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Chemometric discrimination of eight *citrus* plants utilizing chromatographic and spectroscopic techniques and insights into their biological potentials

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ABSTRACT

Citrus sinensis balady orange, *C. sinensis* navel orange, *C. paradisi*, *C. limon*, *C. sinensis* bloody orange, C. *sinensis* sweet orange, *C. aurantium* var. *amara* and *C. reticulata* were successfully discriminated using chromatographic and spectroscopic techniques coupled with chemometrics. Ultraviolet spectroscopy (UV), and nuclear magnetic resonance spectroscopy (NMR) managed to discriminate the alcohol extract samples to six and five clusters respectively on exposing the obtained data to Principle component analysis (PCA). High performance liquid chromatography (HPLC) was utilized for differentiating the different samples based upon their rutin content where *C. aurantium* demonstrated the highest rutin content (0.795 mg/mL). LC-ESI-MS led to the identification of 35 compounds belonging mainly to flavonoids and limonoids. *In vitro* biological investigations including DDPH, ABTS, FRAP and enzyme inhibitory activities revealed the promising antioxidant, neuroprotective, anti-hyperglycaemic and skin-lightning potentials of *citrus* samples that were correlated with the total phenol and flavonoid contents. In *silico* ADME/TOPKAT reflected the acceptable pharmacokinetic, pharmacodynamic and toxicity properties of the identified secondary metabolites.

1. Introduction

The *Citrus* genus, comprising diverse species of flowering plants belonging to the Rutaceae family, holds immense value both economically and culturally worldwide. Renowned for their luscious fruits, citrus trees are cultivated in various regions across the globe, from tropical to subtropical climates (Spiegel-Roy and Goldschmidt, 1996). The genus includes iconic fruits such as oranges, lemons, limes, grapefruits, and mandarins, each prized for its unique flavor, aroma, and nutritional benefits. Besides being favourable foods, *Citrus* fruits revealed a variety of health benefits related to its multiple phytoconstituents particularly flavonoids, limonoids, coumarins, essential minerals and vitamins (Gironés-Vilaplana et al., 2014; Tripoli et al., 2007). Significant enhancement in blood circulation, antiviral, anti-allergic and anti-cancer effects have been ascribed to *Citrus* juices especially to oranges and grapefruits (Kumari et al., 2023). *Citrus* fibers as well proved to exhibit regulatory mechanisms relevant to cardiovascular disorders, type 2 diabetes mellitus, and cancer (Liu et al., 2024). Promising antioxidant, antibacterial and anticancer activities were demonstrated for *Citrus* peels probably related to their volatile constituents like α-pinene,

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S-limonene, and cis-terpinene (Job et al., 2024).

Chemical variability among *Citrus* species is a well-documented phenomenon that stems from differences in genetics, environmental factors, and agronomic practices. This variability manifests in the composition and concentration of various phytochemicals, including flavonoids, phenolic compounds, essential oils, and organic acids, which contribute to the distinctive aroma, flavor, and health properties of different *Citrus* cultivars (Ben Hsouna et al., 2023; Chen et al., 2021). Through combining advanced chromatographic, spectroscopic, and spectrometric techniques, researchers can delve into the intricate chemical makeup of citrus extracts, identifying and quantifying individual compounds with precision (Castañeda et al., 2024; El-Din et al., 2023). Besides, the emergence of chemometric techniques combined with diverse chromatographic and spectroscopic methods shows promise as a valuable tool for distinguishing between related species, offering enhanced precision and depth in classification (Aboulwafa et al., 2018; Gamal El-Din et al., 2022).

Leaves are often an underutilized and undervalued part of *Citrus* plants despite their rich potential. While much attention is traditionally given to the fruits for their economic and culinary significance, the *Citrus* leaves hold a treasure trove of bioactive compounds that remain largely untapped. The morphological features of *Citrus* leaves offer valuable discriminative characteristics, aiding in the differentiation of *Citrus* species and cultivars (Riahi et al., 2024). Meanwhile, exploring the chemical profiles of *Citrus* leaves presents an opportunity to unlock their hidden potential for nutraceutical, and medicinal applications. Furthermore, analysing the unique composition of bioactive compounds present in the leaf extracts serves as a powerful tool for taxonomic classification, facilitating more accurate delineation between closely related species and varieties, and hence aid in varietal authentication and quality control. Utilizing *Citrus* leaves could likewise provide added value to *Citrus* orchards *via* creating alternative revenue streams and reducing waste.

Hence, the current study aimed to discriminate between the leaves of eight *Citrus* varieties abundant in Egypt namely *C. sinensis* balady orange, *C. sinensis* navel orange, *C. paradisi*, *C. limon*, *C. sinensis* bloody orange, C. *sinensis* sweet orange, *C. aurantium* var. amara *and C. reticulata* utilizing Ultra Violet spectroscopy (UV), High-performance liquid chromatography (HPLC) and Nuclear Magnetic Resonance spectroscopy (NMR) analyses coupled with multivariate data analyses. Besides, comprehensive metabolic profiling of the eight *Citrus* extracts using LC-ESI-MS followed for enriching the arsenal of tools available for differentiating the different *Citrus* varieties. Additionally, comparative investigation of the antioxidant, neuroprotective, anti-hyperglycaemic and skin-lightning effects of the different *citrus* extracts was accomplished utilizing various *in vitro* assays. Moreover, the pharmacokinetic, pharmacodynamic and toxicity properties of the major secondary metabolites were investigated.

2. Materials and methods

2.1. Plant material

Eight fresh *Citrus* species and varieties namely *C. sinensis* balady orange (CSB1), *C. sinensis* navel orange (CSN2), *C. paradisi* (grapefruit) (CP3), *C. limon* (L.) Burm. (lemon) (CL4), *C. sinensis* bloody orange (CSO5), C. *sinensis* sweet orange (CSS6), *C. aurantium* var. *amara (Bitter* orange) (CAA7) and *C. reticulata* (mandarin orange) (CR8) (Rutaceae) were obtained from the Research Station of the Faculty of Agriculture, Benha University, Egypt. The different species and varieties were kindly authenticated and ascertained by Professor B. Houlyel, professor of pomology, Faculty of Agriculture, Benha University. Voucher specimens of the leaves of the collected plants were kept under the code number of CSBa07211, CSN07212, CP07213, CL07214, CSBb07215, CSS07216, CA07217, and CR07218, respectively in the Department of Pharmacognosy, Faculty of Pharmacy, Menoufia University, Shibin Elkom, Egypt.

2.2. Preparation of the different citrus species leaf extracts

The air-dried leaves (10 g) of the eight *Citrus* species were subjected to coarse crushing followed by three successive macerations in neat methanol till exhaustion (300 mL L × 3). Filtration, evaporation of filtrates at 45 °C under reduced pressure in Rotvap (Heidolph Instruments, model Laborota 4001, Viertrieb, Germany) and final lyophilization followed to yield dried extracts of 1.53 g, 1.02 g, 1.67 g, 0.97 g, 1.32 g, 1.73 g, 1.48 g and 1.28 g of CSB1, CSN2, CP3, CL4, CSO5, CSS6, CAA7 and CR8, respectively.

2.3. Sample preparation of different citrus species samples and instrumentation

2.3.1. Ultraviolet spectroscopy

A stock solution of each *Citrus* sample was prepared by macerating 0.5 g of each dried *Citrus* extract in 10 mL of methanol (HPLC grade) to obtain a concentration of 50 mg/mL. For ultraviolet (UV) spectroscopic analysis, 1 mL of the stock solution was withdrawn, diluted with methanol to 25 mL using a stoppered glass volumetric flask to yield a concentration of 2 mg/mL. The prepared samples were individually analyzed by V-630 UV–Visible spectrophotometer (JASCO, Shimadzu, Japan). Measurements were in triplicates and the spectra was assessed in the UV region from 200 to 400 nm. An excel sheet was construction for a matrix comprising the total sum of samples and their triplicates multiplied by 200 variables for the multivariate data analysis. The entire spectrum ranging from 200 to 400 nm was taken into account to define PC1 and PC2 (Aboulwafa et al., 2018; Gad and Bouzabata, 2017).

2.3.2. High performance liquid chromatography (HPLC)

Analysis using High Performance Liquid Chromatography (HPLC) was performed following the previously validated described method (Gad and Bouzabata, 2017; Gómez-Mejía et al., 2019). Calibration solutions of 0.20, 0.40, 0.60, and 1.00 mg/mL were prepared for rutin standards in HPLC grade MeOH. The determination of the working range was guided by previous studies reporting rutin quantitation from citrus extracts (Bilbao et al., 2007; Gómez-Mejía et al., 2019). Samples were prepared in triplicates, individually filtered through a PTFE membrane millipore filters (25 mm,0.45 μm) into HPLC vials and aliquots 20 μL of each standard solution were injected into C18 reversed-phase column (150 mm, 4.6 mm, 5 μm) coupled with a quaternary pump in the LC system, Agilent 1200 series HPLC-DAD (Agilent Technologies, Santa Clara, CA, USA). The mobile phase consisted of water as solvent A and methanol as solvent B. The concentration of methanol (solvent B) was incrementally increased by 10% every 3 min until reaching 100% in 30 min. This gradient elution technique was carried out at a flow rate of 1 mL/min. The eluted samples were detected at the maximum wavelength absorption (λ max) of 278 nm (Aboulwafa et al., 2018). A calibration curve was generated for rutin by plotting the peak area against the concentration, which was the average of three measurements. Linearity was assessed by calculating the linear regression from the peak area versus concentration plot of standard solutions using the linear least squares methodology. The limits of detection (LOD) and quantification (LOQ) were determined mathematically based on the standard deviation (sd) of the calibration curve and its slope (S), using multipliers specified in the ICH standard (International Conference on Harmonization, FDA, USA). The LOD and LOQ were calculated using the equations: LOD = [3.3 * sd/S] and LOQ = [10 * sd/S].

For HPLC chromatographic analysis of *Citrus* extracts, 1 mL of the prepared stock solutions of different *Citrus* varieties (described in section 2.3.1) was diluted to 50 mL using neat methanol (HPLC grade) to attain concentrations of 1 mg/mL. HPLC investigations were performed in triplicates where 20 μL aliquots of each sample were injected into the LC system, with elution carried out as previously described with rutin

standards. Determination of rutin was done quantitatively for each tested sample utilizing calibration curve for standard rutin solutions (Youssef et al., 2023).

2.3.3. Nuclear magnetic resonance spectroscopy (NMR)

^{1}H NMR analyses were performed on a Bruker 400 MHz NMR spectrometer at the operating frequency of 400.13 Hz. 10 mg of each sample were accurately weighed in 1.5 mL reaction tube followed by the addition of 1 mL of deuterated DMSO-D6 (Sigma-Aldrich, Germany). The mixture was then mixed thoroughly and sonication followed for 15 min in an ultrasound bath, and then kept to stand followed by centrifugation for 10 min at 14,000 rpm. The supernatant (800 μL) was transferred to a 5 mm diameter NMR spectroscopy tube, and NMR analysis was performed (Gad and Bouzabata, 2017; Youssef et al., 2017, 2021a). ^{1}H NMR spectra of the examined samples were imported to the ACD Lab software where signals in the range 0–8 ppm were utilized for chemomertic analysis. Exclusion of the residual solvent signals for DMSO (2.65–2.45 ppm) and water (3.60–3.10 ppm) was done followed by importing the data to an excel sheet for multivariate analysis.

2.3.4. Liquid chromatography coupled with mass (LC-ESI-MS) metabolic profiling of citrus species

Metabolic profiling of the methanol extracts for the eight *Citrus* species was done on Agilent 1100 Series using Knauer (250 × 2 mm, ID) that is pre-packed with Eurospher 100-5 C18, and an integrated pre-column. 10 μL were used as an injection volume and the mobile phase was composed of two solvents; solvent A composed of water with 0.1 % formic acid whereas solvent B is acetonitrile (ACN) containing 0.1 % formic acid. Elution was done in a gradient manner starting from 10% of solvent B till 100% at 28 °C. The flow rate was set at 0.2 mL min^{-1}resultig in equilibration time of 26 min for 10 column volumes. A Finnigan LCQ-DECA mass spectrometer was employed and ESI interface both was used both in negative and positive modes where drying and nebulizing gas is N_2, the capillary temperature is 250 °C whereas the spray, capillary and tube lens voltages are 4.48 kV; 39.6 V and 10.00 V, respectively with full scan mode in mass range *m/z* 100–2000 m/z. Xcalibur TM 2.0.7 software (Thermo Scientific, Karlsruhe, Germany) was used for data acquisitions as well as chromatogram integration (Thabet et al., 2018).

2.3.5. Determination of total phenolic and flavonoid contents

Total phenols and flavonoids contents for the different *Citrus* species leaf extract were evaluated using spectrophotometric assays with Folin-Ciocalteu and $AlCl_3$, respectively as previously reported where the results obtained from analyzes were computed and expressed as mg GAE (gallic acid equivalent)/g, and mg Re (rutin equivalent)/g for total phenolics and flavonoids, respectively (Kljakić et al., 2023; Uysal et al., 2017; Youssef et al., 2022).

2.4. In vitro biological evaluation of citrus species

2.4.1. In vitro evaluation of the antioxidant activity

In vitro evaluation of the antioxidant activity of the eight *Citrus* plants was done using free radical scavenging 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), ferric-reducing antioxidant power (FRAP), reducing-power cupric-reducing antioxidant capacity (CUPRAC), metal-chelating (MCA) and phosphomolybdenum (PHD) assays as previously described (Apak et al., 2016). Trolox equivalent (TE/g extract) was used to express the results that are carried three times and the differences among the examined samples were calculated by ANOVA assays (Tukey's test) whereas metal-chelating activity was expressed as the EDTA equivalent (mg EDTA/g extract).

2.4.2. In vitro evaluation of the neuroprotective activity

In vitro neuroprotective activity of the eight *Citrus* plants was evaluated *via* the determination of acetyl cholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory potential as previously determined by Aktumsek et al. (2013) with certain modification as described by Mamadalieva et al. (2022). Galantamine equivalent (mg GALAE/g) was used to express the results that are carried three times and the differences among the examined samples were calculated by ANOVA assays (Tukey's test).

2.4.3. In vitro evaluation of the anti-hyperglycaemic activity

In vitro anti-hyperglycaemic activity of the eight *Citrus* plants was determined *via* estimation of amylase inhibitory activity using Caraway–Somogyi iodine/potassium iodide (IKI) method as previously reported by Lazarova et al. (2015) but with some modifications as described by Mamadalieva et al. (2022). Acarbose equivalent (mg ACAE/g) was used to express the results that are carried three times and the differences among the examined samples were calculated by ANOVA assays (Tukey's test).

2.4.4. In vitro evaluation of the skin-lightning activity

In vitro skin-lightning activity was determined *via* the calculation of tyrosinase inhibition potential adopting dopachrome assay with slight modification as previously described (Mamadalieva et al., 2022; Zengin et al., 2014). Kojic acid equivalent (mg KAE/g) was used to express the results that are carried three times and the differences among the examined samples were calculated by ANOVA assays (Tukey's test).

2.5. Discrimination of the eight citrus species using multivariate data analysis

Multivariate data analyses were done using unsupervised pattern recognition technique comprising principle component analysis (PCA) and Hierarchical cluster analysis (HCA) depending upon the reported data from the different chromatographic and spectroscopic techniques. Both PCA and HCA were done by CAMO's Unscrambler® X 10.4 software (Computer-Aided Modeling, As, Norway) as previously reported (Altyar et al., 2020; Gamal El-Din et al., 2022; Youssef et al., 2020). PCA provide a notable discrimination for all observations gathered from the assessed samples with concomitant discrimination into separate clusters based upon their variation in the quantity and quality of the secondary metabolites that certainly reflected on their discriminant biological behavior. Meanwhile, HCA was done using the entire linkage approach designed for the group classification of groups (Bouzabata et al., 2022).

2.6. Pharmacokinetic, pharmacodynamic and toxicity prediction

Pharmacokinetic, pharmacodynamic and toxicity determination of the predominating compounds in *Citrus* species was done using the ADME/TOPKAT (absorption, distribution, metabolism, excretion, and toxicity) protocol carried by Discovery Studio 4.5 software (Accelrys Inc., San Diego, CA, USA). The assessed ADME parameters comprises human intestinal absorption, aqueous solubility, plasma protein binding prediction (PPB), inhibition potential of cytochrome P450 2D6, blood-brain barrier penetration (BBB) and liver toxicity effect. TOPKAT prediction descriptors include Rat oral LD50, chronic LOAEL (lowest observed adverse effect level), female and male rats carcinogenicity based on National Toxicology Program (NPT), Ames mutagenic potential, skin and eye irritation, and toxicity to hepatocellular carcinoma (Bouzabata et al., 2022; Elhady et al., 2021; Mamadalieva et al., 2021; Youssef et al., 2021b).

3. Results

3.1. Ultraviolet spectroscopy for the discrimination of citrus species

The methanol extracts of the eight *Citrus* plants exhibited UV absorption bands within the UV region in the range of 200–400 nm. The UV spectroscopic data were exposed to unsupervised Principle

component analysis (PCA) employing cross validation technique after mean centering processing for the data. PCA score plot illustrated in Fig. 1A resulted in a successful discrimination of the eight *Citrus* plants into six main clusters with PC1 and PC2 accounted for 91% and 7% of whole data variance, respectively. *C. paradisi* (grapefruit) and *C. sinensis* bloody orange are clustered together in one cluster that is allocated in the upper right quadrant showing positive values for both PC1 and PC2 and thus reflecting their similarities in their secondary metabolites with UV absorbance potential. However, *C. limon* is greatly discriminated from the rest of tested samples which is located in the upper left quadrant showing negative values for PC1 (91% of variance) and positive values for PC2. Meanwhile, C. *sinensis* sweet orange and *C. aurantium* var. *amara* were placed together in one cluster occupying the centre of the plot approaching that of *C. sinensis* navel orange. Regarding, *C. sinensis* balady orange and *C. reticulata* (mandarin orange), they exhibited discriminate clusters where the former lies in the lower right quadrant showing positive values for PC1 and negative values for PC2 whereas the latter occupies the lower left quadrant with negative values for both PCs. Moreover, the loading plot illustrated in Fig. S1 showed that all of the metabolites showing absorption in the region of 200–400 nm (Fig. 1B) serve as discriminatory signals that led to the distinctive classification of the tested samples into discriminate clusters. Results of PCA were further consolidated by HCA where the dendrogram illustrated in Fig. 1C revealed three main clusters namely cluster I, II, and III evidenced by the short distance between the grouped clusters. *C. limon* constituted a separate cluster (Cluster I) whereas cluster II included *C. sinensis* navel orange, C. *sinensis* sweet orange and *C. aurantium* var. *amara* that are very close distance to each other in addition to *C. reticulata* (mandarin orange) that is slightly further from the other. Regarding cluster III was further subdivided into two sub-clusters one containing *C. paradisi* and *C. sinensis* bloody orange meanwhile the other contains *C. sinensis* balady orange.

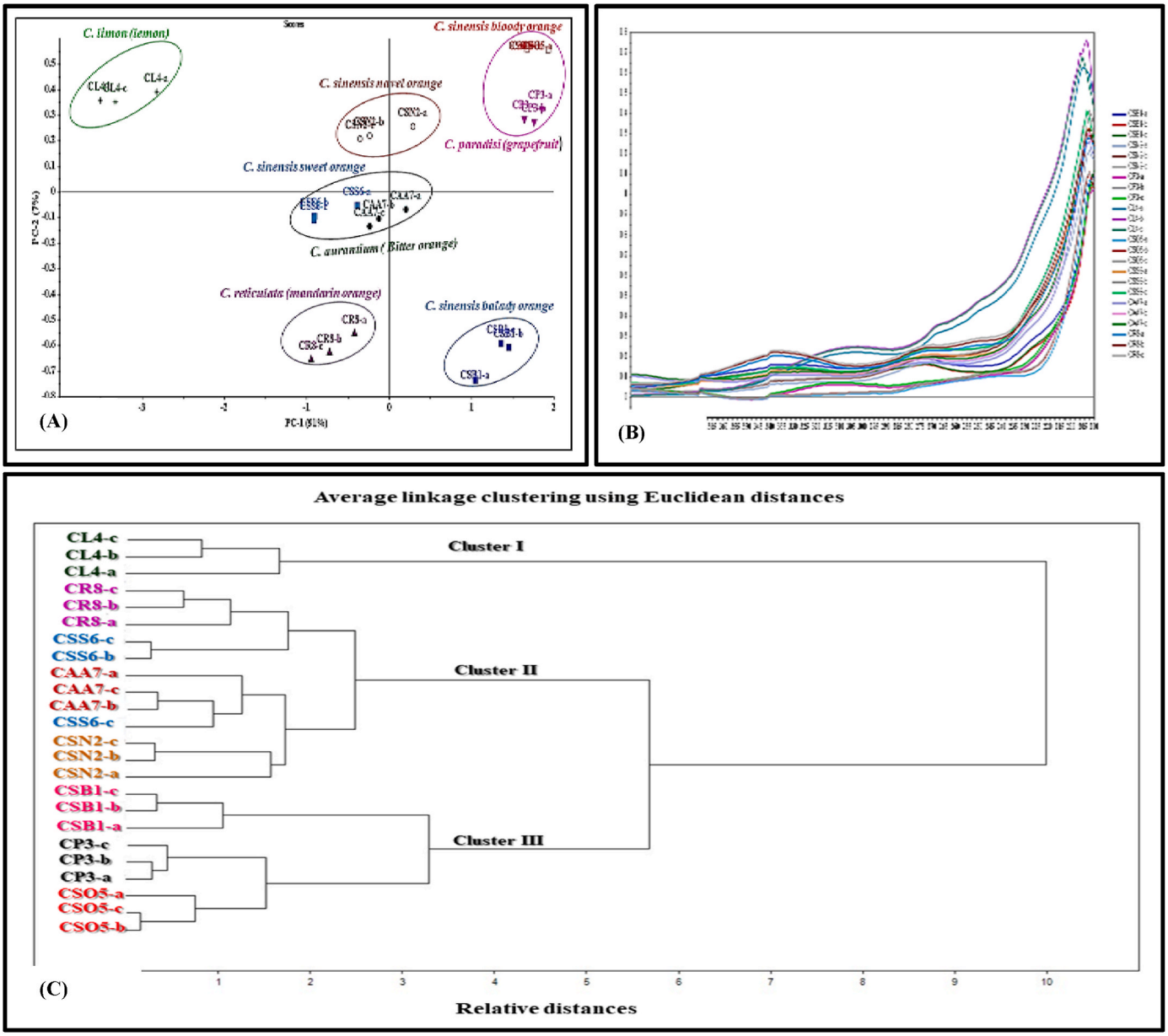

Fig. 1. UV-based multivariate data analysis of eight *Citrus* plants. **(A)** Score plot; **(B)** overlapped UV-spectra; x-axis represents wavelengths in nm and y-axis represents the absorbances **(C)** HCA.

3.2. High Performance Liquid Chromatography (HPLC) for the discrimination of citrus species

Herein, HPLC analysis was done to further discriminate the eight *Citrus* plants based upon their rutin content in the methanol extracts of their leaves where the % of rutin (Fig. 2SA) was estimated in the *Citrus* samples relying upon a validated HPLC analysis *via* the construction of a standard rutin calibration curve (Fig. 2SB). The content of rutin in each *Citrus* sample was deduced from the produced peak area where each sample was measured in triplicates (Figs. S3 and S4) and summarized in Table 1. Results reveal the highest concentration of rutin in *C. aurantium* var. *amara*)Bitter orange) (CAA7) estimated by 0.795 mg/mL in contrast to *C. limon* (lemon) (CL4), that showed the lowest concentration of rutin estimated by 0.137 mg/mL. Besides, *C. reticulata* (mandarin orange) (CR8) and *C. sinensis* bloody orange (CSO5) showed almost similar rutin content estimated by 0.393 mg/mL. Rutin calibration curve exhibited a good linearity in the assessed range of concentration (0.2–1 mg/mL) with $R^2 > 0.995$, with confidence interval of 95% for the slope (m) and y-intercept (b) estimated to be 22888.85–41197.45 and −5808.52-5625.19 respectively. The limit of detection and limit of quantification

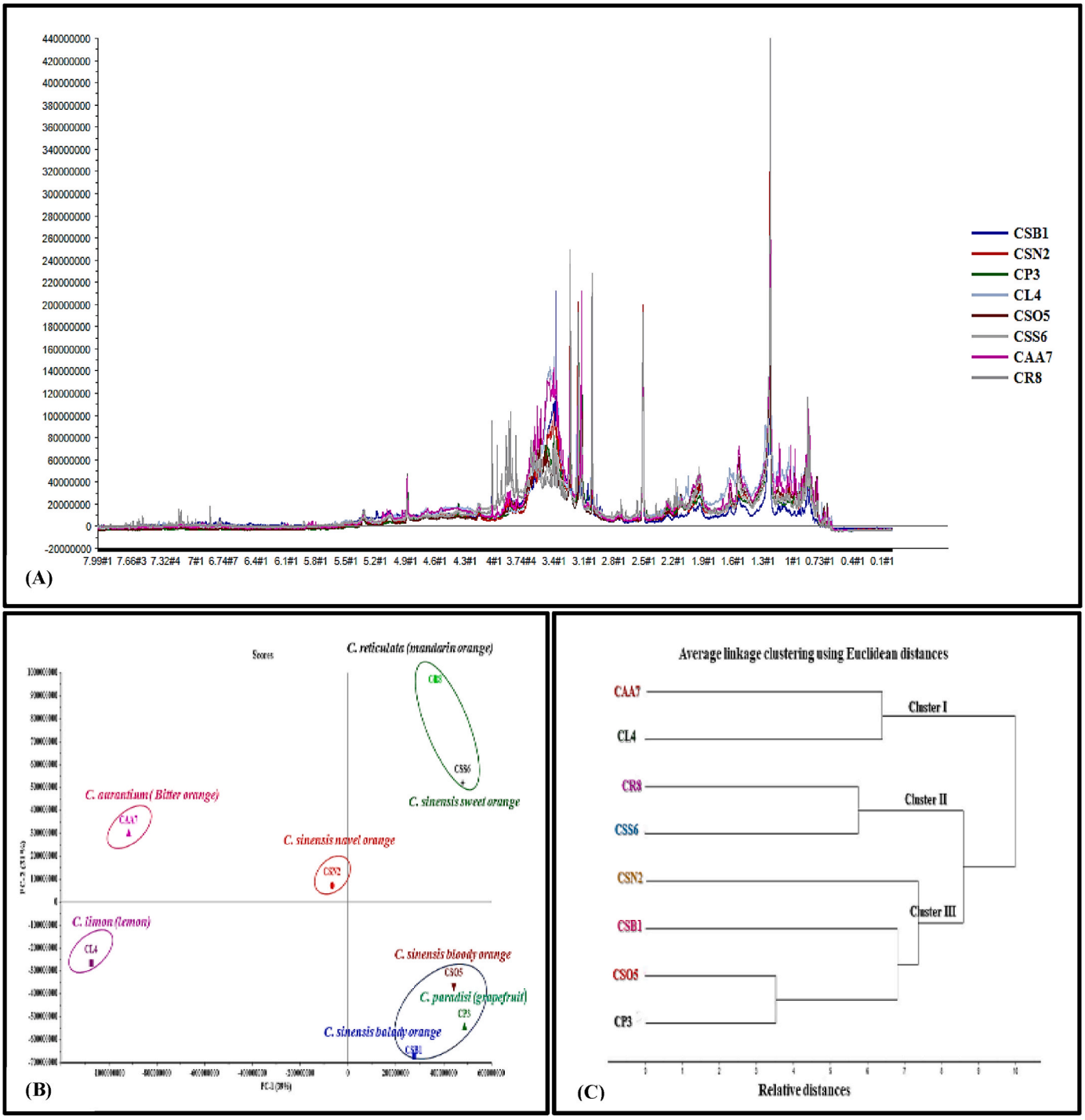

Fig. 2. ^{1}H-NMR-based multivariate data analyses of eight *Citrus* plants. **(A)** overlapped ^{1}H-NMR -spectra; x-axis represents chemical shift in ppm and y-axis represent the relative signal intensity **(B)** Score plot; x-axis represents PC-1 that constitutes 39% of total variance and y-axis represents PC-2 that constitutes 31% of total variance; **(C)** HCA; each sample is the mean of three replicates.

Table 1
Estimation of rutin content (mg/mL) in the methanol extracts present in the leaves of eight different Citrus plants.

Sample	Rutin content (mg/mL)
C. sinensis balady orange (CSB1)	0.611 ± 0.022
C. sinensis navel orange (CSN2)	0.297 ± 0.011
C. paradisi (grapefruit) (CP3)	0.181 ± 0.006
C. limon (lemon) (CL4)	0.137 ± 0.011
C. sinensis bloody orange (CSO5)	0.393 ± 0.017
C. *sinensis* sweet orange (CSS6)	0.716 ± 0.034
C. aurantium var. *amara*) Bitter orange) (CAA7)	0.795 ± 0.022
C. reticulata (mandarin orange) (CR8)	0.393 ± 0.011

*Values are expressed as the mean ± standard error where the margin of error was calculated using the t-distribution with a 95% confidence level.

were calculated to be 0.0274 and 0.0829 mg/mL, respectively.

3.3. Nuclear magnetic resonance spectroscopy (NMR) for the discrimination of citrus species

^{1}H NMR overlapped spectra (Fig. 2A) of the eight *Citrus* plants revealed the presence of two main regions, the first that is greatly intense and existed upfield from δ1H 0.0–5.0 ppm indicating the existence of primary metabolites, sugar parts of the glycosides in addition to non-polar to medium polarity metabolites as well as limonoids. However, the second region is less intense and is located in the downfield region in the range δ^{1}H from 5.0 to 8.0 ppm assigning the existence of phytoconstituents with aromatic nucleus in particular flavonoids and phenolic acids. Data obtained from the entire ^{1}H NMR spectral region were further subjected to multivariate data analysis using Principle component analysis (PCA). The score plot represented in Fig. 2B showed a discriminant classification of the eight citrus plants into five clusters with PC1 and PC2 accounted for 39% and 31% of whole data variance, respectively. *C. sinensis* balady orange, *C. sinensis* bloody orange and

Table 2
Characterization of chemical constituents of the methanol extracts of the leaves of eight *Citrus* plants using LC-ESI-MS in positive and negative modes.

No.	R_t	Compound	$[M-H]^-$	$[M+H]^+$	CSB1	CSN2	CP3	CL4	CSO5	CSS6	CAA7	CR8	Ref.
Flavones													
1.	5.66	Apigenin 7-O-neohesperidoside-6-C-glucoside	739	–	–	–	–	–	–	–	–	+	Brito et al. (2014)
2.	7.09	Veronicastroside (Luteolin-7-O-rhamnoside)	593	595.5	–	–	+	–	–	+	–	+	Roowi and Crozier (2011)
3.	7.89	Isorhoifolin (Apigenin 7-O-rutinoside)	–	579	+	–	+	+	+	–	+	+	Gattuso et al. (2007)
4.	7.99	Diosmin (diosmetin-7-O-rutinoside)	607	–	–	–	–	–	+	+	+	+	Horowitz (1956)
5.	9.08	Vitexin	431	–	–	–	–	–	–	–	+	+	Adewole and Ishola (2021)
6.	9.26	Isovitexin	431	–	–	–	–	–	–	–	–	+	
7.	9.93	Isoscutellarein	285	–	–	–	+	+	+	–	–	+	Tong et al. (2018)
8.	20.06	Diosmetin	–	301	+	+	–	+	–	–	+	–	Roowi and Crozier (2011)
9.	20.56	Chrysoeriol 7-O-neohesperidoside	607	–	–	–	–	–	+	+	+	+	Barreca et al. (2016)
10.	21.94	Chrysoeriol 8-C-glucoside (Scoparin)	461	–	–	–	+	–	–	+	+	+	Barreca et al. (2014)
11.	27.77	Rhoifolin (Apigenin 7-O-neohesperidoside)	–	579.5	+	+	+	+	+	+	+	–	Gattuso et al. (2007)
12	27.72	Quercetogetin	–	319	–	–	–	–	+	+	+	+	Del Río et al. (1998)
Flavanones													
13.	6.75	Neoeriocitrin	595.5	–	–	–	+	–	–	+	–		Masao et al. (1971)
14.	6.95	Isosakuranetin-7-O-Neohesperidoside (Poncirin)	593	–	+	–	+	–	–	–	–	+	Zhu et al. (2013)
15.	7.33	Naringenin-7-O-neohesperidoside (Naringin)	579.1	581.3	–	+	+	–	+	–	+	+	El-Beltagi et al. (2022)
16.	7.68	Neohesperidin	609	–	+	+	–	–	+	–	+	+	El-Beltagi et al. (2022)
17.	7.69	Taxifolin	303	–	+	–	–	+	+	–	–	+	Feng et al. (2018)
18.	8.97	Brutieridin	753		–	–	–	–	+				Di Donna et al. (2009)
-													
19.	9.93	Isosakuranetin	285	287	–	–	+	+	+	–	+	+	He et al. (2021)
20.	17.69	Hesperidin	611	–	+	+	–	–	+	–	+	+	Garg et al. (2001)
21.	19.71	Naringenin	271	–	–	–	–	+	+	+	–	+	Di Donna et al. (2013)
22.	20.87	Melitidin	707	–	–	–	–	–	–	–	–	+	Barreca et al. (2011)
23.	21.14	Naringenin 7-O-rutinoside (Narirutin)	579	581	–	+	+	–	+	–	+	+	Masao et al. (1971)
24.	24.66	Prunin	–	435	+	+	–	+	+	+	+	+	Medina-Remón et al. (2011)
Flavonols													
25.	21.62	Isoquercetrin	463	–	+	+	+	+	+	+	+	+	Medina-Remón et al. (2011)
26.	23.22	Rutin	609	611.5	+	+	+	+	+	+	+	+	Soares et al. (2015)
Limonoids and Others													
27.	6.45	Citrusin F	519	–	–	+	–	–	–	–	–	–	Matsubara et al. (1991)
28.	6.91	Citric acid	191	–	+	+	–	+	–	+	–	+	Füzfai and Molnár-Perl (2007)
29.	9.73	Limonin	469	–	–	–	–	+	+	–	–	+	Gualdani et al. (2016)
30.	10.43	Obacunone	453	455	+	–	–	+	–	–	–	–	Manners et al. (2000)
31.	15.55	Deacetylnomilin	471	–	+	–	–	–	–	–	–	–	Tian and Schwartz (2003)
32.	20.21	Nomilin	–	515.5	–	–	–	+	+	+	+	+	Tian et al. (2003)
33.	27.38	Obacunoic acid-hexoside	–	653	+	+	–	–	–	–	–	–	Schoch et al. (2001)
34.	28.30	Obacunone-hexoside	-	635	–	+	+	–	–	–	–	–	Schoch et al. (2001)
35.	28.56	Nomilinic acid-hexoside	–	713	–	+	–	–	–	–	–	+	Jayaprakasha et al. (2011)

C. paradisi (grapefruit) were grouped in one cluster in the lower right quadrant showing positive values for PC1and negative values for PC2 and thus reflecting certain similarities in their entire metabolic profile. Additionally, C. *sinensis* sweet orange and *C. reticulata* (mandarin orange) are clustered together in the upper right quadrant with positive values for both PCs. Meanwhile, *C. sinensis* navel orange, *C. aurantium* var. *amara*)Bitter orange) and *C. limon* (lemon) are scattered in the plot where *C. sinensis* navel orange occupied the centre of the score plot. Concerning *C. aurantium* var. *amara,* it was located in the left upper quadrant with negative values for PC1 and positive values for PC2 whereas *C. limon* (lemon) was positioned in the lower left quadrant displaying negative values for both PCs. This discrimination relied upon the discriminatory NMR signals as showed in the loading plot (Fig. S5). Furthermore, this was also confirmed by HCA (Fig. 2 C) that classified the samples into three main clusters in which cluster I contains *C. aurantium* var. *amara* Bitter orange) and *C. limon* (lemon) whereas cluster II comprises C. *sinensis* sweet orange and *C. reticulata* (mandarin orange). Concerning cluster III, it is sub-clustered into three sub-clusters; the first for *C. sinensis* balady orange, the second for *C. sinensis* navel orange however the third is for *C. sinensis* bloody orange and *C. paradise.*

3.4. Liquid chromatography coupled with mass (LC-ESI-MS) metabolic profiling and discrimination of citrus species

LC-ESI-MS metabolic profiling of the eight *Citrus* species led to the tentative identification of thirty-five compounds belong mainly to flavones, flavanones, flavanols as well as limonoids and other compounds as showed in Table 2. The assignment of peaks was done relying upon the retention times, the mass spectral data in both the positive and negative ionization modes. Besides, comparison of the ESI-MS/MS spectral data was done with previously published literature on Citrus plants together with various online databases (Table 2). The interpreted compounds identified in our LC-ESI-MS analysis served as variables for defining PC1 and PC2 in the principal component analysis (PCA). This allowed for the assessment of metabolite profile variation among different Citrus species.

Chemical characterization of the tested samples revealed that diosmetin, citric acid and deacetylnomilin constitute the major metabolites in *C. sinensis* balady orange (CSB1) however diosmetin, citric acid, citrusin are abundant in *C. sinensis* navel orange (CSN2). Besides, luteolin-7-*O*-rhamnoside, apigenin 7-O-rutinoside, apigenin 7-*O*-neohesperidoside, neoeriocitrin, isosakuranetin-7-O-neohesperidoside, naringenin-7-*O*-neohesperidoside and naringenin 4′-methyl ether) represent the major metabolites in *C. paradisi* (grapefruit) (CP3).

Regarding *C. limon* (lemon) (CL4), nomilin, citric acid, obacunone, showed a great abundance meanwhile in *C. sinensis* bloody orange (CSO5), apigenin 7-O-rutinoside, apigenin 7-*O*-neohesperidoside, naringenin-7-*O*-neohesperidoside, neohesperidin, brutieridin, hesperidin, naringenin, naringenin 7-*O*-rutinoside, pruning, limonin and nomilin are present in a notable abundance. For C. *sinensis* sweet orange (CSS6), naringenin, citric acid and nomilin are the most abundant whereas in *C. aurantium* var. *amara*)Bitter orange) (CAA7) apigenin 7-*O*-rutinoside, chrysoeriol 8-C-glucoside, apigenin 7-*O*-neohesperidoside) and nomilin are the most abundant. Regarding, *C. reticulata* (mandarin orange) (CR8) apigenin 7-*O*-neohesperidoside-6-C-glucoside, diosmin, vitexin, chrysoeriol 7-*O*-neohesperidoside, neohesperidin, hesperidin, naringenin, citric acid and nomilin constitute the predominant metabolites.

It is worthy to mention that rutin and isoquercetin exist in all of the examined samples. By subjecting LC/MS data subjected to chemometric analysis revealed the great clearly obvious that the samples are great scattering among samples as illustrated in the score plot (Fig. 3A) with PC1 and PC2 accounted for 32% and 17%, respectively of the whole variance. By comprehensive examining of the plot, two clusters were mainly observed with *C. paradise and C. sinensis* navel orange allocated in the right upper quadrant declaring positive values for both PCs whereas *C. sinensis* sweet orange and *C. aurantium* var. *amara* were grouped in one cluster in the left upper quadrant with negative values for PC1 and positive values for PC2. However, the rest of the samples were greatly segregated in the plot. Besides, the existence of various secondary metabolites as displayed in the loading plot (Fig. 3B) act as discriminatory markers that further segregated the samples.

3.5. Determination of total phenolic and flavonoid contents

The total phenolic and flavonoid contents in the tested *Citrus* samples were analyzed using colorimetric methods where the results are summarized in Table S1. The highest level of the total phenolics was determined in *C. sinensis* balady orange with 32 ± 1 mg GAE/g, followed by *C. limon*, *C. sinensis* navel orange and *C. sinensis* bloody orange (24 ± 1 mg GAE/g extract). The lowest level of the total phenolic was determined in *C. sinensis* sweet orange with 12 ± 0.1 mg GAE/g extract. With regard to the total flavonoid content, *C. sinensis* balady orange was again found to be the richest sample with 38 ± 0.4 mg RE/g extract, followed by *C. reticulata* (22.3 mg RE/g extract) and *C. sinensis* navel orange (22 ± 0.2 mg RE/g extract).

3.6. In vitro biological evaluation of citrus species

3.6.1. In vitro evaluation of the antioxidant activity

The antioxidant properties of the tested *Citrus* species were assessed using various chemical assays, including free radical scavenging (ABTS and DPPH), reducing power (CUPRAC and FRAP), metal chelation, and phosphomolybdenum assays. Results of the performed different assay are demonstrated in Table S2 where *C. sinensis* balady orange exhibited the highest scavenging ability in both ABTS (65 mg TE/g) and DPPH (25 mg TE/g) radical scavenging assays, while *C. paradisi* showed no scavenging ability against DPPH. Among the tested samples, *C. sinensis* sweet orange displayed the lowest scavenging ability in the ABTS assay (1011 mg TE/g). The CUPRAC and FRAP assays assess the electron-donation ability of antioxidant compounds through the transformation of Cu2+ to Cu+ and Fe3+ to Fe2+, respectively. As shown in Table S2 *C. sinensis* balady orange exhibited the highest reducing ability in both assays (CUPRAC: 95 ± 1 mg TE/g; FRAP: 55 ± 4 mg TE/g). Conversely, C. sinensis sweet orange showed the weakest reducing ability (CUPRAC: 42 ± 0.4 mg TE/g; FRAP: 19 ± 0.3 mg TE/g). The phosphomolybdenum assay, which involves the reduction of Mo (VI) to Mo (V) by antioxidant compounds under acidic pH, is widely recognized as a measure of total antioxidant capacity. In contrast to other antioxidant assays, the highest ability was observed in *C. limon, C. sinensis* balady orange and *C. paradisi* (2 ± 0.1 mmol TE/g). The metal chelating ability was evaluated by the ferrozine method. Remarkably, four samples (*C. paradis-i, C. limon, C. sinensis* navel, and balady orange) showed significantly higher abilities, with no statistically significant differences observed between them ($p > 0.05$). Conversely, the weakest metal chelating abilities were observed in three Citrus samples (*C. sinensis* sweet orange, *C. aurantium* var. *aurantium*, and *C. reticulata*).

3.6.2. In vitro evaluation of the neuroprotective activity

The inhibitory potentials of the *Citrus* samples against cholinesterase, were investigated, and the results were presented in Table S3. *C. limon* demonstrated the strongest inhibitory effect on both AChE (2.50 ± 0.06 mg GALAE/g) and BChE (2.20 ± 0.28 mg GALAE/g). Conversely, the weakest AChE inhibitory effect was observed in *C. aurantium* var. *amara* (2.09 ± 0.11 mg GALAE/g), while *C. reticulata* exhibited the lowest BChE inhibitory effect (1.45 ± 0.09 mg GALAE/g).

3.6.3. In vitro evaluation of the anti-hyperglycaemic activity

In the amylase inhibition assay, *C. limon* demonstrated the highest inhibition with 0.27 ± 0.01 mmol ACAE/g, followed by *C. paradisi* and *C. sinensis* navel orange both with 0.25 ± 0.01 mmol ACAE/g). Furthermore, *C. sinensis* navel orange exhibited the most potent inhibitory activity against glucosidase with 2.44 ± 0.13 mmol ACAE/g, while

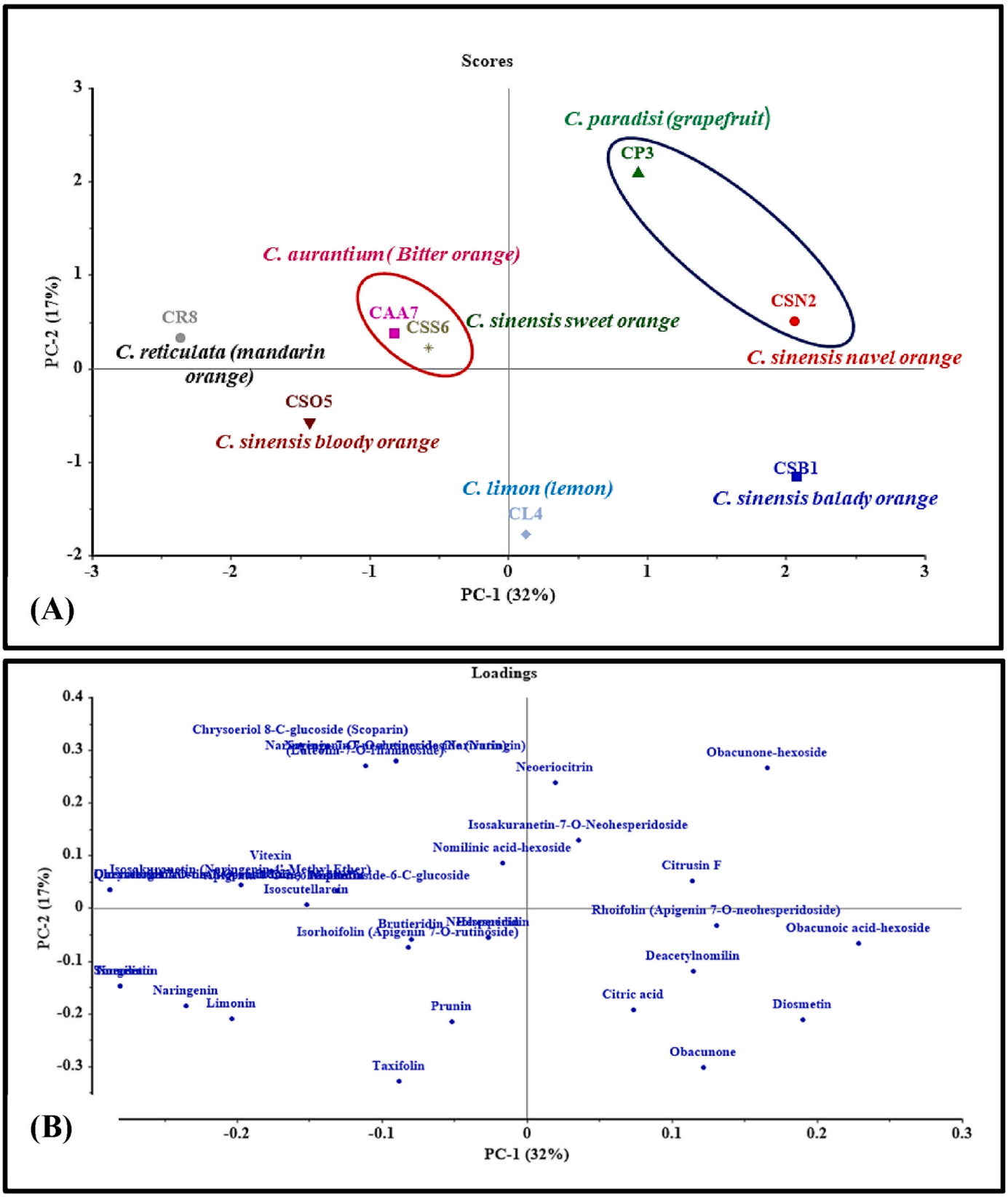

Fig. 3. LC/MS-based multivariate data analyses of eight *Citrus* plants. (A) score plot; (B) loading plot; where x-axis represents PC-1 that constitutes 32% of total variance and y-axis represents PC-2 that constitutes 17% of total variance; each sample is the mean of three replicates.

C. paradisi displayed the weakest glucosidase inhibitory ability (0.13 ± 0.01 mmol ACAE/g) (Table S3).

3.6.4. *In vitro evaluation of the skin-lightning activity*

In terms of tyrosinase inhibitory effects, *C. aurantium* var. *amara* displayed the highest ability with 58.95 ± 0.86 mg KAE/g, followed by *C. reticulata* (55.05 mg KAE/g) and *C. limon* (54.96 ± 1.15 mg KAE/g). The remaining *Citrus* species exhibited comparable tyrosinase inhibitory effects (43.59–47.20 mg KAE/g) without statistically significant differences ($p > 0.05$) (Table S3).

3.6.5. *Discrimination of citrus species based upon the biological activity*

The consistency between the obtained antioxidant results and the total content of phenols and flavonoids in the extracts is evident. This observation is further supported by the Pearson correlation analysis revealing a strong correlation between radical scavenging and reducing power assays with the total bioactive compounds (Fig. 4A). Meanwhile, by subjecting the results obtained from the biological assays to chemometric analysis, it was clearly notable that the samples are scattered in the plot as illustrated in the score plot (Fig. 4B) with PC1 and PC2 accounted for 71% and 18%, respectively of the whole variance. By comprehensive examining of the plot, five main clusters were clearly observed with *C. paradise* and *C. sinensis* bloody orange occupying one cluster in the left upper quadrant; *C. sinensis* navel orange and *C. limon* are located together in the right upper quadrant meanwhile *C. sinensis* sweet orange and *C. aurantium* var. *amara* are allocated in one cluster with a nearby cluster of *C. reticulate* in the left lower quadrant *however C. sinensis* balady orange was allocated alone in the right lower quadrant. This clustering is most similarly to the UV based discrimination which explained that the biological activity of these samples is greatly correlated to their metabolites with UV absorption potential. The various assays showed their impact as discriminatory signals as revealed in Fig. 4C.

3.7. *Pharmacokinetic, pharmacodynamic and toxicity prediction*

Pharmacokinetic, pharmacodynamic and toxicity prediction of the major secondary metabolites identified in the leaves of different *Citrus* species was established using ADME/TOPKAT (absorption, distribution, metabolism, excretion and toxicity) protocol by Discovery Studio 4.5 software. Results of ADME prediction illustrated in Table S4 showed that all the examined compounds possess low to good solubility taking the level 2 and 3 respectively with the exception of rutin that showed low but possible solubility (level 1). Regarding intestinal absorption, most of the tested metabolites showed either good absorption (level 0) and thus lies within the 99% absorption ellipse or very low absorption (level 3)

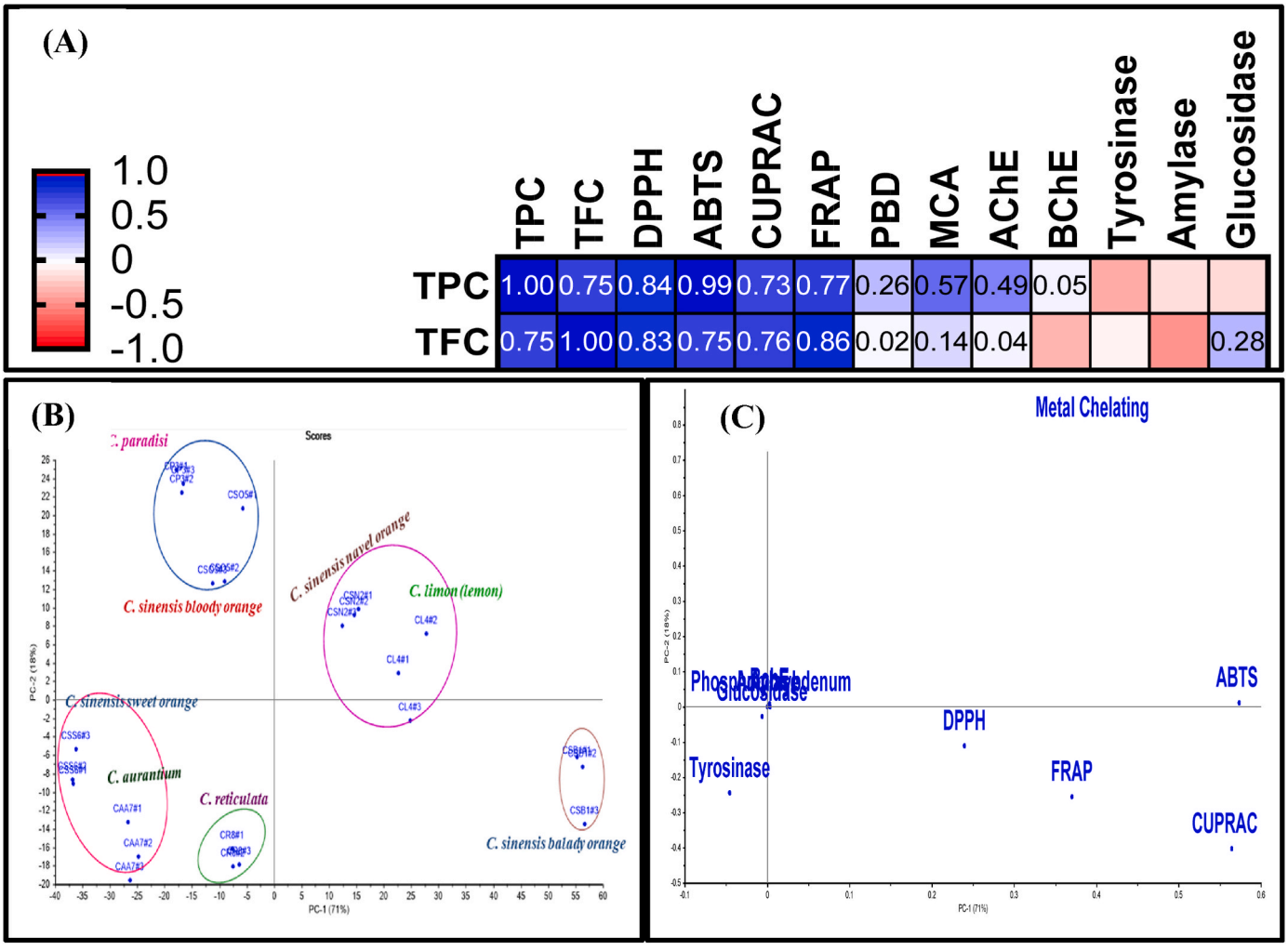

Fig. 4. Pearson correlation between total bioactive compounds and biological activities of samples (TPC: Total phenolic content; TFC: Total flavonoid content; MCA: Metal chelating assay; PBD: Phosphomolybdenum) (A); biological assays-based multivariate data analyses of eight *Citrus* plants (B) score plot; **x-axis represents PC-1 that constitutes 71% of total variance and y-axis represents PC-2 that constitutes 18% of total variance** (C) loading plot; x-axis represents PC-1 that constitutes 71% of total variance and y-axis represents PC-2 that constitutes 18% of total variance; each sample is the mean of three replicates.

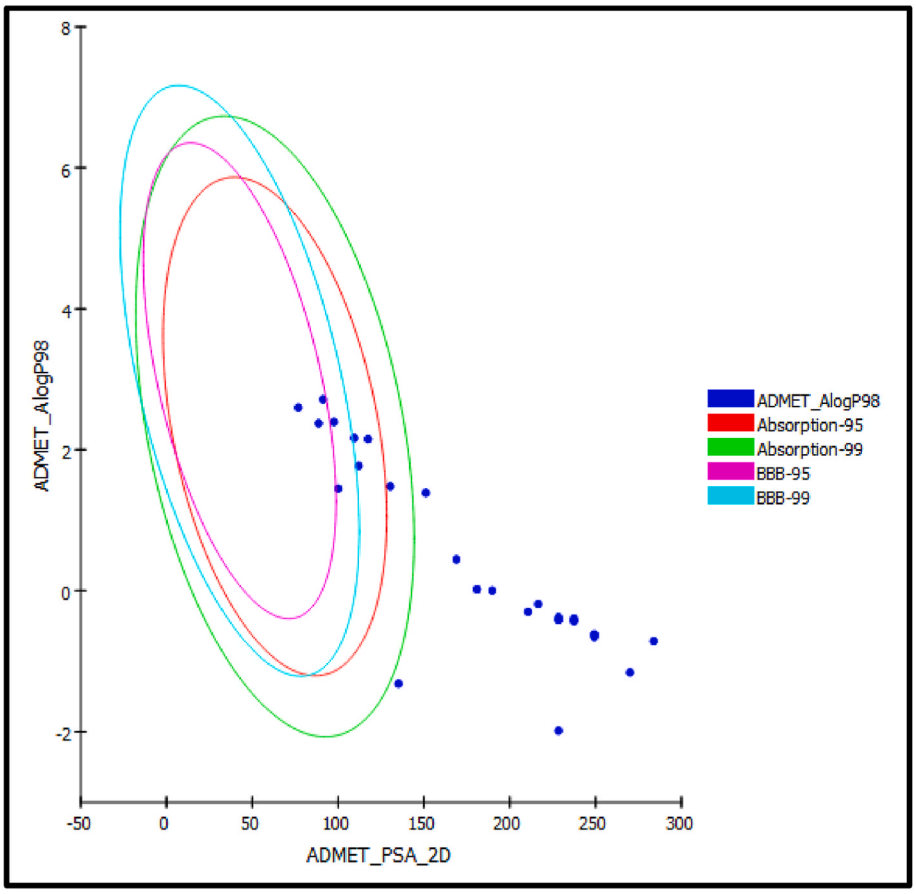

Fig. 5. ADMET Plot of major secondary metabolites identified from the leaves of *Citrus* species methanol extracts revealing 95% and 99% confidence limit ellipses regarding human intestinal absorption and the blood-brain barrier (BBB) model.

and hence allocated outside the 99% absorption ellipse as revealed in the ADMET plot (Fig. 5) with the exception of taxifolin that showed moderate absorption and citric acid that showed low absorption. Regarding BBB (blood brain barrier) penetration level, most of the examined compounds demonstrated undefined BBB penetration level (level 4) and thus allocated outside the 99% BBB confidence eclipse except diosmetin, isosakuranetin, limonin, naringenin and obacunone that showed low BBB penetration level and thus lied within the 99% BBB confidence eclipse (Fig. S4). Furthermore, most of the tested metabolites showed less than 90% PPB (plasma protein binding) except deacetylnomilin, diosmetin, limonin, nomilin and obacunone that showed more than 90% PPB. Additionally, among the tested metabolites only diosmetin, isosakuranetin and naringenin displayed certain inhibition to CPY2D6 in addition they varied in their toxicity on liver where some of them are non-toxic in contrast to others that revealed certain toxicity as displayed in Table S5.

4. Discussion

Citrus is an important genus of flowering plants in the Rutaceae family, commonly known for its juicy flavorful fruit. Discriminating between different citrus species is crucial for various reasons, including agriculture, horticulture, culinary applications, and understanding their nutritional and medicinal properties. Hence the current study was directed towards the discrimination between the leaf extracts of eight Citrus plants abundant in different regions of Asia and Africa.

Ultraviolet spectroscopy acts as a simple tool, inexpensive tool for the discrimination of herbal products. It was successful in the discrimination of *Citrus* species in the current study where the alcohol extracts of the eight *Citrus* plants exhibited UV absorption bands within the UV region in the range of 200–400 nm owing to their content of polyphenolic secondary metabolites in particular flavonoids and limonoids. These secondary metabolites are characterized by the existence of multiple chromophores and conjugated systems that serve as UV absorbing systems (Khettal et al., 2017; Zhang et al., 2020). Flavonoids and their glycosides that predominate the *Citrus* plants displayed UV absorbance at spectral range from 240 to 380 nm with two major peaks termed Band I that appeared with λ_{max} in the region of 300–380 nm that is mainly due to UV absorbance by B-ring cinnamoyl moiety meanwhile Band II showed λ_{max} in the range of 240–280 nm that is mainly attributed to the UV absorbance of A-ring benzoyl system (Mabry et al., 1970). Besides, limonoids exhibited strong absorption bands at 203 nm as well as and 286 nm in the UV spectrum (Abdelgaleil et al., 2004). Subjecting the obtained UV data to chemometric analysis using PCA and HCA, it was found that *C. paradisi* (grapefruit) and *C. sinensis* bloody orange are clustered together in one cluster Meanwhile, C. *sinensis* sweet orange and *C. aurantium* var. *amara* were placed together in one cluster occupying the center of the plot approaching that of *C. sinensis* navel orange and thus reflecting their similarities in their secondary metabolites with UV absorbance potential. However, *C. limon, C. sinensis* balady orange and *C. reticulata* (mandarin orange) were scattered in the plot in which the former is greatly discriminated from the rest of tested samples.

Standardization using a validated HPLC method is highly adopted in the quality control of herbal products (Oluyemisi et al., 2012). Moreover, rutin constitutes one of the dominant compounds present in considerable quantities in the leaves of Citrus plants where HPLC-UV method was previously adopted for quantification of rutin and hesperidin in *Citrus limonia* leaves (Soares et al., 2015). Results of the current HPLC analysis based upon rutin contents showed that *C. aurantium* var. *amara*)Bitter orange) comprises the highest concentration of rutin in

contrast to *C. limon* (lemon), that showed the least rutin concentration. In addition, *C. reticulata* (mandarin orange) and *C. sinensis* bloody orange showed almost similar rutin content.

^{1}H NMR spectra of the *Citrus* plants revealed the presence of two main regions, the first intense upfield region from δ1H 0.0–5.0 ppm related to limonoids, primary metabolites, sugar parts of the glycosides and the second less intense downfield region in the range δ^{1}H from 5.0 to 8.0 ppm assigned for the phytoconstituents with aromatic nucleus most probably, flavonoids and phenolic acids. Discrimination using ^{1}H NMR spectra is considered an attractive and successful screening method for the effective segregation of related samples relying upon its simplicity, rapidity as well as the huge amount of information afforded by ^{1}H NMR. It was previously adopted for the discrimination of orange juice from that pulp wash (Le Gall et al., 2001). ^{1}H NMR data coupled with chemometrics demonstrated the close similarity in the metabolic profiles of *C. sinensis* balady orange, *C. sinensis* bloody orange and *C. paradisi* (grapefruit) being grouped into one cluster. Similarly, C. *sinensis* sweet orange and *C. reticulata* (mandarin orange) were clustered together in another group. Meanwhile the variant metabolic profiles of *C. sinensis* navel orange, *C. aurantium* var. *amara* (Bitter orange) and *C. limon* (lemon) was deduced from their scattering in the score plot. It is noteworthy to mention that although secondary metabolites are less intense compared to primary metabolites as displayed by NMR spectra, they do exhibit a pronounced role in activity in very small quantities comparable to primary metabolites with the concomitant appearance of notable role as phytopharmaceuticals (Bourgaud et al., 2001).

LC-ESI-MS metabolic profiling affords one of the best combinations of selectivity and sensitivity, and thus seems indispensable in various Plant metabolomics approaches. It covers a great mass range and targets many classes of compound classes, expressing the entire biochemical categories of plants (t'Kindt et al., 2008). Besides, it is greatly useful for the detection of various categories of plant secondary metabolites such as phenolic acids, alkaloids, phenylpropanoids, saponins, flavonoids, polyamines and glucosinolates, relying upon the used stationary phase (De Vos et al., 2007). LC-ESI-MS of the eight *Citrus* species led to the tentative identification of thirty-five compounds belong mainly to flavaones, flavanones, flavanols as well as limonoids and other compounds that greatly vary in their major metabolites but with isoquercetrin and rutin existing in all the examined samples. By subjecting LC/MS data to chemometric analysis, it was clearly obvious that the samples are greatly scattered in the plot with *C. paradise and C. sinensis* navel orange were allocated in the right upper quadrant whereas C. *sinensis* sweet orange and *C. aurantium* var. *amara* were grouped in one cluster in the left upper quadrant. However, the rest of the samples were greatly segregated in the plot.

The antioxidant activity of plant extracts is always of significant importance due to its potential health benefits in reducing oxidative stress and its implication in further biological disorders including inflammation, cancer, metabolic disorders, immunity and aging (El-Din et al., 2022). Besides, cholinesterase inhibitors are highly used to ameliorate dementia including Alzheimer's disease that is characterized by irreversible neurological disorder that particularly elevated with age (Mitić and Lazarević-Pašti, 2021). Moreover, α-amylase is vital in carbohydrate digestion resulting in a pronounced increase in blood glucose level, and thus its inhibition could significantly reduce postprandial glucose level with concomitant controlling of postprandial glucose level in diabetic patients (Park and Han, 2018). Furthermore, tyrosinase enzyme is important for the formation of melanin resulting in hyperpigmentation, and hence its inhibition might control dark skin patches (El-Nashar et al., 2021). Hence, biological evaluation of the antioxidant, neuroprotective, anti-hyperglycaemic and skin-lightning potential of the eight *Citrus* plants was performed *in vitro* using different popular assays with the aim of further differentiation of *Citrus* plants based on their bioactivities.

Results revealed significant variations in the total phenolic and flavonoid content among the tested *Citrus* samples ranging between 12.07 and 32.46 mg GAE/g *and* 2.66–38 mg RE/g. These findings are consistent with previous studies that reported different levels of phenolic compounds from various *Citrus* species. Abeysinghe et al. (2007) reported total phenolic levels ranging from 184 to 916 mg chlorogenic acid equivalent (CAE)/100 g fresh material in the edible tissues of four *Citrus* species. Meanwhile, Azman et al. (2019) found variations in the total phenolic content of fresh and frozen *Citrus* peels, ranging from 72.01 to 136.48 mg GAE/100 g fresh material. Interestingly, Adnan et al. (2014) reported higher phenolic and flavonoid contents in the alcohol leaf extract of *C. paradisi* compared to other studied *Citrus* species contrasting the results of the current study as *C. paradisi* exhibited the lowest level of these compounds among the tested *Citrus* species. This variation is probably attributed to the difference in the other studied species of Citrus in addition to geographical and climatic factors, including annual rainfall and daily sun exposure.

The consistency between the obtained antioxidant results and the total content of phenols and flavonoids in the extracts is evident and supported by the Pearson correlation analysis revealing a strong correlation between radical scavenging and reducing power assays with the total bioactive constituents. These findings align with previous studies that have reported a significant correlation between total bioactive compounds and antioxidant properties in certain *Citrus* species (Adnan et al., 2014; Chen et al., 2021). Notably, *C. sinensis* balady orange and *C. limon* exhibited superior antioxidant properties compared to other *Citrus* species. Table 2 highlights the presence of specific compounds such as obacunone and deacetylnomilin in these two *Citrus* species, which may contribute to their observed antioxidant properties. These compounds have been previously identified as potent antioxidants by several researchers (Magurano et al., 2021; Xu et al., 2016; Zhou et al., 2022). Furthermore, the antioxidant abilities may also be attributed to certain flavonoids such as diosmetin and taxifolin (Topal et al., 2016; Wójciak et al., 2022). Results agree with the different literature reporting the antioxidant properties of various *Citrus* species (Adnan et al., 2014; Manchanda et al., 2023; Sultana et al., 2015).

In the enzyme inhibitory assays, all samples exhibited inhibitory potentials. Unlike the antioxidant results, the obtained enzyme inhibitory abilities did not show a strong correlation with the total phenolic and flavonoid contents in the extracts. This can be attributed to the complex nature of phytochemicals and their different interactions with the active or allosteric sites of enzymes. Results agree with the reported *in vivo* and *in vitro* inhibitory activities of different *Citrus* species on acetylcholinesterase (Carvalho et al., 2013; Senol et al., 2016).

The antidiabetic effects of different parts of *C. media* were studied by Menichini et al. (2011), where the leaves displayed significant amylase and glucosidase inhibitory effects among the tested parts. Leporini et al. (2020) reported a notable amylase inhibitory effect of *C. clementina* leaves with lower IC50 values, as well as significant tyrosinase inhibitory effects. Additionally, Khettal et al. (2017) described the polyphenol oxidase inhibition potentials of seven *Citrus* species, highlighting *C. limon* and *C. aurantium* as the most active species among those tested. Meanwhile, by subjecting the results obtained from the biological assays to chemometric analysis, it was clearly notable that the samples are clustered in a manner most similarly to the UV based discrimination which explained that the biological activity of these samples is greatly correlated to their metabolites with UV absorption potential.

In silico ADME/TOPKAT prediction reflected the acceptable pharmacokinetic, pharmacodynamic and toxicity properties of the major secondary metabolites identified in the leaves of different *Citrus* species.

5. Conclusions

The current study represents the first report of chemometric discrimination of eight *Citrus* plants namely *C. sinensis* balady orange, *C. sinensis* navel orange, *C. paradisi* (grapefruit), *C. limon* (L.) Burm. (lemon), *C. sinensis* bloody orange, C. *sinensis* sweet orange, *C. aurantium* var. *amara*)Bitter orange) and *C. reticulata* (mandarin orange) utilizing

UV, HPLC, NMR and LCMS. The study additionally encompassed the evaluation of the *in vitro* antioxidant, neuroprotective, anti-hyperglycemic, and skin-lightning potential of the investigated *Citrus* varieties. Furthermore, the correlation between the total content of phenols and flavonoids in the different *Citrus* extracts and their different biological activities was investigated using Pearson correlation analysis. Interestingly, most of the utilized chromatographic and spectroscopic analytical methods successfully discriminated the different specimens based upon the similarities and differences in their chemical constituents that dependently reflected on their biological behavior. Strong correlations were observed between the total phenolic and flavonoid contents and the evident potent antioxidant and enzyme inhibitory activities. In addition, *in silico* ADME/TOPKAT prediction reflected the acceptable pharmacokinetic, pharmacodynamic and toxicity properties of the identified secondary metabolites. It is worthy noted that the different analytical and spectroscopic methods coupled with chemometrics adopted in the current study can be implemented in pharmaceutical industries for better monitoring of the quality of herbal preparations containing the studied *Citrus* species. Further clinical studies are recommended for guaranteeing the safety of incorporating the studied *Citrus* species and varieties in pharmaceutical dosage forms for the relief of Alzheimer, hyperglycaemia and hyperpigmentation.

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Credit authorship contribution statement

Conceptualization, F.S.Y.; Methodology, F.S.Y., S.S.E. and G.Z.; Software, F.S.Y. and G.Z.; Validation, F.S.Y., G.Z. and M.L.A.; Formal analysis, F.S.Y., G.Z. and M.I.G.; Investigation, F.S.Y., S.S.E. and G.Z.; Resources, S.S.E., M.O.L. and D.I.H.; Writing – original draft, F.S.Y., G.Z. and M.I.G.; Writing – review & editing, S.S.E., M.O.L., D.I.H. and M.L.A.; Supervision, M.L.A.; Project administration, F.S.Y., S.S.E. and M.L.A.; Funding acquisition, S.S.E. and M.O.L. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crfs.2024.100803.

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