

Implementing metabolomics techniques in the acceleration of the discovery of new antidiabetic bioactive metabolites obtained from *Quercus coccifera*

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Abstract

Metabolomics is a technique used to compare the chemical profiles of different extracts. Natural sources like plants can be used, after being linked with metabolomics, to enhance the ability of drug discovery. A multivariate analysis and metabolomics profiling were performed on *Quercus coccifera* leaf extracts using LC-HRMS and NMR raw data using the SIMCA 14.0 program. All multivariate analyses were guided by the results of the α -amylase and -glucosidase inhibitory activity of the produced extracts. Both boiled water and methanolic extracts were selected to be active, with a percentage of inhibition higher than 80% upon using 1 mg/ml of each extract. The rest of the extract was found to be inactive and failed to obtain biological activity greater than 40%. Only methanolic extract was found to have a unique chemical profile and was found as an outlayer in both the supervised and unsupervised PCA scatter plots. The rest of the extracts were found to exert a known chemical profile and were found inside the domain. In addition, the OPLS-DA loading plot and heat map indicate the presence of a highly diverse chemical profile in methanol, with higher and lower chemical shifts and molecular weights. Both boiled water and methanol extracts were found to exert similar activity, but metabolomics profiling shows that methanolic extract contains a unique chemical profile and should be selected for a further fraction for the possible discovery of a new antidiabetic compound.

Keywords

antidiabetic, metabolomics, multivariate analysis, drug discovery, analysis

Introduction

Natural sources used to be an important source of novel bioactive compounds and played a major role in the treatment of different diseases like diabetes mellitus (DM) (Jaber 2023, 2024). Thus, pharmaceutical companies and different research facilities are putting effort into enhancing the isolation ability of bioactive compounds from

different ecosystems through different techniques like metabolomics. Metabolomics is a science dealing with different metabolomic profiles of the primary and secondary metabolites produced by the ecosystem to maintain the normal functions of the cells (Aminov 2022). Biological systems encompass a wide range of entities, including cells, biofluids, tissues, organisms, and ecosystems. In metabolomics, the focus is on the secondary metabolites

with a molecular weight lower than 1500 Da (Fiehn 2002; Miggiels et al. 2019). The chemical profile of metabolites that are produced from any ecosystem is largely affected by many factors like epigenetic and post-translational modification, interconnected metabolite relationships, and environmental conditions (Aminov 2022).

Quercus coccifera was reported to have different chemical classes of metabolites known for their biological activity against various diseases, such as alkaloids, glycosides, tannins, and terpenoid compounds (Jaber 2024). In addition, the strong antioxidant activity of *Q. coccifera* extracts indicates their importance in the treatment of various types of diseases (Morales 2021). *Q. coccifera* (Oaktree) leaf extract was found to exert anti-diabetic activity *in vivo* in a mouse model and *in vitro*, especially in boiled water and methanolic extracts (Jaber 2023). In another study in 2022, the phenolic content of the African *Q. coccifera* acorns was found to have promising antidiabetic activity after being tested *in vitro* (Mezni et al. 2022). Moreover, in 2021, Mingxia Wu and his team found the *Q. coccifera* extracts to have a strong activity on dipeptidyl peptidase IV, an enzyme important for regulating blood glucose (Wu et al. 2021). In the field of natural products, the metabolomics approach can play a critical and vital role in the prioritization of extract fractionation in the drug discovery process. By applying the metabolomics technique to liquid chromatography coupled with high-resolution mass spectroscopy (LC-HRMS) and nuclear magnetic resonance (NMR) results, assisted by biological assay results, chemical profiling was done to select the most unique chemical profile containing extract for further fractionation (Fiehn 2002; Miggiels et al. 2019). Through the analysis of metabolite profiles, researchers can effectively compare different plant extracts and prioritize those that exhibit unique or enriched metabolite profiles associated with desired therapeutic effects (Aminov 2022). Such prioritization will aid time management, which is considered a critical factor that has a major contribution to the drug discovery process. Thus, the resources can be managed, and the ability to isolate a new bioactive metabolite will increase. In this research, *Quercus coccifera* leaf extracts were subjected to both NMR and LC-HRMS and *in vitro* (α -glucosidase and α -amylase) to confirm the uniqueness of the methanolic extract when compared to other produced extracts. This will be done by integrating the mentioned analysis techniques into the metabolomics approach to be used as evidence for selecting methanolic extract for further fractionation.

Materials and methods

Plant material collection

Quercus coccifera plant leaves were collected on the 4th of May, dried, and stored in a canvas bag, and the leaves were identified before, according to Jaber (Jaber 2023).

Extraction Procedure

Each extract was prepared by soaking 100 mg of powdered leaves in suitable solvents (n-hexane, chloroform, methanol, boiled water, and microwaved water) for 24 h under the fume hood. All organic solvents were HPLC-grade and were obtained from Sigma-Aldrich, USA. The extraction procedure for all extracts was done under similar conditions to avoid any variability, as it has a huge effect on metabolomics profiling and multivariate analysis (Kumar and Misra 2019).

NMR spectroscopy analysis

5 mg/600 μ l of each extract solution was prepared after being dissolved in DMSO-d⁶ purchased from Sigma-Aldrich, USA. Proton (¹H) NMR analysis was conducted by transferring each extract dissolved in DMSO-d⁶ to a 5 mm tube, followed by placing each tube in a 500 MHz Bruker NMR instrument. Each ¹H NMR spectra produced for the analysis of each extract was processed using MestReNova 14.2 (Mnova 14.2), purchased software from MesterLab Research SL, USA. Various adjustments were applied, including smoothing with Whittaker Smoother, baseline correction with Whittaker Smoother, apodization with Gaussian 1.00, and manual phase correction, all within MestReNova. Subsequently, the spectra from the extracts were stacked to visualize the differences between the extracts.

Mass spectroscopy analysis

A total of 1 mg/ml of each extract was prepared by dissolving 1 mg of extract in 1 ml of HPLC-grade methanol obtained from Sigma-Aldrich, USA. Each prepared sample was placed in LC-HRMS, and these samples were performed using an Accela HPLC system (Thermo Scientific, Germany) coupled with an orbitrap ascend tribrid mass spectrometer (Orbitrap, Germany) and an electrospray ionization source, following the procedure described (Cheng et al. 2015). LC-HRMS analysis was conducted on both positive and negative modes, covering ranges between 150 and 200 m/z. During the experiment, an ACE5 Excel 3 Super C18 column (5 μ m \times 150 mm \times 3 mm) obtained from Hichrom Limited, Reading, UK, was used with a flow with an injection volume and flow rate of 10 μ l and 300 μ l/min, respectively. Both ultrapure water with 0.1% formic acid (solvent A) and HPLC-grade acetonitrile with 0.1% formic acid (solvent B) were used as a solvent system following the protocol published previously (Sebak et al. 2020).

In vitro alpha-amylase and alpha-glucosidase activity

To assess the activity of α -amylase, 100 μ l of plant extracts dissolved in a suitable solvent was mixed with

100 μ l of α -amylase enzyme and incubated at 37 °C for 30 minutes (Tamil et al. 2010). Then, a 1% starch solution was added to the mixtures and further incubated for 1 hour at 37 °C (Tamil et al. 2010). The reaction was stopped by adding 200 μ l of dinitrosalicylic (DNS) acid color reagent and boiling the samples for 5 minutes, followed by cooling to room temperature (Tamil et al. 2010). A positive control using acarbose was included, and the percentage of inhibition for all extracts was determined by comparing the absorbance results at 540 nm for the test samples and the control.

Similarly, for the α -glucosidase inhibitory assay, following the Pistia-Brueggeman and Hollingsworth protocol (Pistia-Brueggeman and Hollingsworth 2001), 50 μ l of different plant leaf extracts at final concentrations of 1 mg/ml were added to 96-well plates. To each well, 10 μ l of α -glucosidase (1 U/ml) and 125 μ l of pH 6.8 phosphate buffer were added, and the mixture was incubated at 37 °C for 20 minutes. After 20 minutes, 20 μ l of 1 M pNPG (4-Nitrophenyl-D-glucopyranoside) substrate were added, followed by further incubation for 30 minutes. The reaction was terminated by adding 50 μ l of 0.1 N Na₂CO₃, and the optical density of each well was measured at 405 nm using a microplate reader. Acarbose was used as a positive control, and the activity of the extracts was measured. All results were processed using graph pad Prism 5 for drawing and the calculation of biological activity, while the below equation was used for measuring the biological activity of plant extracts:

$$\text{Extract inhibitory activity} = \left[\frac{Xa - Xb}{Xa} \right] \times 100\%$$

Multivariate profiling using NMR and LC-HRMS

All HRMS results were separated into two sets of positive (Mass+H) and negative (Mass-H) modes using the MassConvert tool designed by Proteowizard (Chambers et al. 2012). These sets of the positive and negative modes were imported to MZmine 2.41 (Pluskal et al. 2010), and the results were processed with the MS/MS library database provided by the instrument. The processing of LCHRMS data with the MS-MS library was done using a multistep protocol published by Cheng et al. (2015) with both modes (Cheng et al. 2015). Upon HRMS data processing, CSV files were generated for both modes and exported to SIMCA 16, designed by Umetrics, Umeå, Sweden, for the generation of both supervised and supervised principal component analysis (PCA) and orthogonal projections to latent structures discriminate analysis (OPLS-DA) for metabolomics profiling. Furthermore, the stacked NMR spectra were used for the generation of a data set between 0 and 15 ppm and processed in the same manner using SIMCA 16 to produce both the supervised and unsupervised PCA scatters and the OPLS-DA loading plot.

Results

Extracts yield

All extracts produced after extraction produced a sufficient yield to perform metabolomic profiling and multivariate analysis processing, as presented in Table 1. All yields were found to be sufficient to prepare NMR samples (at least 5 mg is needed), LCHRMS samples (1 mg from each extract is needed), and biological assay testing (at least 5 mg is needed).

Table 1. Extract yield resulted from multistep extraction.

Solvent	Extract weight (mg)	% yield	Solvent	Extract weight (mg)	% yield
n-Hexane	53.13	0.053	Boiled water	232.47	0.232
Chloroform	30.66	0.031	Microwaved water	259.14	0.259
Methanol	147.67	0.148			

Chemical profiling

NMR profiling

According to the stacked proton NMR spectrum presented in Fig. 1, all extracts show the presence of peaks between 1.25 and 9.00 ppm. The intensity of the peaks for n-hexane and chloroform extracts is extremely low, while the rest of the extracts show a higher peak intensity. The peaks in both n-hexane and chloroform extracts show lower chemical shifts, which is a strong indication of the presence of aliphatic hydrocarbon chains and/or fatty acid compounds. Both water extracts and methanolic extracts show a distribution of peaks with higher chemical shifts (above 6 ppm), indicating the presence of aromatic structures with substitution with electronegative functional groups like nitrogen (N) and/or oxygen (O).

LC-HRMS profiling

According to the LC-HRMS results of the crude extracts presented in Fig. 2, lower molecular weight compounds with a molecular weight below 500 Da are found in n-hexane and

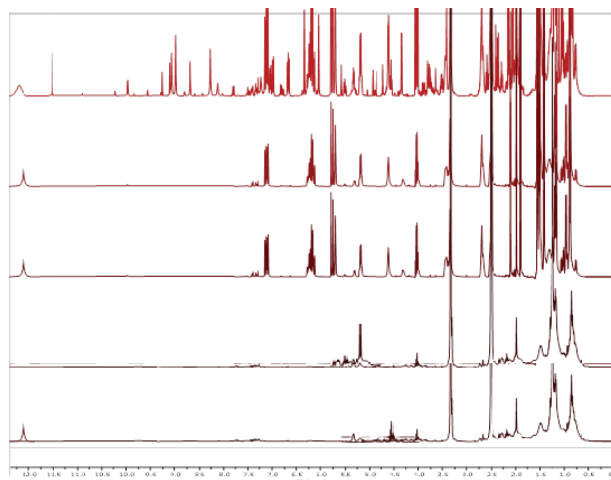


Figure 1. Stacked H¹ NMR spectrum of plant leaf extracts. Highlighted peaks are peaks found in more than one extract.

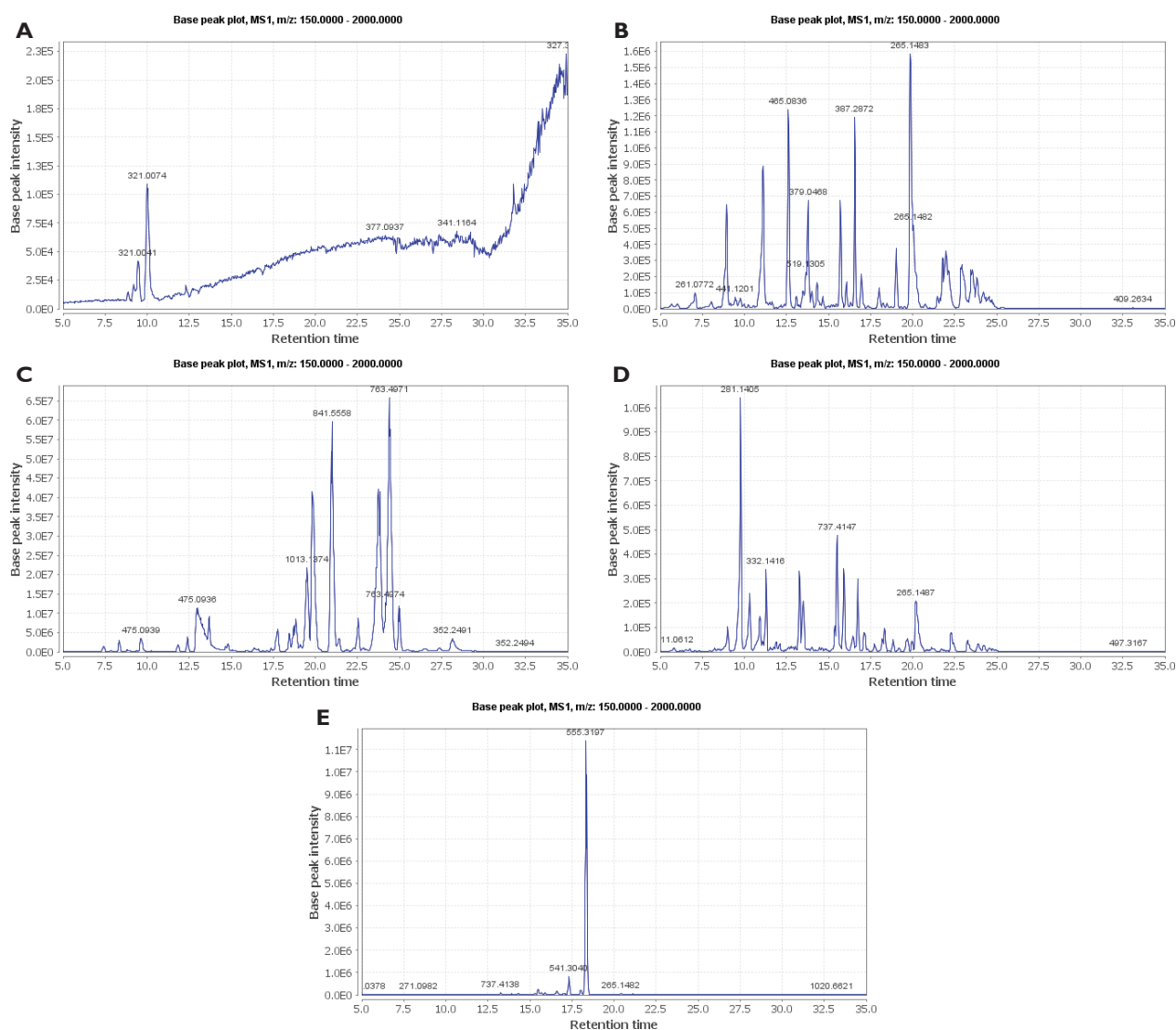


Figure 2. LC-HRMS spectra for plant leaf extract. **A.** n-hexane extract; **B.** Chloroform extract; **C.** Methanol extract; **D.** Boiled water extract, and **E.** Microwaved water extract.

chloroform extracts. N-hexane extract was found to exert a noisy spectrum, while chloroform extract was found to exert a clearer spectrum with a higher number of compounds and molecular weights. On the other hand, ethanol extracts show the presence of a high distribution of compounds with molecules of low and high molecular weight. While most compounds are found in both boiled and microwaved water with a molecular weight of less than 500 Da.

Metabolomics profiling and extract prioritization

According to the α -amylase and α -glucosidase inhibition assay results presented in Fig. 3, both methanol and boiled water extracts were selected to be grouped as active extracts. The percentage of inhibition for both extracts against α -amylase and α -glucosidase was higher than 80%. On the other hand, the rest of the extracts (n-hexane, chloroform, and microwaved water extracts) were grouped as inactive. The selected extracts failed to exert a percentage inhibition of 50% or higher.

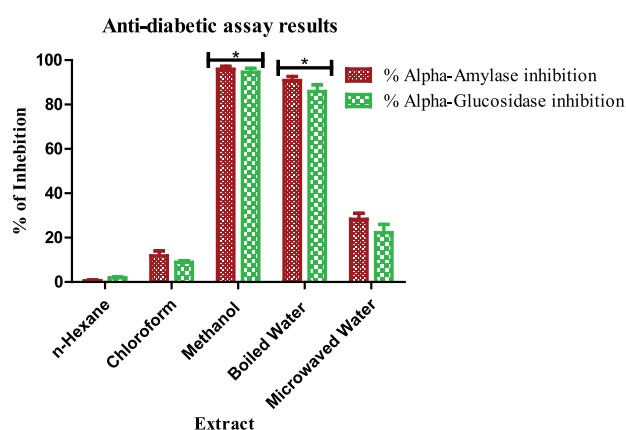


Figure 3. Anti-diabetic activity results of both α -amylase and α -glucosidase used for metabolomics as guidance.

According to the unsupervised PCA scatter plot produced by the analysis of NMR results presented in Fig. 4A, methanol was found to exert a unique chemical profile when compared to other extracts. In addition, both water

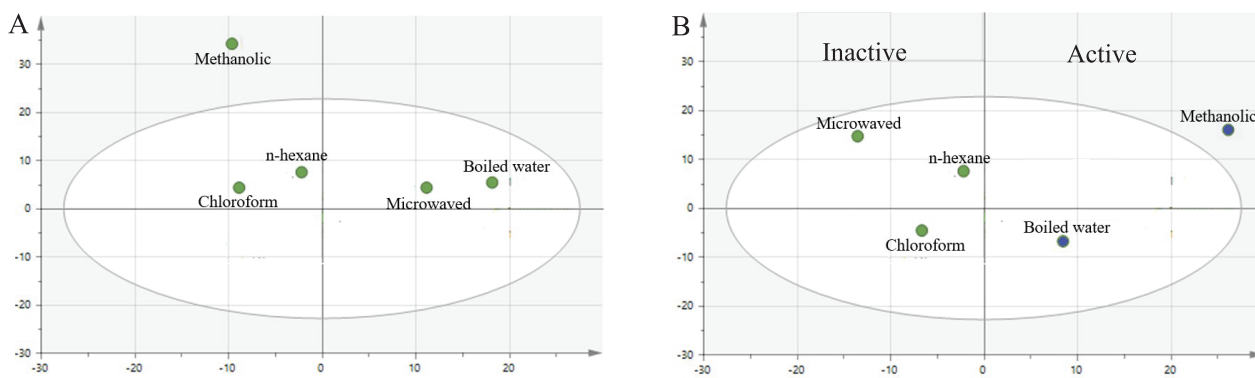


Figure 4. PCA-scatter plot of different extracts using NMR results. **A.** Unsupervised scatter plot, and **B.** Supervised scatter plot.

extracts (boiled and microwaved) and hydrophobic solvent extracts (chloroform and n-hexane) show a complete difference in the chemical profile. On the other hand, when applying the biological activity results to the multivariate analysis presented in Fig. 4B, again, methanolic extract showed a unique chemical profile and was found to be an out layer, while boiled water extract was found to show a known chemical profile based on the chemical shifts of the tested extracts. The inactive extracts (microwaved water, chloroform, and n-hexane) show a known chemical profile with no uniqueness. Both supervised and unsupervised models for the NMR results of the plant leaf extracts were found to be strong, with R^2 and Q^2 values of (0.99 and 0.97) and (0.99 and 0.88), respectively.

According to the OPLS-DA loading plot for the NMR data presented in Fig. 5, most NMR peaks produced

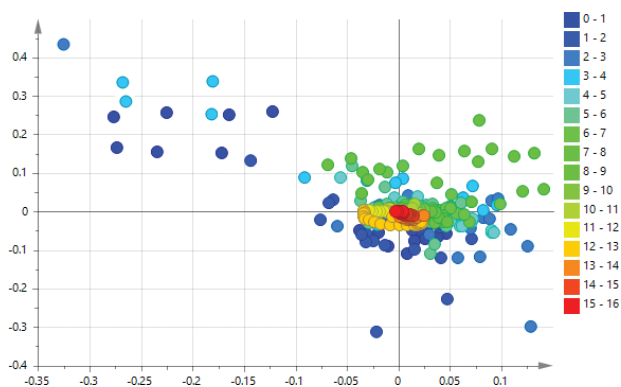


Figure 5. OPLS-DA loading plot for NMR spectral data of different plant leaf extracts.

from the inactive extracts have a chemical shift of 5 ppm or less. A few peaks were found to have a chemical shift higher than five and resulted from the microwaved extract. On the other hand, a wide distribution of the peaks chemical shifts was produced from the active extracts (methanolic and boiled water extracts) between 0–10 ppm.

The unsupervised PCA scatter plot presented in Fig. 6A for LC-HRMS processed data confirmed the previous finding, which indicates a unique chemical profile for methanolic extract when compared to the MS-MS library database and was found as an out layer. On the other hand, both water extracts indicate the presence of similar chemical profiles and are similar for chloroform and n-hexane extracts.

According to the loading plot and the heatmap of the compounds found in plant extracts presented in Fig. 7A, B, respectively, bioactive extracts were found to exert a highly diverse chemical profile, especially in methanolic extract. The most promising compounds found in methanolic extract with a high potential for uniqueness are the compounds circled by red and with a molecular range between 500 and 1000 Da. Again, the heatmap confirms the earlier finding, and methanolic extract was found to have a chemical profile with the highest diversity. All compounds assigned by the red circle were found to exert a P value of less than 0.05 at a confidence interval of 90%.

Again, like the NMR results model, supervised and unsupervised models were found to be strong, with high R^2 and Q^2 values of (0.99 and 0.98) and (0.99 and 0.92).

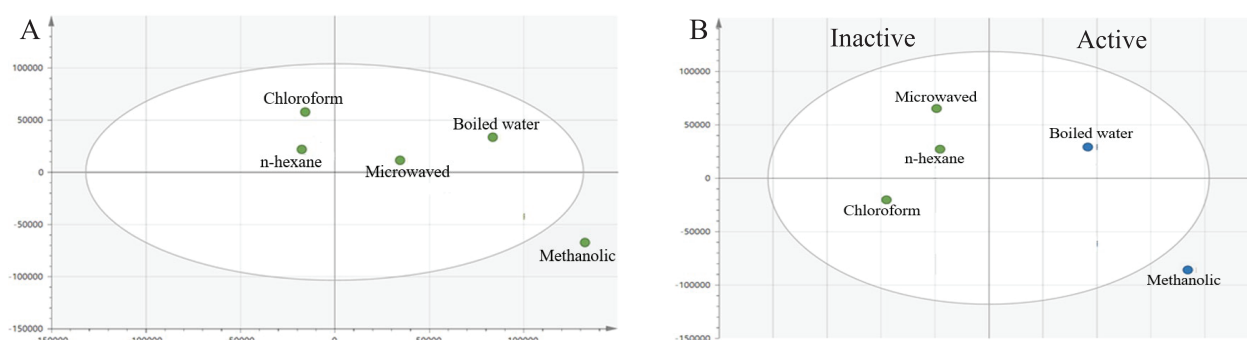


Figure 6. PCA-scatter plot of different extracts using LC-HRMS results. **A.** Unsupervised scatter plot, and **B.** Supervised scatter plot.

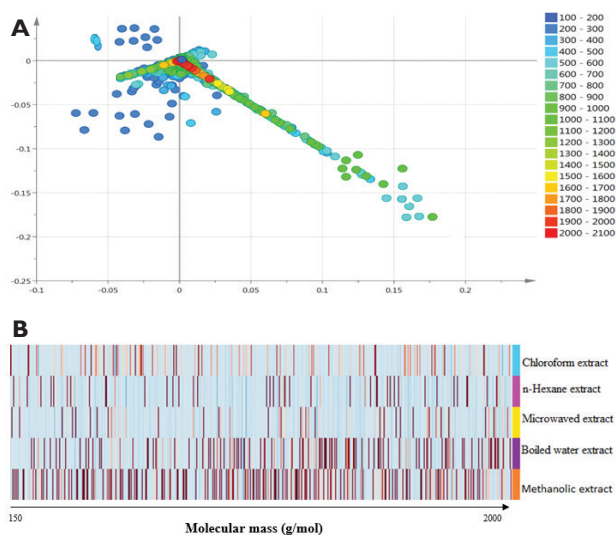


Figure 7. OPLS-DA loading plot and heatmap for LC-HRMS results. **A.** OPLS-DA loading plot, and **B.** Heatmap showing the intensity of molecules based on their molecular weights provided by LCHRMS data (blue shows the low intensity, and red shows the high intensity).

Discussion

The prioritization of plant extracts for future drug discovery processes relies heavily on the metabolomics approach (Mattoli et al. 2023). This approach allows scientists to thoroughly analyze the diverse array of small molecules present in the extracts, enabling a comprehensive understanding of their potential benefits (Mattoli et al. 2023). Biological assay results can be used as guidance for the software to group the extracts according to biological activity to enhance multivariate analysis outcomes. When α -amylase and -glucosidase were used to determine the antidiabetic activity of extracts, both boiled water and methanolic extracts exerted the highest activity of more than 80%. These results were found to be similar to previously published research (Jaber 2023). Thus, the reason for selecting both boiled water and methanolic extracts as active extracts is that the rest are active according to their biological activity. All statistical models for the chemical profiles produced by both NMR and LC-HRMS were found to be strong and perfectly fitted, as R^2 values were found to be higher than 0.98. All statistical models produced by SIMCA are considered to be very representative and strong models, either for the models produced from NMR or LC-HRMS data, due to the presence of higher R^2 values and a difference between R^2 and Q^2 of less than 0.3 (Zanatta et al. 2021; Mazlan et al. 2020). Both NMR and LC-HRMS results of hydrophobic solvents (n-hexane and chloroform) indicated the presence of low-molecular-weight hydrocarbon compounds lower than 500 Da and aliphatic structures with a chemical shift of less than 6 ppm. This was confirmed by the multivariate analysis presented in the loading plots in Figs 5, 7A. Predictably, such compounds as n-hexane and chloroform extracts were previously found to extract hydrocarbon compounds (Gao et al. 2019; Rincón-Cervera

et al. 2020). According to the PCA plots, both extracts were found to contain a known chemical profile without promising biological or unique compounds. On the other hand, both water extracts were found to exert compounds with a higher chemical shift (above 6 ppm) and higher molecular weights (greater than 500 Da), as presented in the NMR and LC-HRMS results in Figs 1, 2, respectively. This could be due to the presence of polyoxygenated and nitrogen-containing compounds. It was found before that water extracts are found to exert a high concentration of polyphenols, which usually have higher chemical shifts and molecular weights than hydrocarbon compounds (Dzah et al. 2020). According to the LC-HRMS, PCA, and heat map plots and graphs presented in Figs 3, 5, 7B, there is a difference between both extracts resulting from the difference in the methods of extraction. It is well known that microwaves can accelerate the degradation of polyphenolic compounds (Doldolova et al. 2021). Thus, the boiled water extract chemical profile shows the highest number of peaks in the LC-HRMS spectrum and a different chemical profile. Both extracts were found to exert a known chemical profile with promising biological activity for water extracts. Finally, methanolic extract was found to exert the most diverse chemical profile, with higher and lower chemical shifts as per NMR results and molecular weights as per LC-HRMS. This resulted from the co-solvency nature of methanol as a solvent, which gives it the ability to extract hydrocarbon, nitrogenated, and oxygenated compounds (Kour et al. 2023). This was confirmed by the multivariate analysis of PCA and heat map plots and graphs. Methanolic extract shows a unique bioactive chemical profile with strong biological activity. Thus, methanolic extract should be selected for further fractionation not only because of its biological activity but also because of the uniqueness of its chemical profile, which could lead to the isolation of a new lead bioactive compound. In addition, methanolic extract NMR and LC-HRMS data indicated the possibility of the isolation of compounds that follow the Lipinsky rule of five and can be used for the design of oral drugs (Zhang and Wilkinson 2007).

Conclusion

The objective of this study was to apply a multivariate analysis technique for the prioritization of *Quercus coccifera* leaf extracts for further fractionation and purification. The aim was the isolation of new, promising bioactive compounds that can be used either as a drug for the treatment of elevated blood glucose level elevation or as a starting point for the design of a new drug for the same reason. According to the biological assay results, both boiled water and methanolic extracts have shown promising results. The PCA scatter plots produced from the analysis of the NMR and LC-HRMS spectra indicate the presence of a unique chemical profile in methanolic extracts when compared to other extracts. On the other hand, OPLS-DA loading plots and heat maps confirm the diversity of

methanolic extract over other extracts, including boiled water extract. Thus, the methanolic extract should be chosen for further fractionation over the boiled water extract, as it is an active extract and has a unique chemical profile

over the other active extracts. By doing so, a time reduction will be needed during the drug discovery process by prioritizing methanolic extracts using metabolomics and multivariate analysis techniques.

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