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# Royal jelly fatty acids bioprofiling using TLC-MS and digital image analysis coupled with chemometrics and non-parametric regression for discovering efficient biomarkers against melanoma†

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A comprehensive approach of untargeted and targeted fatty acid bioprofiling of different royal jelly commercial and pharmaceutical products based on HPTLC-image analysis and melanoma cytotoxic activity together with chemometric analysis was applied in this study for discovering efficient biomarkers. Principal component analysis based on HPTLC-image analysis fingerprints of fatty acid loading plots were used to determine the chemical markers responsible for classification of royal jelly samples into fresh and lyophilized ones. These markers were identified using the HPTLC-MS technique as 8-hydroxyoctanoic acid, 3,10-dihydroxydecanoic acid, 10-hydroxy-2-decenoic acid, decanedioic acid and 10-hydroxydecanoic acid. These discriminating markers were quantified *via* the HPTLC-imaging technique for targeted profiling using two different methods: parametric and non-parametric regression. The non-parametric regression method exhibited superiority in terms of linearity, accuracy and precision. Biomarkers were determined from the 3D-loading plot of orthogonal projection to latent structures model based on the fatty acid quantitative data together with the melanoma cytotoxic activity data. 10-Hydroxy-2-decenoic acid showed the greatest reduction in melanoma cell viability followed by decanedioic acid then 8-hydroxyoctanoic acid. The present study is considered the first attempt to discriminate fresh and lyophilized royal jelly samples based on their holistic lipidomic profile to discover efficient fatty acid reducing melanoma cell viability.

## 1. Introduction

Royal jelly is a honey bee milky-white secretion generated from the hypopharyngeal and mandibular glands of three to twelve day-old nurse bees (*Apis mellifera* L., family: Apidae). It is the only food source to the queen bee during her life, but it is fed to early-instar larvae of workers only for their first three days.[1] Due to this special food, queen bees are distinct from worker bees in their size, production of eggs and life longevity.[2] Numerous dietary supplements containing royal jelly are available in the market. These supplements are claimed to be anti-oxidant,[3] immunostimulant,[4] anti-aging,[5] anti-hypercholesterolemic,[6] anti-inflammatory,[7] anti-tumor,[8] wound-healing,[9] antibacterial[10] and skin whitening agents.[11]

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It is believed that these pharmacological claims are due to different constituents existing in royal jelly such as proteins, carbohydrates, fatty acids, vitamins, and trace minerals.[12] The most distinctive constituents in royal jelly are its fatty acids.[13] Whereas animal and plant fatty acids are mainly composed of 14–20 carbon atoms and are present in the form of esterified triacylglycerols, the royal jelly fatty acids are medium-length chain (8–12 carbon atoms) free fatty acids, either hydroxylated, or dicarboxylated, saturated or mono-unsaturated at the 2-position.[14] Many of the biological properties of royal jelly are attributed to its unusual bioactive fatty acid components. The major royal jelly fatty acid is 10-hydroxy-2-decenoic acid (10-HDA), also known as queen bee acid.[15,16] The quantity of 10-HDA in royal jelly differs based on the origin of the jelly and the features of the bee.[17] 10-HDA is not present in any other natural product, even other bee products. Therefore, it can be used as a marker for the validation of royal jelly quality.[18,19]

Many studies were published describing the assay of 10-HDA in pure royal jelly and royal jelly products using liquid chromatography methods.[19,20] In addition, HPLC and UPLC

methods as well as capillary zone electrophoresis (CZE) and HPLC techniques were compared for the quantitation of 10-HDA.[17,21] Also, gas chromatography-mass spectrometry (GC-MS) was applied in the study of royal jelly lipidome.[22,23] However, GC-MS techniques require the derivatization of free fatty acids into their corresponding trimethylsilyl esters. Therefore, other researches were carried out to study the fatty acid content of royal jelly using LC-MS.[24] Most recently, a stability-indicating HPTLC-densitometric assay was developed for the determination of 10-HDA content in royal jelly products marketed in Egypt.[25]

Two different approaches of analysis are applied in the study of natural products metabolomes: untargeted and targeted analytical approaches. Untargeted approach aims to maximize the number of detected metabolites. Hundreds to thousands of metabolites can typically be measured. The chemical identity of each metabolite is not known at the start of the study. Data acquired during the data acquisition is applied to identify metabolites after data acquisition. However, one single analytical method cannot detect all of the metabolites. It's therefore desirable to combine multiple analytical approaches to maximize the number of metabolites detected and increase coverage of the metabolome. Whereas, targeted studies analyze a relatively small and specific number of metabolites. These metabolites are chemically characterized and biochemically annotated at the start of the study before data acquisition is performed.[26,27]

High performance thin-layer chromatography (HPTLC) is a very beneficial technique for developing fingerprint profiles of the extracts because the whole chromatogram is regarded as a multidimensional vector, without the need to identify single spots.[28] The hyphenation to mass spectrometry (MS) allows the identification of the components on the TLC plates[29] which can then be quantified using different regression methods. Among these methods are the parametric and non-parametric regression.[30]

Parametric regression method presumes that examined data apply the normal (Gaussian) distribution. Therefore, it uses the arithmetic mean as the measure of central tendency of these data. It also supposes that the errors are only present in the $y$-values and $x$-values are error-free.[30] This method can be applied only if these assumptions are true, but can give misleading results if they are not true; thus the least squares parametric regression method is considered to be not robust to violations of its assumptions. Another major disadvantage in parametric regression method is that it is highly influenced by the outlying results.[30] On the other hand, in non-parametric regression, the median is usually applied instead of the mean, because in many circumstances it is a more realistic measure of central tendency.[30] Non-parametric regression overcomes the demerits of the parametric one, as it does not presume that either the $x$- or $y$-direction errors follow the normal distribution and it does not have the assumption that all the errors are in the $y$-direction. Also, it is not influenced by the existence of outliers.[30]

Melanoma is the most serious type of skin cancer. It develops in the melanocytes that produce melanin, the pigment that gives the skin its color and protects it from UV light damage.[31] Melanoma *in vitro* model using B16F10 melanoma cell culture was used in this study to investigate the melanoma cytotoxic effect of royal jelly and its bioactive fatty acids.

The aim of this work was to carry out untargeted and targeted fatty acid profiling of different royal jelly commercial and pharmaceutical products based on HPTLC-image analysis, HPTLC/MS and anti-melanoma activity testing with the use of chemometric analysis. Principal component analysis (PCA) based on HPTLC-image analysis fingerprints of fatty acids loading plots were used to determine the chemical markers responsible for the classification of royal jelly samples. These chemical markers were then identified using HPTLC/MS technique. The identified markers were then quantified *via* HPTLC-imaging technique for targeted profiling using two different regression methods: parametric and non-parametric regression. Biomarkers were determined from the coefficients plot of orthogonal projection to latent structures (OPLS) model based on the fatty acids quantitative data together with the anti-melanoma activity data. It is worth-mentioning that; this is the first time to study royal jelly fatty acids using HPTLC-image analysis and HPTLC/MS and to apply multivariate analysis to identify the efficacy associated markers against melanoma.

## 2. Material and methods

### 2.1. Royal jelly samples collection and preparation

Eighteen individual fresh and lyophilized royal jelly commercial and pharmaceutical samples (RJ1–RJ18) were collected during summer 2020 from different apiary stores, pharmaceutical companies and community pharmacies in Egypt. Details about the source of each sample are available in Table S1.† After testing several extraction solvents; methanol attained the highest extraction yield. 1 g of each sample was separately extracted with 2 mL methanol using ultrasonic bath for 30 minutes. The obtained extracts were then filtered by disk filter (0.20 μm) prior to sample application.

### 2.2. Standard solutions preparation

Accurate weights from each individual reference standard; 8-hydroxyoctanoic acid, 3,10-dihydroxydecanoic acid, 10-hydroxy-2-decenoic acid (10-HDA), decanedioic acid and 10-hydroxydecanoic acid were dissolved in methanol. Standards solution mixture (STD MIX-5) was prepared by mixing the appropriate amounts of individual standards stock solutions. The standards concentrations used for calibration were 1–30 μg μL$^{-1}$.

### 2.3. Chromatographic parameters and conditions

15 μL of royal jelly samples solutions as well as standards solutions mixture (STD MIX-5) were applied with a 100 μL syringe using Linomat V automated spray-on band applicator Camag (Muttenz, Switzerland) controlled with WinCats manager software. The application settings were 15 mm from the margins and 15 mm from the bottom of the plate with bandwidth 8 mm and inter-band spaces 5 mm. Application was

performed on silica gel 60 F254 (10 × 20 cm) HPTLC plates (Merck, Darmstadt, Germany).

Several chromatographic solvent systems were tried to maximize the resolution for the developed spots corresponding to royal jelly fatty acid. Finally, toluene : ethyl acetate (7.5 : 2.5 v/v) was found to be the optimum developing solvent. The plates were then developed over a distance of 85 mm using 40 mL of the mobile phase in a vertical (20 × 10 cm) twin trough CAMAG glass chamber saturated for 15 min before the development.

### 2.4. Post-chromatographic derivatization

After development, TLC plates were sprayed with a freshly prepared solution of 0.5 mL *p*-anisaldehyde in 50 mL glacial acetic acid and 1 mL 97% sulfuric acid and then heated to 105 °C until maximum visualization of spots. The plates were captured under white light using digital camera (14 megapixel, Fujifilm, Japan). The images were then stored as jpg for further processing.

### 2.5. Image processing and multivariate data analysis

**2.5.1. Data acquisition for untargeted metabolic profiling.** Images of the plates have been photo-edited and collected in one image by Adobe Photoshop® then had been processed with ImageJ 1.51 h; a free Java-based image analysis software (Wayne Rasband, NIH, USA). A two-dimensional plot of pixels' intensities against the distance along a fixed line was generated for each sample track by "Plot Profile" algorithm built in the ImageJ. Chromatographic data matrix comprised of 18 rows (number of royal jelly samples) and 189 columns (values of pixels' intensities in each sample's chromatogram) was constructed and transferred into Microsoft Office Excel 2013. Preprocessing of data using Standard Normal Variate correction (SNV) was applied. Multivariate analysis of the processed HPTLC profiles using "principal component analysis (PCA)" was performed for dimensionality reduction of the dataset by searching the direction of maximum variance using small number of latent variables. PCA was performed using SIMCA 14.1 software (Umetrics, Umea, Sweden).

Heat maps and "Hierarchical Clustering Analysis (HCA)" for exploring class membership using agglomerative approach were performed by Gitools 2.3.0 software (Biomedical Research Park, Barcelona).

**2.5.2. HPTLC-MS analysis.** The identification of the determined variables according to the PCA loading plots was performed through hyphenation of high-performance thin-layer chromatography with mass spectrometry (HPTLC-MS) *via* the TLC-MS-Interface Advion Plate Express™ automated TLC plate reader, NY (USA), Nawah Research Center, Cairo, Egypt. The compounds were directly eluted from the HPTLC plate through a semi-automatic piston using a solvent consisting of a mixture of chloroform and methanol (1 : 2) at a flow rate of 0.25 mL min⁻¹. MS analysis was performed on Advion Compact Mass Spectrometer (CMS), NY (USA). Data acquisition was performed in negative mode using ESI ion source in the mass range 100–700 Da.

**2.5.3. Targeted fatty acids profiling using a novel validated HPTLC method.** Peak areas corresponding to 8-hydroxyoctanoic acid, 3,10-dihydroxydecanoic acid, 10-hydroxy-2-decenoic acid (10-HDA), decanedioic acid and 10-hydroxydecanoic acid were determined by Sorbfil TLC Videodensitometer V 2.5 from JSC Sorbpolymer. These peak areas were used for quantitative evaluations.[32]

**2.5.4. Parametric and non-parametric regression analysis.** Both parametric (P) and non-parametric (NP) regression analysis were applied in this study. Regarding non-parametric (NP) regression, various methods can be applied to fit a straight line to a group of points. The easiest of these methods is Theil's "incomplete" method[30] which is utilized in this study. Comparison was carried out between the best fit straight line gained *via* Theil's method and that gained *via* the least squares parametric regression method. ICH guidelines[33] were followed to validate the developed HPTLC-image analysis method. The following validation parameters were investigated:

*2.5.4.1. Linearity and sensitivity.* The evaluation of the linearity of the suggested HPTLC method was carried out by the analysis of various concentrations of standards solution mixture (STD MIX-5). In accordance with ICH guidelines, minimally five concentrations should be analyzed. By the use of both parametric and non-parametric regression methods, regression equation, correlation coefficient ($r$), intercept ($a$), slope ($b$), standard deviation of residuals ($S_{y/x}$), standard deviation of intercept ($S_a$), and standard deviation of slope ($S_b$) were studied. Variance ratio ($F$) and sensitivity in terms of limit of detection (LOD) and limit of quantitation (LOQ) were calculated as well.

*2.5.4.2. Accuracy and selectivity.* The accuracy and selectivity of the parametric regression method were evaluated by applying recovery studies, which were carried out *via* standard addition method. Preparation of samples solutions of a representative sample (RJ13) methanolic extract, in which the concentrations of royal jelly fatty acids were formerly calculated, was carried out. After that, known concentrations of standards solution mixture (STD MIX-5) were added to them. The experiment was followed as formerly mentioned. Each solution was prepared in triplicate and analyzed. Percentage mean recovery and percentage relative error ($E_r\%$) for royal jelly fatty acids were used for determination and expression of accuracy. In case of non-parametric method, the accuracy and selectivity were determined as in parametric regression method except that the percentage mean recovery and percentage relative error ($E_r\%$) calculations relied on the intercepts and slopes gained *via* the non-parametric regression method. To test for the significant difference in accuracy among the studied types of regression: parametric (P) and non-parametric (NP) regression, Student's *t*-test for comparing sample means was carried out.

*2.5.4.3. Precision.* The analytical method precision gives an idea on the random error. It demonstrates the nearness of agreement among a group of measurements gained by multiple sampling of the same homogenous sample under the previously

specified conditions. It is comprised of repeatability (intra-day precision) and intermediate precision (inter-day precision).[33]

*2.5.4.3.1. Repeatability (intra-day precision).* The intra-day difference in the determination of royal jelly fatty acids was demonstrated by the analysis of three different concentrations of standards solution mixture (STD MIX-5) within the range of linearity using of the same equipment, the same analyst and the same analytical procedure in the same laboratory. Method repeatability was determined from RSD% value gained by repetition of the experiment three times for each concentration on the same day.

*2.5.4.3.2. Intermediate precision (inter-day precision).* Determination of the method inter-day precision was carried out by analyzing standards solution mixture (STD MIX-5) prepared at the same levels of the intra-day method but on three successive days. Percentage relative standard deviation (RSD%) was used to express the precision. *F* test was carried out to test for the significant difference in variance among the two studied regression types: parametric (P) and non-parametric (NP) regression.

### 2.6. *In vitro* mouse melanoma cytotoxicity assay

Mouse melanoma cells (B16-F10) were obtained from Nawah Scientific Inc., (Mokatam, Cairo, Egypt). Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM). These media were supplemented with 100 mg mL$^{-1}$ of streptomycin, 100 units per mL of penicillin and 10% of heat-inactivated fetal bovine serum in humidified, 5% (v/v) $CO_2$ atmosphere at 37 °C. Cell viability was assessed by sulforhodamine B (SRB) assay.[34,35] Aliquots of 100 μL cell suspension ($5 \times 10^3$ cells) were in 96-well plates and incubated in complete media for 24 h. Cells were
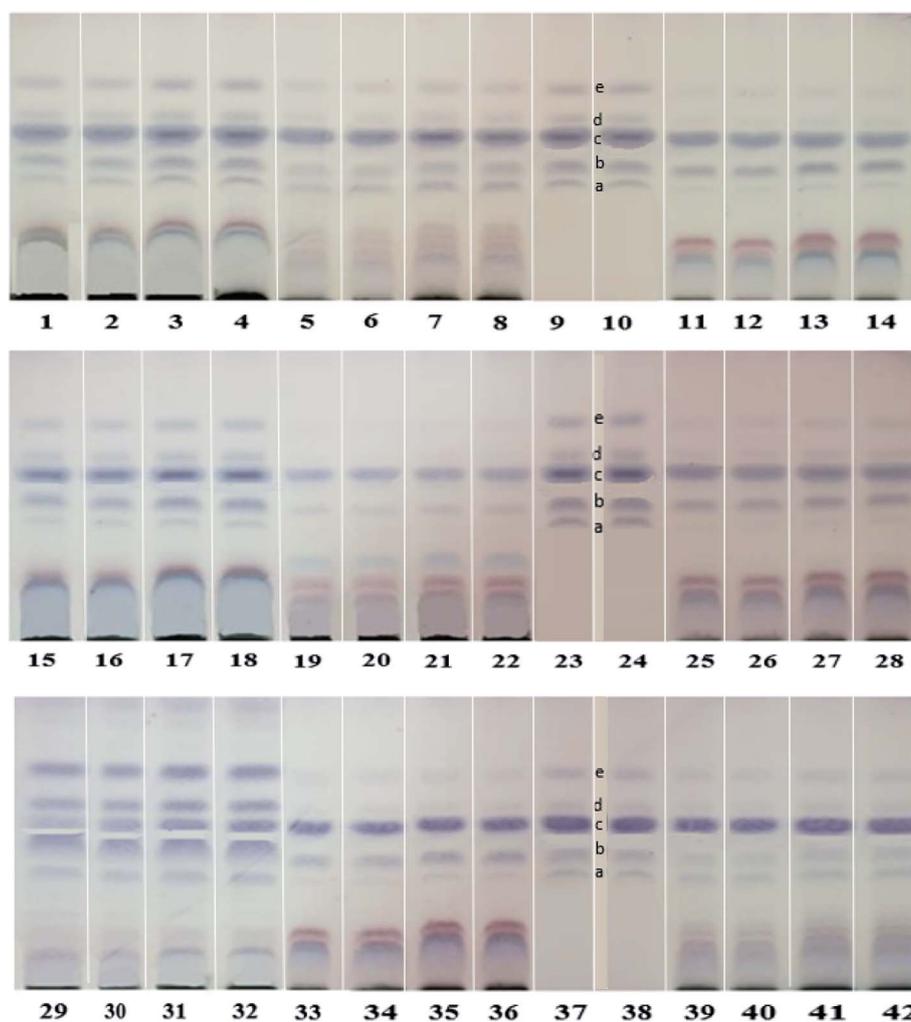


**Fig. 1** The chromatogram of the different RJ samples viewed under white light after post-chromatographic derivatization with anisaldehyde/sulfuric acid spray reagent. Tracks 1, 2; sample RJ1, tracks 3, 4; sample RJ2, tracks 5, 6; sample RJ3, tracks 7, 8; sample RJ4, tracks 11, 12; sample RJ5, tracks 13, 14; sample RJ6, tracks 15, 16; sample RJ7, tracks 17, 18; sample RJ8, tracks 19, 20; sample RJ9, tracks 21, 22; sample RJ10, tracks 25, 26; sample RJ11, tracks 27, 28; sample RJ12, tracks 29, 30; sample RJ13, tracks 31, 32; sample RJ14, tracks 33, 34; sample RJ15, tracks 35, 36; sample RJ16, tracks 39, 40; sample RJ17, tracks 41, 42; sample RJ18, tracks 9, 10, 23, 24, 37 and 38; STD MIX-5, where (a) 8-hydroxyoctanoic acid, (b) 3,10-dihydroxydecanoic acid, (c) 10-hydroxy-2-decenoic acid (10-HDA), (d) decanedioic acid and (e) 10-hydroxydecanoic acid.

treated with another aliquot of 100 µL media containing drugs at various concentrations. After 72 h of drug exposure, cells were fixed by replacing media with 150 µL of 10% trichloroacetic acid (TCA) and incubated at 4 °C for 1 h. The TCA solution was removed, and the cells were washed 5 times with distilled water. Aliquots of 70 µL SRB solution (0.4% w/v) were added and incubated in a dark place at room temperature for 10 min. Plates were washed 3 times with 1% acetic acid and allowed to air-dry overnight. Then, 150 µL of Tris (10 mM) was added to dissolve protein-bound SRB stain; the absorbance was measured at 540 nm using a BMG LABTECH®-FLUOstar Omega microplate reader (Ortenberg, Germany).

## 2.7. Orthogonal projection to latent structures (OPLS) model for discovering efficient biomarkers against melanoma

Bio-profiling of the different extracts was attempted through determination of the biomarkers by OPLS analysis using SIMCA 14.1 software (Umetrics, Umea, Sweden). The data matrix of the previously determined concentrations of 8-hydroxyoctanoic acid, 3,10-dihydroxydecanoic acid, 10-hydroxy-2-decenoic acid (10-HDA), decanedioic acid and 10-hydroxydecanoic acid in each sample was used as X-matrix while the cytotoxic activity against mouse melanoma cells was used as the Y-matrix. This was followed by a regression step where the decomposition of X was used to predict Y through another two matrices of scores
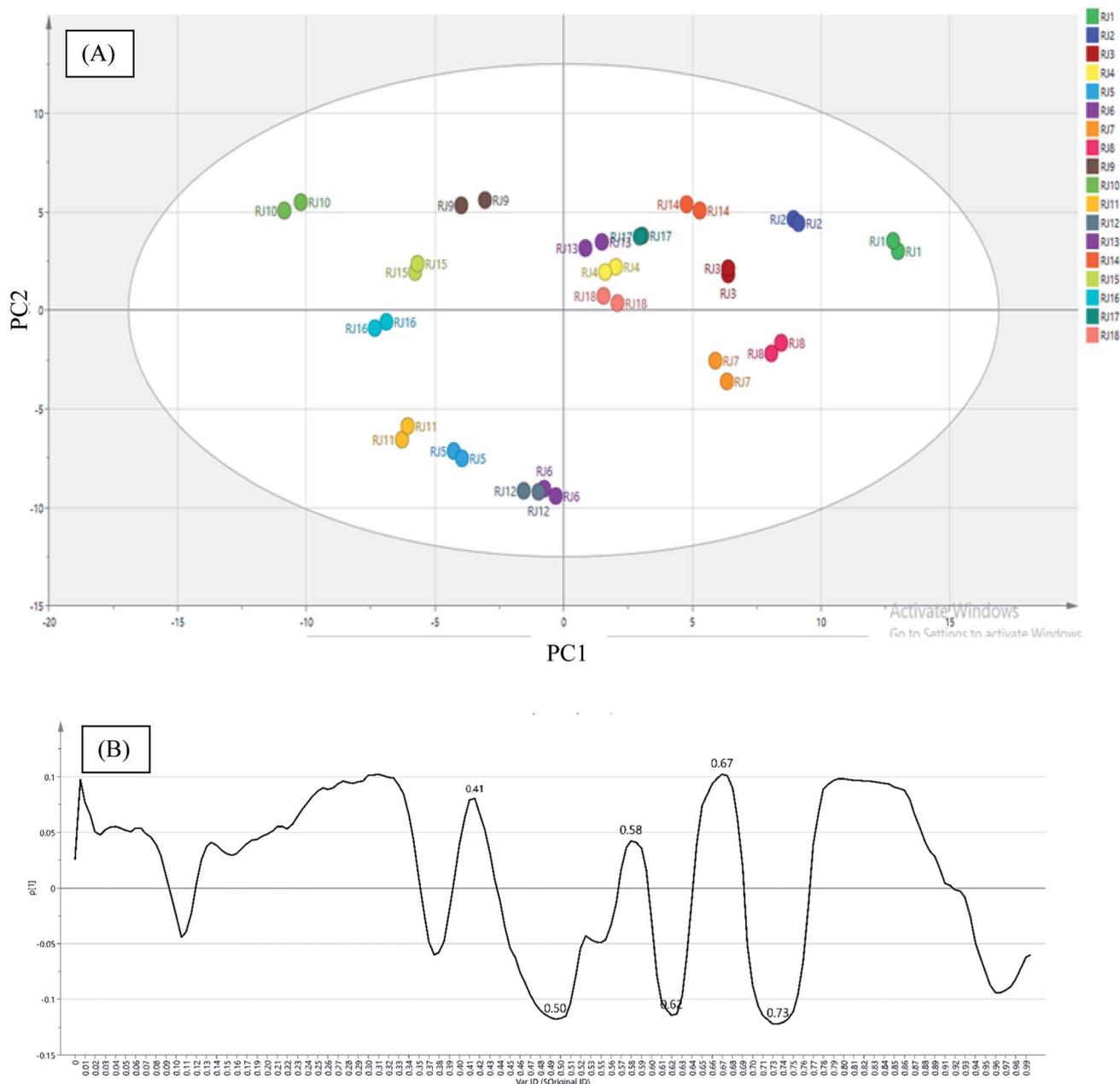


Fig. 2 PCA score scatter plot of RJ samples based on intensities of pixels for the chromatogram after post-chromatographic derivatization with anisaldehyde/sulfuric acid (A). Loading line plot of PCA (B).

and loadings. Meanwhile, variable importance for the projection (VIP) values were utilized for determination of the X-variables significance for the model.

# 3. Results and discussion

## 3.1. Untargeted fatty acids profiling of royal jelly HPTLC fingerprints

The lipidome of royal jelly (RJ) consists of medium-chained (8–12 carbon atoms) free fatty acids.

Many of the biological properties of RJ are attributed to its unusual bioactive fatty acid components.[36] HPTLC coupled with chemometric techniques was devoted for the first time for rapid comparative fingerprint profiling of fatty acids in the different lyophilized and fresh RJ samples collected from various sources.

Analysis time for each TLC plate (14 samples per plate) required 20 min from application to development for a total of 60 min for all sample plates (18 samples in duplicates). This is more time saving than the reported HPLC methods[37] which require at least 30 min for the elution of each separate sample for a total of 1080 min for 18 samples in duplicates.

A previous study specified the amount of 10-HDA in fresh and lyophilized RJ to be 1.4% and 3.5%, respectively.[38] However, the study relied solely on a single fatty acid as a quality marker rather than the holistic lipidomic profiling adopted in this work.

In the present investigation, HPTLC fingerprinting was applied for initial screening of RJ for discrimination of the investigated samples. Image capturing was performed using video densitometry. The HPTLC chromatogram for 18 RJ samples obtained at white light after post-chromatographic derivatization with anisaldehyde/sulfuric acid reagent and
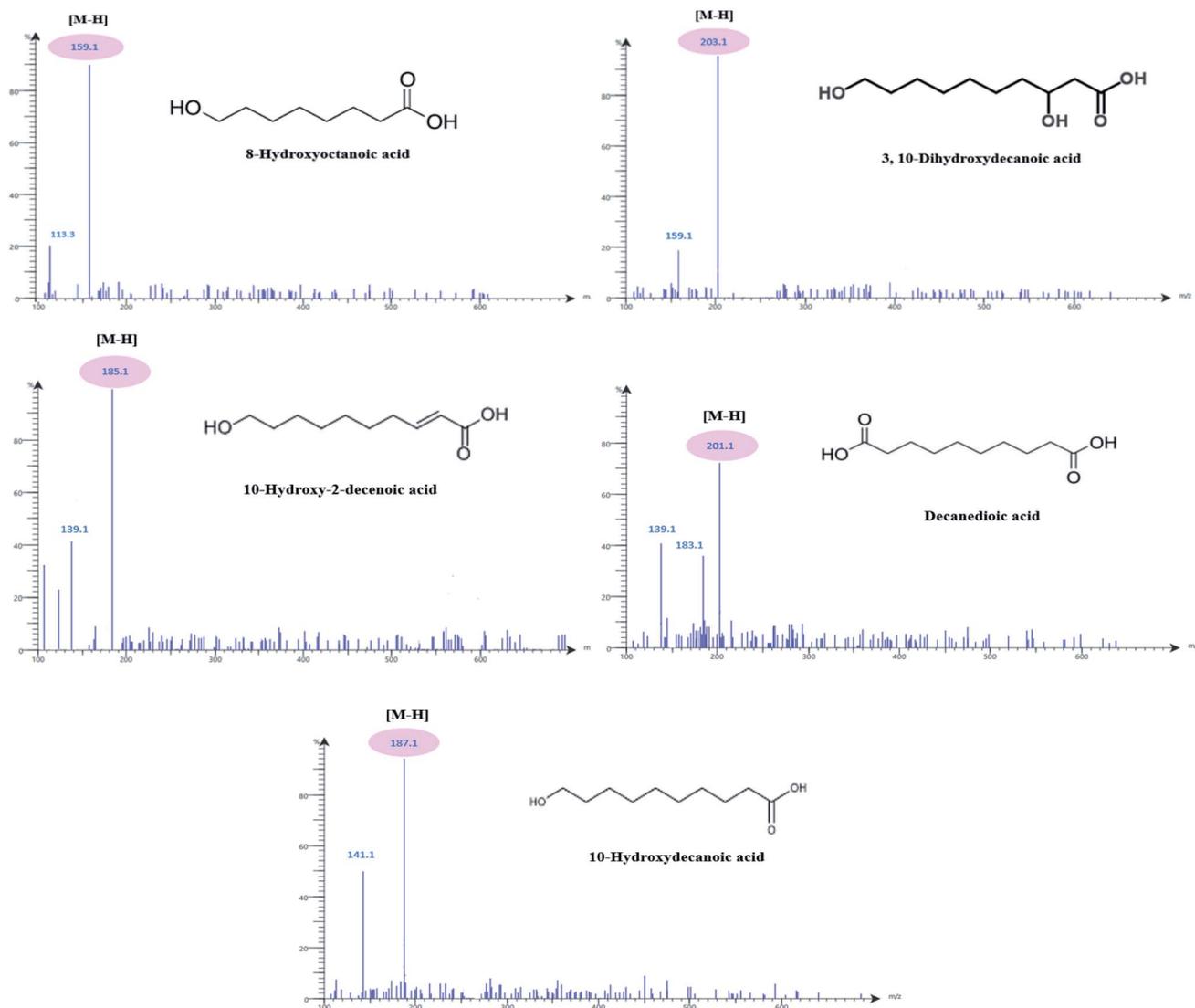


Fig. 3   HPTLC-ESI-MS analysis of 8-hydroxyoctanoic acid, 3,10-dihydroxydecanoic acid, 10-hydroxy-2-decenoic acid (10-HDA), decanedioic acid and 10-hydroxydecanoic acid in different RJ samples.
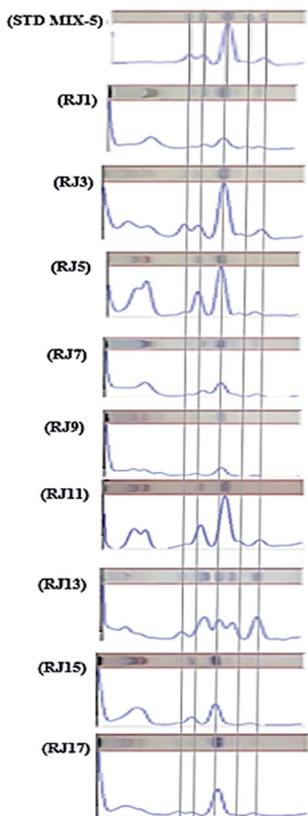
**Fig. 4** Superimposed chromatograms obtained for; standards solution mixture (STD MIX-5) and representative samples from each source; RJ1, RJ3, RJ5, RJ7, RJ9, RJ11, RJ13, RJ15 and RJ17 viewed in white light after post-chromatographic derivatization with anisaldehyde/sulfuric acid spray reagent.

subsequent heating to 105 °C is presented in (Fig. 1). The different RJ samples exhibited different chromatographic patterns which could be detected visually through examination of different relative bands intensities among samples tracks. The data set obtained from chromatogram contained 189 pixels length profile for each sample. Untargeted analysis was applied on the matrix constructed from 18 samples × 189 variables. Principal component analysis (PCA) was performed to provide an outline of the ability of the HPTLC variables to discriminate different RJ samples. Mutual projections of factor scores for the first two PCs described 58.2% (PC1) and 24.5% (PC2) of the variability in the data (Fig. 2A). The score scatter plot for the first two components revealed that lyophilized RJ samples (RJ 1, 2, 3, 4, 7, 8, 13, 14, 17 and 18) collected from different sources were successfully clustered along the positive side of PC1, while fresh samples (RJ 5, 6, 9, 10, 11, 12, 15 and 16) on its negative side. This neat separation between the two clusters indicates the difference in the fatty acids profiles between fresh and lyophilized RJ. This highlights the criteria of exploiting the holistic lipidomic profile of RJ rather than considering 10-HDA only as a quality marker.

The PC1 loading plot (Fig. 2B), revealed that bands at $R_f$ values 0.41, 0.5, 0.58, 0.67 and 0.73 are the variables that are the most influential on PC1 and PC2 directions. These bands were

scrapped and analyzed using mass spectrometry analysis. The zones at $R_f$ values of 0.41, 0.5, 0.58, 0.67 and 0.73 were identified as 8-hydroxyoctanoic acid, 3,10-dihydroxydecanoic acid, 10-hydroxy-2-decenoic acid (10-HDA), decanedioic acid and 10-hydroxydecanoic acid, respectively by comparison with reference substances and mass spectral libraries (Fig. 3).

## 3.2. Targeted fatty acids profiling and heat map of RJ samples

A novel digitally-enhanced HPTLC image analysis method using both parametric and non-parametric regression analyses was then developed and validated.[33] This method was applied for quantification of the five significant fatty acids identified from the fingerprint profiling chromatograms in different RJ samples (Fig. 4) namely; 8-hydroxyoctanoic acid, 3,10-dihydroxydecanoic acid, 10-hydroxy-2-decenoic acid (10-HDA), decanedioic acid and 10-hydroxydecanoic acid. The $R_f$ values and peak areas of the bands corresponding to the five significant fatty acids are shown in Table S2.†

**3.2.1. Parametric (P) and non-parametric (NP) regression analyses.** Comparison was carried out between the best fit straight line gained *via* non-parametric Theil's method[30] and that gained *via* the least squares parametric regression method.

*3.2.1.1. Linearity and sensitivity.* Linearity of the method was remarkably enhanced upon applying the non-parametric regression as indicated by higher value of correlation coefficient ($r$) and smaller intercept ($a$) when compared to parametric regression (Table 1). Moreover, the smaller values of ($S_{y/x}$), ($S_a$) and ($S_b$) and greater the values of ($F$) indicated that the regression line was steeper and the experimental points were less scattered around it in NP method than in P one (Table 1). The higher sensitivity of NP regression was indicated by the lower values of (LOD) and (LOQ). This signifies that the linearity ranges for the determination of royal jelly fatty acids could be extended to lower limits of quantitation upon applying the NP regression method.

*3.2.1.2. Accuracy and selectivity.* The accuracy of the method gave a satisfying mean recovery with low percentage relative standard deviation (RSD%) and percentage relative error ($E_r$%) (Table 2). This proves the high accuracy of the developed HPTLC-image analysis method for the determination of royal jelly fatty acids, and the absence of interfering spots with the chromatographic determination of these fatty acids in the samples. The percentage mean recovery was found to be higher and the percentage relative error got lower in the non-parametric regression method in comparison with the parametric one demonstrating better accuracy of the former method (Table 2). Upon applying Student's *t*-test for comparing sample means, it was found that the obtained *t* test values were higher than the critical ones and that indicated significant difference among their means (Table 2).

*3.2.1.3. Precision.* Evaluation of the repeatability (intra-day precision) and intermediate precision (inter-day precision) for the determination of royal jelly fatty acids by the developed HPTLC-image analysis method showed a good agreement among the individual test results data (Table S3†). It was

**Table 1** Parametric (P) and non-parametric (NP) linear regression and statistical parameters for the determination of royal jelly fatty acids by the developed HPTLC-image analysis method[a]

| | | 8-Hydroxyoctanoic acid | 3,10-Dihydroxydecanoic acid | 10-Hydroxy-2-decenoic acid | Decanedioic acid | 10-Hydroxydecanoic acid |
|---|---|---|---|---|---|---|
| Regression equation | P | $y = 2\,515\,60x + 1222.3$ | $y = 187\,378x + 2826.8$ | $y = 118\,014x + 27\,439$ | $y = 218\,883x + 650.23$ | $y = 121\,387x + 483.22$ |
| | NP | $y = 250\,836.9x + 718.2813$ | $y = 185\,537.9x + 2249.5$ | $y = 116\,388.9x + 29\,428.88$ | $y = 217\,409.7x + 596.2756$ | $y = 122\,901.4x + 22.125$ |
| $r$ | P | 0.914 | 0.914 | 0.968 | 0.985 | 0.989 |
| | NP | 0.998 | 0.999 | 0.998 | 0.999 | 0.999 |
| $a$ | P | 1222.3 | 2826.8 | 27 439 | 650.23 | 483.22 |
| | NP | 718.281 | 2249.5 | 29 428.88 | 596.276 | 22.125 |
| $b$ | P | 251 560 | 187 378 | 118 014 | 218 883 | 121 387 |
| | NP | 250 836.9 | 185 537.9 | 116 388.9 | 217 409.7 | 122 901.4 |
| $S_{y/x}$ | P | 1359.94 | 1678.986 | 4159.753 | 183.260 | 879.742 |
| | NP | 251.837 | 188.198 | 1109.376 | 70.017 | 375.978 |
| $S_a$ | P | 1604.025 | 1980.333 | 4906.352 | 483.330 | 2320.231 |
| | NP | 302.328 | 225.930 | 1331.795 | 84.055 | 451.358 |
| $S_b$ | P | 64 635.31 | 47 879.35 | 17 793.44 | 21 774.99 | 10 349.61 |
| | NP | 11 969.31 | 5366.811 | 4745.382 | 8319.475 | 4423.149 |
| $F$ | P | 15.148 | 15.316 | 43.989 | 101.043 | 137.562 |
| | NP | 658.773 | 1792.768 | 902.344 | 1024.371 | 1158.089 |
| LOD[b] | P | 0.021 | 0.035 | 0.137 | 0.007 | 0.063 |
| | NP | 0.004 | 0.004 | 0.038 | 0.001 | 0.012 |
| LOQ[b] | P | 0.064 | 0.106 | 0.416 | 0.022 | 0.191 |
| | NP | 0.012 | 0.012 | 0.114 | 0.004 | 0.037 |

[a] $r$: correlation coefficient, $a$: intercept, $b$: slope; $S_{y/x}$: standard deviation of residuals, $S_a$: standard deviation of intercept, $S_b$: standard deviation of slope, $F$: variance ratio; equals the mean of squares due to regression divided by the mean of squares about regression (due to residuals), LOD: limit of detection, LOQ: limit of quantitation. [b] Concentration mg per spot.

**Table 2** Parametric and non-parametric evaluation of the accuracy for the determination of royal jelly fatty acids in spiked samples by the developed HPTLC-image analysis method

| | Recovery%[b] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 8-Hydroxyoctanoic acid | | 3,10-Dihydroxydecanoic acid | | 10-Hydroxy-2-decenoic acid | | Decanedioic acid | | 10-Hydroxydecanoic acid | |
| Nominal fatty acid concentration[a] (µg per spot) | P | NP | P | NP | P | NP | P | NP | P | NP |
| 10 | 97.59 | 99.58 | 97.14 | 99.02 | 96.95 | 98.99 | 97.63 | 100.15 | 96.45 | 99.45 |
| 20 | 96.55 | 99.84 | 98.13 | 99.45 | 97.58 | 99.15 | 97.77 | 99.45 | 97.23 | 99.32 |
| 30 | 97.95 | 100.05 | 97.33 | 100.14 | 98.45 | 99.89 | 98.45 | 99.99 | 97.14 | 99.98 |
| Mean%[c] | 97.3633 | 99.8233 | 97.5333 | 99.5366 | 97.66 | 99.3433 | 97.95 | 99.8633 | 96.94 | 99.5833 |
| SD[d] | 0.72700 | 0.23544 | 0.52538 | 0.56500 | 0.75319 | 0.48013 | 0.43863 | 0.36678 | 0.42673 | 0.34961 |
| RSD (%)[e] | 0.74669 | 0.23586 | 0.53867 | 0.56763 | 0.77124 | 0.48331 | 0.44781 | 0.36729 | 0.44020 | 0.35108 |
| $E_r$%[f] | 2.63666 | 0.17666 | 2.46666 | 0.46333 | 2.34 | 0.65666 | 2.05 | 0.13666 | 3.06 | 0.41666 |
| $t$-Test[g] | — | 9.65749 | — | 7.78965 | — | 5.65367 | — | 10.0388 | — | 14.3747 |

[a] The fatty acid content in the sample + fatty acid content in standard fatty acids mixture added to the sample. [b] The mean recovery of three determinations at each concentration level of the fatty acid. [c] The mean of all recoveries at different concentration levels of the fatty acid. [d] Standard deviation of the mean of recoveries at different concentration levels of the fatty acid. [e] Percentage relative standard deviation. [f] Percentage relative error. [g] $t$-Test for comparison between two sample means, $t$ critical = 2.12 ($n_1 = n_2 = 9$, $P = 0.05$).

observed that, the RSD% got lower in the non-parametric method in comparison with the parametric one, demonstrating better precision of the former method. The obtained $F$ test values were higher than the critical ones and that proved the significant difference among the variance and precision of both methods (Table S3†).

**3.2.2. Quantification of RJ fatty acids and HCA-heat map.** The developed and validated HPTLC method was then applied for the quantification of the five discriminating fatty acids namely; 8-hydroxyoctanoic acid, 3,10-dihydroxydecanoic acid, 10-hydroxy-2-decenoic acid (10-HDA), decanedioic acid and 10-hydroxydecanoic acid in different RJ samples (Table 3). It was

Table 3  Assay results of royal jelly fatty acids in some royal jelly commercial and pharmaceutical products by the developed HPTLC-image analysis method

| | | | RJ1 | RJ2 | RJ3 | RJ4 | RJ5 | RJ6 | RJ7 | RJ8 | RJ9 | RJ10 | RJ11 | RJ12 | RJ13 | RJ14 | RJ15 | RJ16 | RJ17 | RJ18 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 8-Hydroxyoctanoic acid | P | % w/w[a] | 2.021 | 0.522 | 0.613 | 0.588 | 0.054 | 0.475 | 0.186 | 0.224 | 0.0003 | 0.031 | 0.035 | 0.319 | 0.959 | 0.753 | 0.057 | 0.189 | 0.588 | 2.598 |
| | | RSD%[b] | 3.15 | 2.18 | 3.44 | 1.15 | 2.63 | 3.47 | 2.87 | 2.62 | 1.23 | 1.45 | 2.96 | 3.56 | 4.15 | 3.55 | 2.14 | 4.25 | 1.06 | 2.88 |
| | NP | % w/w[a] | 2.067 | 0.550 | 0.655 | 0.617 | 0.094 | 0.503 | 0.227 | 0.251 | 0.040 | 0.058 | 0.076 | 0.347 | 1.002 | 0.782 | 0.097 | 0.216 | 0.630 | 2.633 |
| | | RSD%[b] | 1.02 | 0.962 | 0.854 | 0.752 | 1.120 | 2.141 | 0.532 | 0.632 | 0.523 | 0.334 | 0.874 | 0.465 | 1.062 | 2.018 | 0.821 | 0.654 | 0.721 | 0.698 |
| 3,10-Dihydroxydecanoic acid | P | % w/w[a] | 4.699 | 1.301 | 0.658 | 0.713 | 1.197 | 2.510 | 1.304 | 1.288 | 0.125 | 0.291 | 0.840 | 1.39 | 6.203 | 4.484 | 1.660 | 1.557 | 0.621 | 2.769 |
| | | RSD%[b] | 1.023 | 2.036 | 0.947 | 3.547 | 4.235 | 2.587 | 1.879 | 0.965 | 0.874 | 1.854 | 2.369 | 3.147 | 4.258 | 0.987 | 1.014 | 1.587 | 2.854 | 3.698 |
| | NP | % w/w[a] | 4.808 | 1.355 | 0.726 | 0.761 | 1.271 | 2.576 | 1.379 | 1.342 | 0.189 | 0.335 | 0.911 | 1.452 | 6.326 | 4.569 | 1.739 | 1.614 | 0.689 | 0.838 |
| | | RSD%[b] | 0.587 | 0.658 | 0.365 | 1.321 | 1.258 | 0.987 | 0.789 | 0.587 | 0.258 | 1.025 | 0.981 | 1.547 | 1.694 | 0.258 | 0.369 | 0.598 | 0.982 | 1.025 |
| 10-Hydroxy-2-decenoic acid | P | % w/w[a] | 17.566 | 7.193 | 5.130 | 4.698 | 2.755 | 6.053 | 6.186 | 5.918 | 0.286 | 0.670 | 3.334 | 5.061 | 2.233 | 1.593 | 4.573 | 4.179 | 4.815 | 10.861 |
| | | RSD%[b] | 2.369 | 3.215 | 3.178 | 4.125 | 2.587 | 2.569 | 3.2158 | 1.025 | 0.954 | 0.9236 | 0.821 | 1.698 | 2.145 | 3.874 | 4.325 | 1.365 | 2.146 | 0.954 |
| | NP | % w/w[a] | 17.469 | 7.066 | 4.860 | 4.536 | 2.451 | 5.910 | 5.930 | 5.772 | 0.632 | 0.451 | 3.039 | 4.904 | 2.606 | 1.387 | 4.295 | 4.010 | 4.540 | 10.785 |
| | | RSD%[b] | 0.965 | 0.785 | 1.012 | 1.085 | 0.987 | 0.783 | 0.654 | 0.521 | 0.322 | 0.654 | 0.527 | 0.964 | 1.036 | 1.113 | 2.698 | 0.540 | 1.369 | 0.725 |
| Decanedioic acid | P | % w/w[a] | 0.356 | 0.272 | 0.329 | 0.220 | 0.164 | 0.154 | 0.125 | 0.104 | 0.086 | 0.139 | 0.120 | 0.081 | 1.848 | 1.122 | 0.376 | 0.229 | 0.170 | 0.186 |
| | | RSD%[b] | 3.265 | 2.587 | 3.254 | 1.458 | 1.154 | 2.487 | 3.258 | 2.147 | 0.982 | 0.977 | 0.821 | 1.023 | 1.778 | 0.951 | 1.235 | 1.587 | 2.016 | 3.487 |
| | NP | % w/w[a] | 0.364 | 0.278 | 0.337 | 0.225 | 0.170 | 0.159 | 0.130 | 0.108 | 0.092 | 0.143 | 0.126 | 0.084 | 1.865 | 1.133 | 0.384 | 0.234 | 0.176 | 0.191 |
| | | RSD%[b] | 1.087 | 1.005 | 0.954 | 0.752 | 0.541 | 1.547 | 1.004 | 1.054 | 0.725 | 0.654 | 0.587 | 0.885 | 0.964 | 0.798 | 0.652 | 0.752 | 0.654 | 1.651 |
| 10-Hydroxydecanoic acid | P | % w/w[a] | 9.726 | 2.187 | 1.424 | 1.148 | 0.558 | 2.333 | 1.338 | 1.581 | 0.093 | 0.136 | 1.281 | 2.672 | 5.368 | 4.942 | 0.668 | 0.615 | 0.875 | 4.802 |
| | | RSD%[b] | 4.258 | 3.547 | 3.115 | 2.014 | 2.020 | 3.008 | 1.965 | 2.547 | 3.547 | 4.087 | 1.887 | 2.698 | 3.254 | 4.125 | 3.998 | 2.465 | 1.982 | 0.954 |
| | NP | % w/w[a] | 9.681 | 2.210 | 1.482 | 1.184 | 0.626 | 2.355 | 1.397 | 1.611 | 0.167 | 0.184 | 1.340 | 2.689 | 5.377 | 4.931 | 0.735 | 0.657 | 0.939 | 4.793 |
| | | RSD%[b] | 1.254 | 1.115 | 0.654 | 0.964 | 0.447 | 2.541 | 1.008 | 1.567 | 2.695 | 2.487 | 0.924 | 1.254 | 1.874 | 2.146 | 2.541 | 1.054 | 1.231 | 0.574 |

[a] The average mg % w/w of triplicate determinations. [b] Percentage relative standard deviations.
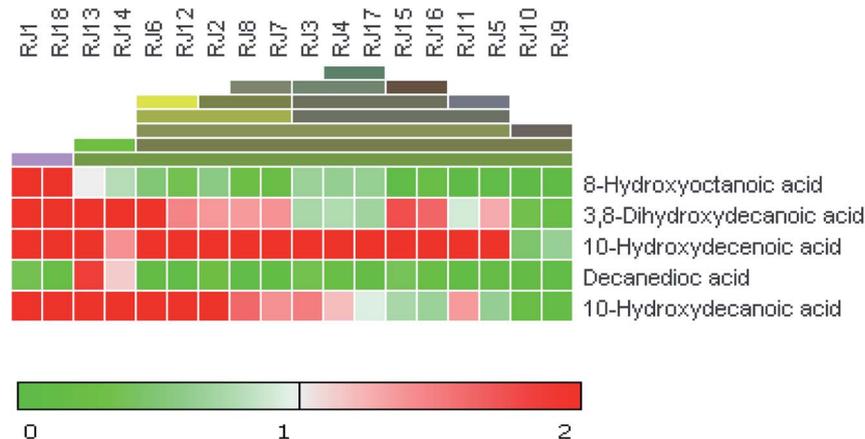
**Fig. 5** HCA–heat map of the 18 RJ samples using the five discriminating fatty acids.
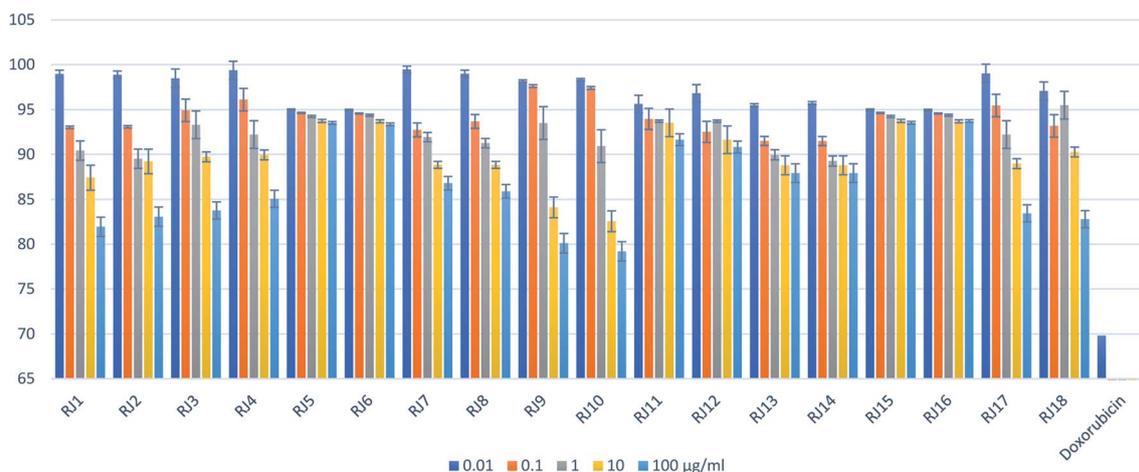


**Fig. 6** B16F1 melanoma cells cytotoxic activities of the 18 RJ samples and doxorubicin as a positive control (results are average of three determinations ± SD).

noticed that RSD% between samples replicates was lower in NP than P method indicating its superiority, therefore, it was adopted in further hierarchical clustering analysis (HCA)-heat-map (Fig. 5). HCA was used as an agglomerative approach for class membership depending on the concentrations of the preselected fatty acids using Ward's algorithm.

### 3.3. OPLS for efficient anti-melanoma biomarker determination

The investigation of the entire RJ fatty acids profile has not been previously reported. For the sake of that, an OPLS model for bioefficacy-directed discrimination of RJ samples was constructed. The model was built in order to correlate the chromatographic lipidomic profile with the B16F1 melanoma cell cytotoxic activity. The multivariate model X matrix (18 RJ samples × five variables) was composed of the predetermined concentrations of the five fatty acids using the NP Theil's regression method, while the B16F1 melanoma cytotoxic activities of the 18 samples (Fig. 6) served as Y matrix. The score scatter plot of the built OPLS model is presented in (Fig. 7A).

The OPLS 3D loading plot (Fig. 7B) represent the Y-variables which are closely correlated to X-variables positioned near them. The first latent variable explained 62.7% of the variation followed by 6.3% variances of the second latent variables, respectively. It was noticed that the queen bee fatty acid (10-HDA) was the closest fatty acid to B16F1 melanoma cytotoxicity data point. This result was further confirmed *via* variable importance for the projection (VIP) plot (Fig. 7C). VIP plot highlighted that 10-HDA followed by decanedioic acid then 8-hydroxyoctanoic acid as the most influencing chemical markers on the OPLS model by possessing a threshold value greater than one. These results came in agreement with the study performed by Peng, *et al.*[11] where 10-HDA exhibited significant reduction in B16F1 melanoma cells viability at a concentration of 1 mM. However, the study attributed the anti-melanoma activity of RJ to a single fatty acid (10-HDA) rather than considering the entire fatty acids profile. The present study is considered the first attempt to discriminate fresh and lyophilized RJ samples based on their holistic lipidomic profile to discover the efficient fatty acids reducing melanoma cell viability.
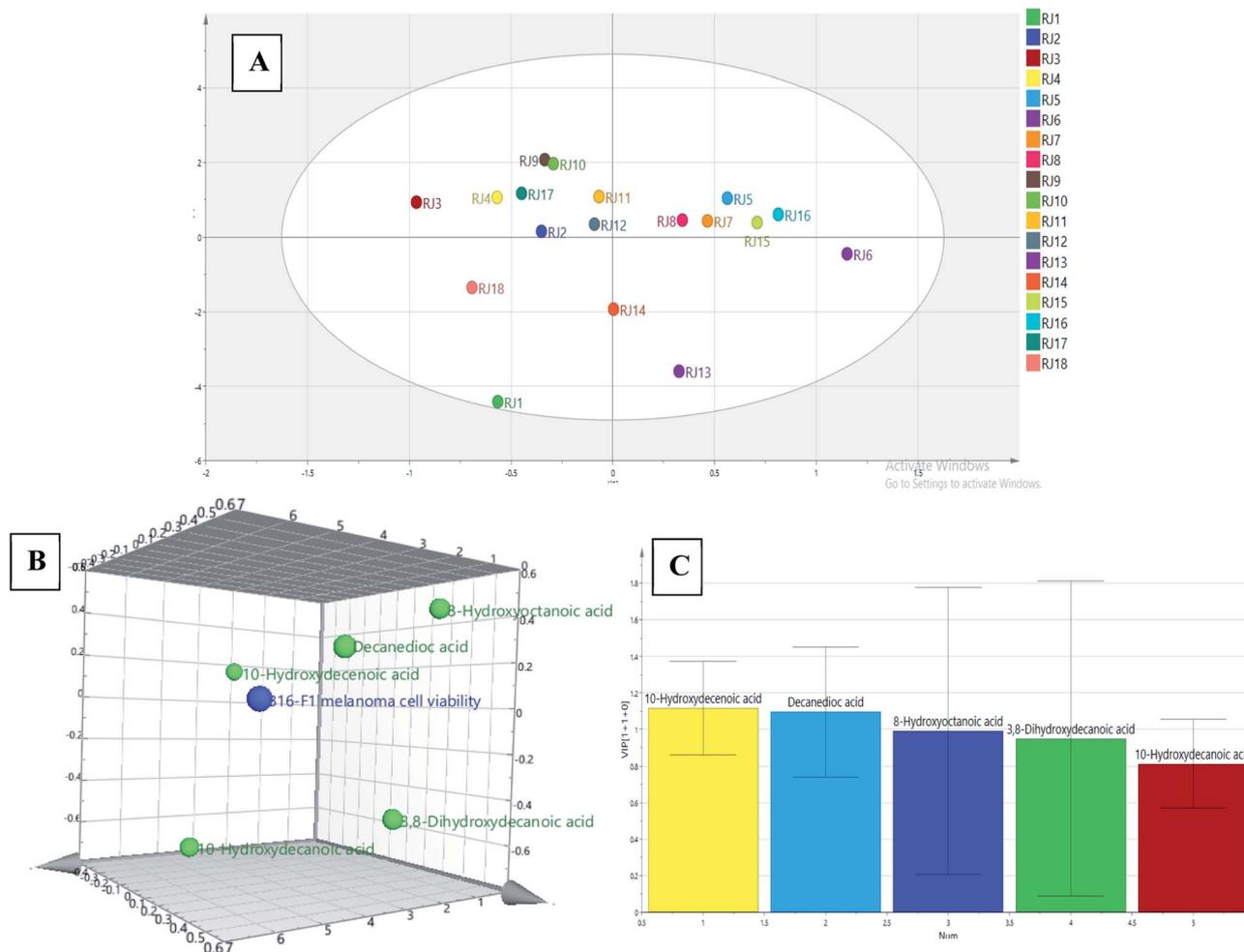
Fig. 7 OPLS score scattered plot of the 18 RJ samples (A), 3D loading scatter plot of the five discriminating fatty acids and B16F1 melanoma cells cytotoxic activities (B) and VIP plot of the OPLS model (C).

## 4. Conclusion

Untargeted and targeted fatty acid profiling of different royal jelly commercial and pharmaceutical products based on HPTLC-image analysis, HPTLC-MS and anti-melanoma activity testing with the use of chemometric analysis was successfully applied in this study. 8-Hydroxyoctanoic acid, 3,10-dihydroxydecanoic acid, 10-hydroxy-2-decenoic acid, decanedioic acid and 10-hydroxydecanoic acid were identified as the chemical markers responsible for the classification of royal jelly samples into fresh and lyophilized samples using principal component analysis (PCA). The parametric and non-parametric regression methods were applied for quantification of these chemical markers. The non-parametric method was better than parametric one in terms of linearity, accuracy and precision. OPLS model showed that 10-HDA was the closest fatty acid to B16F1 melanoma cytotoxicity data point, followed by decanedioic acid then 8-hydroxyoctanoic acid. This study represents the first attempt to discriminate fresh and lyophilized royal jelly samples based on their holistic lipidomic profile and to discover the efficient fatty acids reducing melanoma cell viability and can be extended for efficacy-directed discrimination of complex matrices of other natural products.

## Conflicts of interest

There are no conflicts to declare.

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