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Finding the actor: Bio-detection in botanicals with a case study on safety and efficacy

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1. Introduction

In vitro assays are crucial for cosmetics research, assessing efficacy and safety while adhering to ethical principles [1]. Botanicals, driven by consumer demand for natural products, are a key focus [2-5]. Established in-chemico assays, like titration and spectrometry, evaluate botanical bioactivity, including antioxidant, enzyme inhibition, and skin sensitization properties. These assays are reproducible, simple, quick, and cost-effective

While in-chemico assays evaluate total extract activity, they don't identify the specific active compounds. Botanical extracts are complex, containing numerous diverse compounds, and their composition varies based on several factors. This complexity makes identifying active compounds crucial but challenging. Advanced techniques like UHPLC-MS and spectroscopic methods are used for this purpose. Bio-guided fractionation, while effective, is time-consuming and costly. Therefore, a pragmatic biochemical approach was developed to identify molecules responsible for sensitization and tyrosinase inhibition.

Recent research has focused on in chemico methods for identifying skin sensitization hazards, using non-animal methods like DPRA and ADRA [6-9]. In this work, we developed a bio-detection approach using NAC (from the ADRA assay) using chromatographic profiling of

Glycyrrhiza glabra (licorice) extract, before and after incubation, to identify sensitizers. Changes in the extract's chromatogram, rather than just the peptide peak, pinpoint reactive molecules, which were subsequently identified by mass spectrometry.

Tyrosinase is key in melanogenesis, and the mushroom tyrosinase assay is crucial for evaluating the inhibitory potential of botanicals, especially for skin whitening [10-12]. Here, we developed a novel LC method to evaluate tyrosinase inhibition, using Camellia Sinensis (tea extract) as an example. Instead of focusing on L-DOPA, in this approach, we profiled the extract before and after incubation with tyrosinase. Post-incubation peak reduction pinpoints tyrosinase-inhibiting molecules, subsequently identified by mass spectrometry.

This research significantly advances the analysis of botanical extracts for cosmetics. While leveraging existing innovative techniques like ADRA and tyrosinase assays, we innovatively focused on changes in the extract's chemical profile after reaction, rather than changes in the reactants. This allows direct visualization of the interacting compounds responsible for bioactivity, streamlining bioactive compound identification and meeting the growing demand for natural ingredients.

2. Materials and Methods

2.1. Bio-detection of sensitizer/s in *Glycyrrhiza glabra* extract

2.1.1. Amino acid derivative reactivity assay (ADRA)

Testing was performed according to the ADRA test method of Wanibuchi et al [17]. Briefly test samples were prepared in triplicate by adding 50 µL of Glycyyrhiza glabra extract (0.5 mg/ml) to 150 µL of the NAC stock solution (6.667 µM) and NAL (α-N-(2-(1-naphthyl)acetyl)-L-lysine) stock solution (6.667 µM). Additionally, control samples without test chemicals (without NAC and NAL) were also prepared. Samples were shaken gently, and then incubated in the dark for 24 h at 25°C. Following incubation, 50 µL of 2.5% trifluoroacetic acid (TFA) in water was added to all test samples. Mobile phase (A) was prepared to 0.1% TFA in water, and mobile phase (B) was prepared to 0.1% TFA in acetonitrile. After incubation, NAC and NAL in all samples and standards were quantified using a LC-20A HPLC system (Prominence,

Shimadzu Corporation, Kyoto, Japan) on a Wakopak Core C18 ADRA (φ3.0 × 150 mm, FUJI-FILM Wako Pure Chemical Corporation, Osaka, Japan). The flow rate was 0.3 mL/min, and the temperature of the column oven was maintained at 40°C while that of the auto-sampler was maintained at 4°C. 10 µL of each sample was injected, with a linear gradient from 30% B to 55% B for 9.5 min for NAC and from 20% B to 45% B for 9.5 min for NAL, followed by a rapid increase to 100% B for 0.5 min and holding 100% B for 3.5 min, then back to the initial conditions (30% B for NAC and 20% B for NAL) for a total analysis time of 20 min per sample. Finally, NAC and NAL were quantified using ultraviolet detection at 281 nm.

2.1.2. Biodetection of sensitizers

The identification of the compounds responsible for the reactivity of *Glycyrrhiza glabra* extract was carried out through HPLC with diode array detection (DAD) followed by mass spectroscopy (MS). 150 µL of the 6.667 µM NAC stock solution was mixed with 50 µL of Glycyrrhiza glabra extract (0.5 mg/mL) to generate the test sample. Additionally, a control sample without NAC was also prepared. After giving the samples a gentle shake, they were incubated at 25 °C for 24 hours in the dark. The mobile phase A used was 0.1% (v/v) formic acid (FC) in Milli-Q water and the mobile phase B was 100% acetonitrile. The flow rate was maintained at 1 mL/min, the temperature of the column at 30°C and that of the sample tray at 25°C. A linear gradient from 20% B to 90% B for 110 minutes, followed by a sharp drop to 20% B for 10 minutes, was applied to 5 µL of each sample. On a Capcell-pack C18 (4.6 mm L. × 250 mm I.D., 5 µm particle size) (Osaka Soda (former Shiseido), Japan), an LC-20A HPLC system (Prominence, Shimadzu Corporation, Kyoto, Japan) was employed. Spectrophotometric detection and monitoring were carried out between 190 and 800 nm. Using a positive ionization mode, mass spectrometric (MS) analysis was performed with a full MS scan range ranging from m/z 150 to 1000 Da. The test sample (*Glycyrrhiza glabra* extract alone/ *Glycyrrhiza glabra* + NAC) of 5 µL was injected into the system. For efficient desolvation, the interface block was kept at 400 °C, and the desolvation line (DL) temperature was maintained at 250 °C. 3 L/min and 15 L/min were the flow rates for nebulizing and drying gas, respectively. The Q1 and Q3 pre-bias voltages were both set to -15 V to optimize ion focusing and transmission, and data

acquisition was conducted in full scan mode, for comprehensive mass profiling of the test samples.

2.2. Bio-detection of the tyrosinase inhibitor/s in *C. sinensis*

2.2.1. Tyrosinase inhibition assay

Mushroom tyrosinase (CAS No. 9002-10-2, ³1000 unit/mg) prepared in phosphate buffer (pH 6.5) was used for the assay. 100µL solution of mushroom tyrosinase solution was mixed with C. sinensis extract (0.6 mg/ml) and incubated for 5 min at 25°C. 100 µL of 0.5 mM L-DOPA solution was added to this mixture and the reaction was allowed to continue for another 10 min at 25°C. Subsequently, the reaction was stopped by adding 50 µL of 0.1N HCl to the reaction mixture. Shimadzu UPLC equipped with an autosampler, quaternary pump, and diode array detector (DAD) was used to quantify L-DOPA in the reaction mixture. A Zorbax XDB C18, 4.6 × 250 mm, 5µm, Agilent column, maintained at 35°C, was utilized. Acetonitrile was utilized as mobile phase B, and 10 mM potassium dihydrogen phosphate + 0.05% orthophosphoric acid in water as mobile phase A. A 10µL sample injection volume and a 0.5 mL/min flow rate were maintained. A step-wise gradient mode of elution with steps of 5% B for 0 to 6 min, 30% B for 6 to 10 min, 50% B for 10 to 15 min and 75% B for 10 to 20 min. 5% B was used for 25 to 35 min as the final step. L-DOPA was quantified at 205 nm.

2.2.2. Biodetection of tyrosinase inhibitors

For the identification of tyrosinase inhibitors in *C. sinensis* extract (0.6 mg/ml), it was chromatographed (LC-MS/MS) with and without tyrosinase on a Sciex QTRAP 4500 mass spectrometer coupled to a Shimadzu HPLC system. A Zorbax XDB C18 column (250 × 4.6 mm; 5µm particle size) (Agilent) was used. There were two mobile phases. Mobile phase A consisted of 0.1% formic acid in water, and mobile phase B was 100% acetonitrile. A step-wise gradient elution mode was used at a flow rate of 0.5 mL/min. The steps were 5% B for 0 to 6 min, 30% B for 6 to 10 min, 50% B for 10 to 15 min and 75% B for 10 to 20 min. 5% B for 25 to 35 min was used as the final step to restore 5% B that was used equilibration of column. The run lasted 35 minutes in total, during which time the column oven's temperature was kept at 25 °C. A triple quadrupole device with an electrospray ionization (ESI) source was used for the MS analysis. A curtain gas of 45, an ion spray voltage of 4500 V, and a source temperature of 500 °C were among the analytical parameters. The initial and second ion source gases were set at 40 and 70, respectively. The de-clustering potential was adjusted to 150, with an entrance potential of 10. The collision gas was set to medium, while the collision energy and the collision cell exit potential were 38 and 17, respectively. Data acquisition and processing were

performed using Sciex Analyst version 3.2 and Sciex OS software, provided by Sciex Corporation, USA.

3. Results

3.1 Bio-detection of sensitizer/s from *Glycyrrhiza glabra*

Glycyrrhiza glabra extract subjected to ADRA and HPLC showed 100% NAC depletion, 25.4% NAL depletion, and a mean NAC+NAL depletion of 62.7%, indicating that the extract was reactive. At retention durations of 8.5 and 5.5 minutes respectively, chromatograms reveal NAC and NAL depletion, which are indicated by blue boxes (Fig. 1 A and B, respectively).

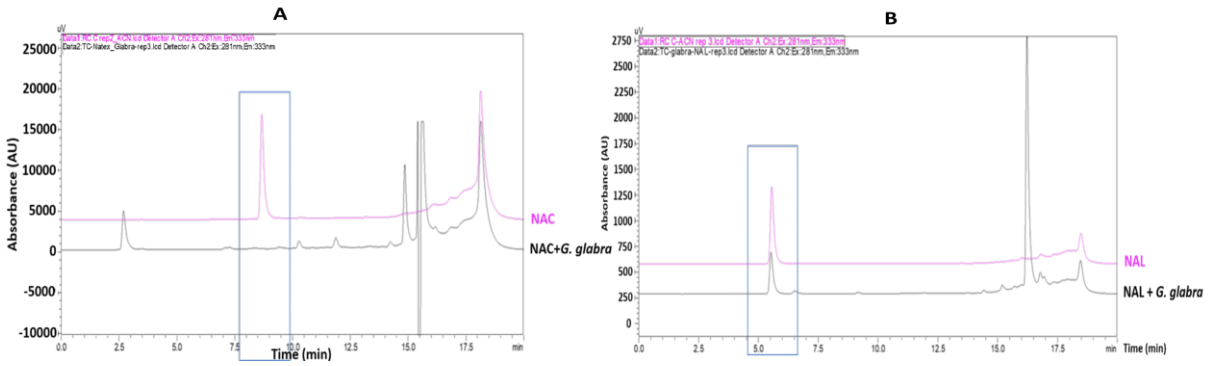

Figure.1. Overlaid HPLC chromatograms following ADRA of A) NAC and *Glycyrrhiza glabra* extract incubated with NAC and B) NAL and *Glycyrrhiza glabra* extract incubated with NAL

A comparative HPLC-DAD-MS study between the *Glycyrrhiza glabra* extract with and without incubation with the NAC peptide noted the depletion of five compounds in the extract incubated with NAC (Fig.2). The five compounds that were depleted were identified as prenylated flavonoids and flavones such as Glabridin (m/z 325), Glabrocoumarin (m/z 337), Hispaglabridin B (m/z 391) Glabrol (m/z 393) and Kanzanol Y (m/z 411). The reactivity of the *Glycyrrhiza glabra* extract seen in ADRA is due to the presence of these compounds.

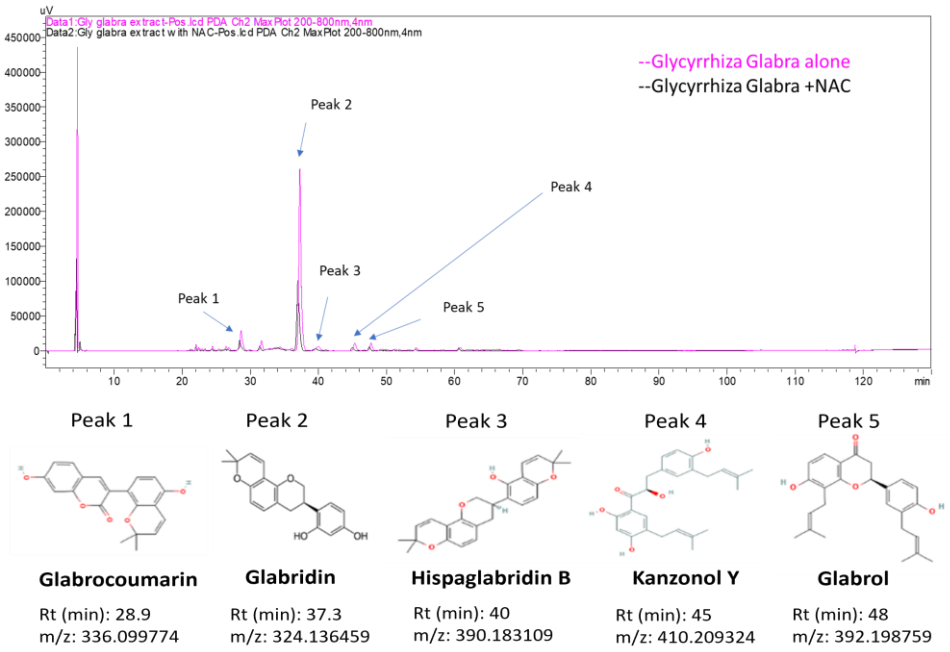

Figure 2. HPLC-MS profile of the *Glycyrrhiza glabra* extract compared to the *Glycyrrhiza glabra* extract incubated with NAC

3.2. Bio-detection of tyrosinase inhibitors from *Camellia sinensis*

The LC-DAD study conducted to detect tyrosinase inhibitors involved the obtaining of chromatograms for the L-DOPA standard, L-DOPA + tyrosinase and L-DOPA + tyrosinase + *C. sinensis* extract and overlaying the three (Fig.3), showed that the retention time of L-DOPA alone is 6.2 min and that the L-DOPA peak disappears completely after incubation with tyrosinase. However, upon incubation of L-DOPA + tyrosinase with the extract, the L-DOPA peak is not as diminished, indicating that the extract inhibits tyrosinase. Calculations revealed that the extract exhibited tyrosinase inhibition of 51.3% at the 0.6 mg/mL concentration.

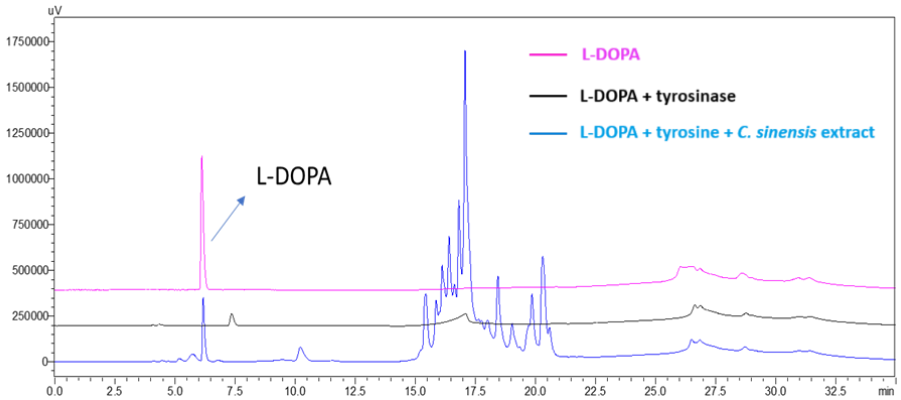

Figure 3. Overlaid HPLC chromatograms of L-DOPA, L-DOPA + tyrosine and L-DOPA + tyrosine + *Camellia sinensis* extract

In the LC-DAD study to identify the compounds responsible for the observed inhibition of tyrosinase in the *C. sinensis* extract, the overlaid chromatograms of L-DOPA alone, *C. sinensis* extract alone and the combination of L-DOPA + tyrosinase + *C. sinensis* extract showed the depletion of several compounds responsible for inhibition of tyrosinase inhibition in the L-DOPA + tyrosinase + *C. sinensis* extract chromatogram. Using MS, three severely depleted peaks at 16.8, 17.1 and 18.1 minutes in TIC were focused and these peaks corresponded to three compounds identified as Epicatechin (m/z 290), Epigallocatechin 3-gallate (m/z 458) and Epicatechin -3-gallate (m/z 442) (Fig.4).

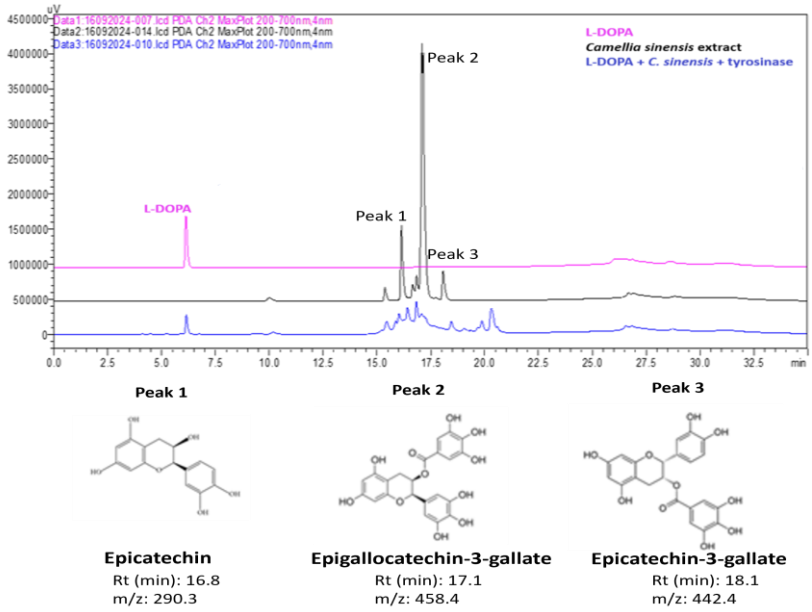

Figure 4. HPLC-MS profile of *Camellia sinensis* extract compared to L-DOPA and *C. sinensis* extract incubated with L-DOPA and tyrosinase

4. Discussion

Analyzing plant compounds for cosmetics using chromatography is challenging due to the complexity and variability of plant materials. Fractionation and identification, while common, is time-consuming, expensive, and not all compounds are suitable for UV detection. Reference standards are also limited. This work demonstrates a simpler method for identifying sensitizers and tyrosinase inhibitors in *Glycyrrhiza glabra* and *C. sinensis* extracts, respectively. The identified sensitizers in *Glycyrrhiza glabra* are known flavonoids, isoflavones, and chalcones with various biological activities, but this study highlights their reactivity to skin proteins. The identified tyrosinase inhibitors in *C. sinensis* are known flavonoids and polyphenols with beneficial properties, including anti-melanogenic activity, further supporting the extract's potential in skin-whitening cosmetic formulations.

5. Conclusions

This study presents a rapid method for identifying bioactive compounds in complex plant extracts, extending in-chemico bio-detection methods. By analyzing chromatographic profile changes before and after reagent interaction, this approach effectively pinpoints sensitizers and tyrosinase inhibitors in *Glycyrrhiza glabra* and *Camellia sinensis* extracts, enhancing the precision of assays like ADRA and tyrosinase-inhibition assays. This streamlined, cost-effective, and ethical method addresses challenges associated with traditional fractionation, supporting the development of safer and more effective cosmetics with natural ingredients. Future work will explore broader applications.

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