49.9 mg/g DW) was observed in media supplemented with high concentration (5.0 mg/l) of NAA. Phenolics accumulation in response to NAA and TDZ gradually declined with increase in hormonal concentration. However, Szopa and Ekiert (2014) observed that PGRs directly influence the production of phenolic compounds in plants in vitro cultures. Among all the PGRs, combined treatment of TDZ + NAA at low concentration exhibited maximum accumulation of TPC in stem-derived calli. Similar trend was observed for TPC in leaf-derived callus culture for which TDZ combined with NAA gave highest accumulation as compared to TDZ or NAA used alone. Faizal et al. (2017) reported that the best treatment for phenolic compounds production in red pitaya callus was 2.0 mg/l NAA + 4 mg/l TDZ, which is consistent with the results of our study. Similarly, Tariq et al. (2014) also highlighted that growth regulators such as NAA and TDZ greatly influence the production of phenolic compounds, flavonoids and antioxidants in *A. absinthium* cultures grown *in vitro* [34].

Antioxidant capacity generally correlated with TPC, thus collinear connection exists between these two variables, as evident from the literature [35,36,37]. Likewise, Khandaker et al. (2012) also indicated that the improved antioxidant activities in apple treated with different PGRs were linked with the increase in TPC [38]. Similar trend was also observed here with the quenching free radical activity. Our data suggests that these plant extracts could serve as a safe antioxidant agent.

Evaluation of antioxidant and anti-aging potential of herbal plant cell culture extracts

A complete screen of antioxidant and anti-ageing capacities of these cell and calli extracts with contrasting phytochemical profiles was

also evaluated in the current project. For in vitro antioxidant screening, antioxidant mechanisms were based on both electron transfer (FRAP and CUPRAC assays) and hydrogen atom transfer (ABTS and ORAC assays). The chelation potential of these extracts was evaluated by both FRAP and metal chelating assays using ferrozine. All of the callus extracts exhibited marked antioxidant and chelation activities. Extract from stem-derived calli grown on 1.0 mg/l TDZ + 3.0 mg/l NAA displayed highest antioxidant activities for all of the assays with values of 1203.7 TEAC for DPPH, 945.8 for ABTS, 733.3 TEAC for ORAC, 535.8 for FRAP, 460.2 for CUPRAC and 54.8 µmol of fixed Fe^{3+}. On the other hand, extract of leaf-derived calli grown on 1.0 mg/l TDZ presented the lowest antioxidant activities with values of 474.4 TEAC for DPPH, 434.5 TEAC for ABTS, 306.7 TEAC for ORAC, 211.9 TEAC for FRAP, 193.3 TEAC for CUPRAC and 23.0 µmol of fixed Fe^{3+}. Whatever the test used, ET-based assays gave higher antioxidant capacities than HAT-based assays. The prominence of this action mode suggested the occurrence of at least one phytochemical involved in this type of antioxidant mechanism in these plant extracts. Here, stem-derived callus extracts displayed higher antioxidant activities than the callus initiated from leaf explants. Combination of NAA and TDZ appeared to further potentiate this biological activity.

The next step involved the evaluation of anti-aging action of these plant extracts (at a fixed concentration of 50 µg/ml) determined as their *in vitro* capacities:

1) to inhibit elastase, hyaluronidase, collagenase, tyrosinase and AGEs

2) to activate SIRT-1 activity. Elastase, hyaluronidase and collagenase have been found to degrade extracellular matrix components in the dermis, thus leading to skin alterations including skin tonus, deep wrinkles and resilience losses [15,50,51]. Tyrosinase dysfunctions advance with aging and can lead to malignant melanoma, as well as, pigmentary disorders such as freckles or melisma [52]. Oxidative stress has been found to be associated with aging and age-related diseases [53] that could lead to the buildup of advanced glycation end products (AGEs) [54]. Therefore, compounds with the ability to inhibit these enzymatic activities or processes have attracted increasing attention in cosmetics. Several studies have challenged the classical radical theory of aging [55], and SIRT1 (a class III deacetylase) have emerged as a new key factor of longevity controlling oxidative stress effects through the stimulation of antioxidant response via FOXOs and p53 pathways [56]. A stimulation of SIRT1 activity has been reported to be crucial in the control of oxidative stress and in the regulation of aging process [56,57]. Interestingly, phytochemicals have been reported to activate SIRT1 homologs and to prolong life span in yeast, drosophila and Caenorhabditis elegans models [58,59,60]. The identification of SIRT1 activators is also of great interest for cosmetic applications.

To better assess the linkage between individual phytochemical and biological activities, Pearson coefficient correlations (PCCs) between these parameters were also calculated. From this analysis, it appeared that the rosmarinic acid is the main contributor towards the antioxidant activities of these extracts, with high (ranging from 0.982 for ABTS assay to 0.997 for DPPH and FRAP assays) and highly significant ($p<0.001$) PCCs. The anti-AGEs activity correlated with the presence of phenolic acids Rosmarinic acid (PCC=0.943, $p<0.001$) and to a less extent Caffeic acid (PPC=0.608, $p=0.036$) could be linked to their well-described antioxidant activity [47]. Furthermore, it was observed that the pentacylic triterpene also significantly contributed towards the antioxidant ORAC assay (PCC=0.604, $p=0.038$). Concerning anti-aging activities, the analysis revealed a more complex linkage. The marked activation of SIRT1 activity and the anti-hyaluronidase activity appeared to be relied on rosmarinic acid and pentacylic triterpenes. Phenolic compounds are known as potent activator of SIRT1 activity [16], whereas both phenolic compounds and triterpenes are described as possible hyaluronidase inhibitors [61]. The marked anti-tyrosinase activity of our sample extracts was significantly linked with *plectranthoic acid* (PCC = 0.622, p = 0.031) and *oleanolic acid* (PCC = 0.603, p = 0.038) but not with the betulinic acid. From a structural point of view, *plectranthoic acid* and *oleanolic acid* originate from the same olenyl cation precursor

[62], which could be one explanation of this observation. The marked anti-collagenase, as well as, less pronounced anti-elastase activities of plant sample extracts were significantly correlated with the pentacylic triterpenes.

4- Conclusion

Our results proved that cell culture protocols provide an excellent reproducible opportunity to optimize and obtain a uniform and high-quality yield of the target compounds. HPLC analyses confirmed the presence of pentacyclic triterpenes namely plectranthoic acid, betulinic acid and oleanolic acid and phenolic acids like caffeic acid and rosmarinic acid in all *in vitro* callus culture conditions. The impact of TDZ and NAA, as well as, the origin of initial explant phytochemical accumulation of these plants species was elucidated and correlated with relevant biological activities. Little is known about the *in vitro* biosynthesis, regulation and accumulation of triterpenes and phenolic compound of these plant species. Hence, present research emphasizes a possible connection with respect to morphology, growth behavior and metabolic activity to produce fast-growing friable calli that is constantly able to generate bulk of the target substances. Results showed the possibility to produce very contracting sample extracts in term of both phytochemical profiles and biological activities relying on simple and reproductive initial conditions. Taking advantage of these contrasting accumulation profiles, we have showed that these in vitro cultures could represent a very promising and sustainable system to produce anti-aging and antioxidant extracts for cosmetic applications. Correlation analysis further helped us to elucidate the complex link connecting phytochemicals accumulated in the callus to the biological activities of the resulting sample extracts. The antioxidant, anti-glycation and SIRT1 activation actions relied on the presence of rosmarinic acid, whereas, anti-tyrosinase, anti-elastase and anti-collagenase activities were found to be linked with the occurrence of pentacylic triterpene derivatives. We anticipate that the methodology employed here could be applied to other health promoting activities of these extracts from in vitro cultures and also to other plant production systems. Our research will facilitate in future to enhance and examine the production of these bioactive metabolites on large-scale cultivation in bioreactors involving several biotechnological strategies like plant cell, tissue and organ cultures.

5- Perspectives of future collaborations with the host laboratory

Dr. Bilal Haider Abbasi has established consortium during his stay in France. This consortium is composed of renowned scientists from different countries. They are hunting some opportunities. However, the active collaboration in provision of plant material for phytochemical and biochemical assays is being exchanged among these labs. Articles published in the framework of the fellowship

6- Articles published in the framework of the fellowship

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