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Multivariate Analysis of Extraction Solvent, Organ, and Habitat Effect on Chemical Profile and Biological Activity of *Eryngium campestre*

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Abstract

Eryngium is the largest and most complex genus in family *Apiaceae*, widely employed in folk medicine as hypoglycemic, antitussive, kidney stone inhibitor, diuretic, aphrodisiac, and anti-inflammatory due to having a considerable amount of secondary metabolites and biologically active chemical compounds such as polyacetylenes, coumarins, flavonoids, phenolics, terpenoids, triterpenoid saponins and steroids. In this study, the effects of extracting solvent, organ selection as well as the habitat of *E. campestre* ecotypes growing in Egypt were evaluated on its chemical profile and antimicrobial activity against six pathogens using Near-Infrared spectroscopy and HPTLC-image analysis, each combined with multivariate analysis. The results showed that different organs of the tested species showed variant metabolomic profiles, together with the geographical origin, being a less profoundly influential factor on the chemical profile; and subsequently the biological activity of the tested samples. Supervised and unsupervised pattern recognition on data of NIR and HPTLC techniques, it was possible to differentiate four clusters: two different habitats, two plant parts (Shoot and Root) as well as the five solvent fractions of each plant sample.

Keywords: Bacterial growth inhibition, ecotypes, multivariate data analysis.

1. Introduction

Since ancient times, herbs have been used to flavor and preserve food as well as treat a variety of infectious ailments (Torras-Claveria et al., 2007). Environmental protection has become of a major concern worldwide. One main element in minimizing environmental pollution is reducing as much as possible the production of synthetic chemicals. Antibiotics in the environment as an important topic has been the interest of several scientists (Abdel Rahman et al., 2011).

Exploring wild flora for plants containing phytochemical compounds with significant antibacterial activity has been an extremely promising alternative (Moradi et al., 2014; Jain et al., 2010). Yet, exploitation and unwise utilization of medicinal plants could lead to their extinction, which is going to be a great loss. This is clearly the case, in particular in fragile habitats such as deserts.

Eryngium L. is a genus of flowering plants with roughly different 250 annual or perennial species that occur primarily in Eurasia, northern Africa, northern and southern America, and Australia. It is the largest and possibly the most taxonomically complicated genus in the family Apiaceae. Some *Eryngium* species' infusions have been traditionally used in European medicine as an antitussive, diuretic, appetizer, stimulant, and aphrodisiac (Zahid et al., 2017; Pirbalouti et al., 2014). The primary earlier identified components from these taxa were

triterpenoid saponins, flavonoids, phenolic acids, coumarins, and polyacetylenes.

Eryngium L. have been regarded as prospective medicinal crops due to phytochemical components and pharmacological capabilities discovered by chemical and biological assessments (Pirbalouti et al., 2014). Since they vary from one another by saponins profile, flavonoids presence, and quantity of active chemicals, it has been reported that the pharmacological characteristics of extract from the plant's leaves and roots are distinctly diverse (de la Luz Cádiz-Gurrea et al., 2019; Pirbalouti et al., 2014).

However, Eryngium species exhibit a wide range of biological and pharmacological activities, including antibacterial, antitumor, antifungal, anti-inflammatory, anti-snake and scorpion venoms, antimalarial, antioxidant, and antihyperglycemic activities . This is because they have a significant amount of secondary metabolites and biologically active chemical compounds, including polyacetylenes (Erdem et al., 2015), coumarins (Vuković et al., 2018; Rjeibi et al., 2017), flavonoids (Vuković et al., 2018), phenolics (Wörz & Diekmann, 2010; Ayuso et al., 2020), terpenoids (Shcherbakova et al., 2018; Kowalczyk et al., 2014), triterpenoid saponins (Zhang et al., 2008), and steroids (Sandvoss et al., 2000; Vauzour et al., 2010).

It has been demonstrated that the extracts of numerous *Eryngium* species have antibacterial activity against a variety of infectious illnesses (Erdem et al., 2015). The present study focused on factors that could affect the antibacterial effectiveness of extracted phytochemicals from *Eryngium campestre,* using different extracting solvents against six pathogenic bacteria. In addition, habitat difference and plant part (shoot/root) were tested as factors, while growth inhibition zone diameter of the six bacterial species was the measured parameter. All data were analyzed with the appropriate statistical tests.

2. Experimental

2.1. Materials

2.1.1.Plant materials and sample preparation

E. campestre plant was sampled from two habitats (A and B) at Borg El-Arab region, 60 Km west of Alexandria city over the years 2021 and 2022 and authenticated by Prof Sania Ahmed (Botany Department, faculty of Science, Alexandria University, Egypt) based on their morphological characters and microscopic features. One habitat (A) was more to the coastal zone and the second (B) was more inland. Aerial parts were separated from roots. Samples were air-dried in darkness (25 °C, 60% relative humidity for 2 weeks), then ground using an electric grinder followed by sieving through 2 mm mesh sieve to obtain fine powder with uniform particle size.

One gram of each sample was used further for NIR analysis.

2.1.2. Plant extraction and fractionation

Twenty grams of each plant sample (shoots and roots separately from each habitat) were mixed with 200 ml 70% ethanol in a conical flask and kept soaked at room temperature for one week. The extracts were filtered, and the solvent was refreshed weekly for two weeks. Filtrate was dried at 70 ˚C under reduced pressure using rotary evaporator and then mixed as a total crude yield. The crude yield of each sample was redissolved in 100 ml 70% ethanol and successively fractionated using petroleum ether, chloroform, ethyl acetate and butanol as fractionation solvents. The resulting fractions were dried at 70 ºC under reduced pressure using rotary evaporator. An aliquot of ten milligrams of each dry fraction was redissolved in 1 ml methanol and the rest was dissolved in DMSO, both were labelled and stored at 4 ˚C until biological and phytochemical investigations (HPTLC), for a total of 20 samples.

2.1.3. Chemicals and supplies

Absolute ethanol, hexane, dimethyl sulfoxide (DMSO), petroleum ether, chloroform, ethyl acetate, butanol, methanol and Merck 20 cm × 10 cm HPTLC silica gel 60F254 (0.25 mm) plates were analytical grade and purchased from Merck, Darmstadt, Germany. Sterile disposable nutrient agar and 12.5 cm plastic petri dishes were purchased from El Gomhuria Company.

2.1.4. Bacterial species

The following bacterial species with their reference numbers were obtained from the faculty of medicine, Alexandria University: *Staphylococcus epidermidis* (ATCC 12228), *Klebsiella pneumoniae* (ATCC 700603), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 29213).

The multi-drug resistant (MDR) strains were isolated from patients at the intensive care unit (ICU), Alexandria general hospital, and identified as *Klebsiella pneumoniae* MDR and *Staphylococcus aureus* MDR.

2.2. Methods

2*.2.1. NIR spectroscopy measurements and spectral pretreatment*

Near infrared analysis was performed on samples of roots and aerial parts of *E. campestre* belonging to habitats A and B, resulting in four sample types. Three samples of each type were analyzed in triplicates. A multi-purpose analyzer (MPA) FT-NIR spectrometer (Bruker Optics GmbH), with an InGaAs detector and an integrating sphere module by scanning over the wavenumber range of 12,000–3,600 cm^{-1} was used to obtain the NIR spectra. While the range between 4000 and 3800 cm1 revealed an

overcrowded noise region, the range between 12000 and 7000 cm1 was non-informative, showing no significant peaks. As a result, these regions were disregarded. Spectra were collected using OPUS spectral acquisition software (ver. 6.5, Bruker Optics Inc.) at a resolution of 16 cm-1 per spectrum by averaging 64 scans. Samples were loaded in glass vessels of diameter 20 mm and height of 50 mm. 1 g of powder were used (covering the bottom of the vessel) for NIR measurements. In order to improve overlapped peaks and improve their resolution, raw NIR spectral data of samples underwent pre-processing based on first order derivative transformation in addition to standard normal variate (SNV) transformation to remove errors that may occur between samples during NIR measurements. Weighted multiple scatter correction (WMSC) was carried out to achieve scatter and baseline drift correction. Regarding the validation parameters, a combination of the aforementioned pre-processing techniques was employed. SIMCA-P + 14.1 software (unmetrics AB, Umea, Sweden) was used to perform the pretreatments and calculations for the multivariate analysis.

2.2.2. Investigating the clustering pattern of samples using unsupervised pattern recognition methods, principal component analysis (PCA) and hierarchical clustering analysis (HCA)

Principal Component Analysis, or PCA, is a type of unsupervised pattern recognition models where a dimensionality-reduction method that is often used to reduce the dimensionality of large data sets, by transforming a large set of variables into a smaller one that still contains most of the information in the large set. Hierarchical Clustering analysis (HCA) is an unsupervised pattern recognition algorithm used to group the data points with similar properties (Bayne & Kramer, 1999; Tobias et al., 1999).

2.2.3. Orthogonal projections to latent structures – discriminant analysis (OPLS-DA) model

To inspect and discriminate unadulterated of different organs from different habitats, OPLS-DA, a supervised method for linear classification, was employed here. The implementation of the right number of latent variables (LVs), helped to create sharp boundaries among the different groups. LV number is considered to be ideal once the prediction error in classification is minimized.

2.2.4 High performance thin layer chromatography

A total of 20 samples (5 fractions for each of the 4 samples listed in Materials, two organs of two different habitats) were dissolved in methanol at a concentration of 10 mg/ml. A mixture of hexane and ethyl acetate at a ratio of 4:1 was used as a mobile phase and a 20 cm × 10 cm HPTLC silica gel plates (0.25 mm) as a stationary phase, with three replica for each sample. A semi-automated TLC applicator (Linomat V) was used to apply 10 µl of each sample on the stationary phase. Plates were developed in TLC jar and were left to dry in air and then sprayed with p-anisaldehyde – sulfuric acid as a derivatizing agent and were heated at 105 °C until colors on the plates appeared. Scanning of all the studied plates was carried out using a flatbed scanner (Canon-MF 4750) (Darwish et al., 2021).

2.2.5 Image processing for untargeted multivariate data analysis of different extracts

Images of the plates were processed with ImageJ 1.51 h image processing program (Wayne Rasband, National Institutes of Health, USA).

Without adding any additional background subtraction, the recorded results were cropped and denoised using the median filter function with a two-pixel width filter. For each sample, the line profile plots were produced using the Plot Profile option. A two-dimensional graph of the pixel intensities along a line is shown in a profile plot. The bUnwarpJ technique included in the ImageJ software was used to align the images (Komsta et al., 2011). In order to build HPTLC profiles of all samples and standard mixtures, obtained data matrices were imported into Microsoft Office Excel 2013 and the RF values and corresponding intensities for each sample's track image were calculated. Principal component analysis (PCA) was used for multivariate analysis of the resulting HPTLC profiles in an effort to minimize the hyper dimensionality of the data. Hierarchical grouping and PCA were performed using SIMCA software.

2.2.6 Antibacterial activity

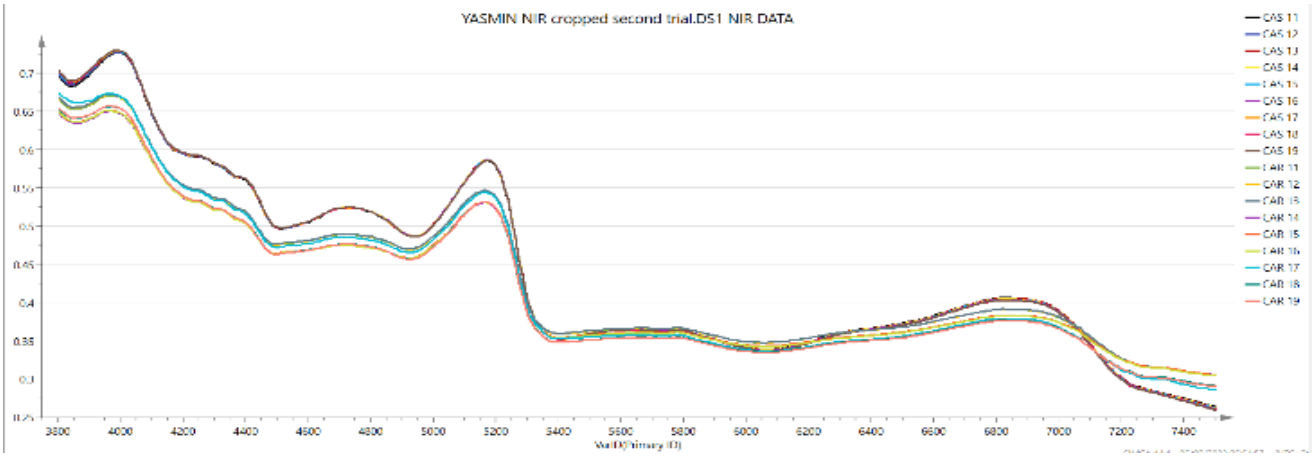
For testing antibacterial activity of plant extract fractions well diffusion method was applied, and diameter of inhibition zone, the average of three replicates for each sample) was determined for each extract against each of the six bacterial species. To assess the antimicrobial activity of plant or microbial extracts, the agar well diffusion method is frequently utilised (Mounyr et al., 2016). The agar plate surface is inoculated using a process similar to the disk-diffusion approach in which a volume of the microbial inoculum is dispersed across the entire agar surface. Next, a volume (20-100 L) of the antimicrobial agent or extract solution is put into the well by aseptically drilling a hole with a diameter of 6 to 8 mm using a sterile cork borer or tip. The test microorganism is then placed on an appropriate agar plate, and the incubation process is continued. The antibiotic ingredient spreads across the agar media and stops the tested microbial strain from growing. The values of inhibition zone diameters were used to create the Y matrix in OPLS-DA models of HPTLC

analyses.

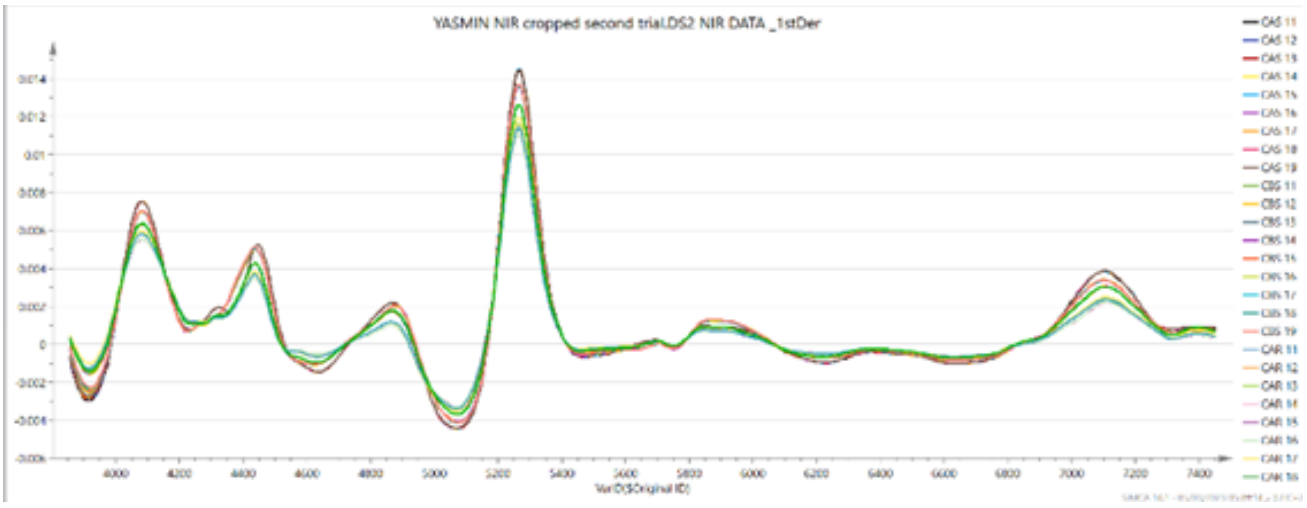
3. Results and Discussion

3.1. NIR spectral analysis of *E. campestre*

The raw comparative spectra of all samples in the given region showed no distinct differences except after pre-processing (Fig 1). The clear differences were observed in the regions: 4700- 4100 cm^{-1} region which indicated the combination of C–C and C–O stretching, and C–H bending vibration which was displayed around 4300 cm^{-1}. At 5630–5800 cm^{-1}–CH aliphatic 1st overtone of the sugar moiety of flavone glycosides were observed. Sesquiterpene lactones displayed overtones bands of symmetric and asymmetric CH_2 stretching at 5800–5700 cm^{-1}, and flavone nucleus –CH aromatic 1st overtone at 5920–5950 cm^{-1}. Meanwhile, the second overtone of the lactone carbonyl group was usually observed in the region 5400–5100 cm^{-1}. And bands that observed at 6000-7000 cm^{-1} were due to the O–H stretching first overtone and N–H stretching.



(A)



(B)

Fig. 1 (A&B): Raw (A) and Preprocessed (B) near infrared spectra (16 cm^{-1} resolution) acquired from 7000-4000 cm-1, of representative samples of *E. campstre* powder.

3.2. Investigating the clustering pattern of E. campstre samples using unsupervised pattern recognition of NIR data

Exploring the PCA results, the score plot showed identical clustering patterns of samples into four clusters relevant to samples of different organs and habitats (Fig 2 A). Hereby, PC 1 (75.4% of variation) separated habitat A samples, on its positive side, from habitat B ones on its negative side. Meanwhile, PC2 (16.4% of variation within data set) separated shoot samples, on its positive side, from root ones on its negative side.

Hierarchical cluster analysis (HCA) was applied as an exploratory multivariate data analysis approach in order to study the grouping trends of the tested samples. In agreement with PCA results, it can be deduced from the dendrogram that samples were clustered into two main clusters, were shoots were distinctly distinguished from root samples. Each cluster of which, was furtherly divided into two subclusters representing samples of different geographical origins (Fig 2 B).

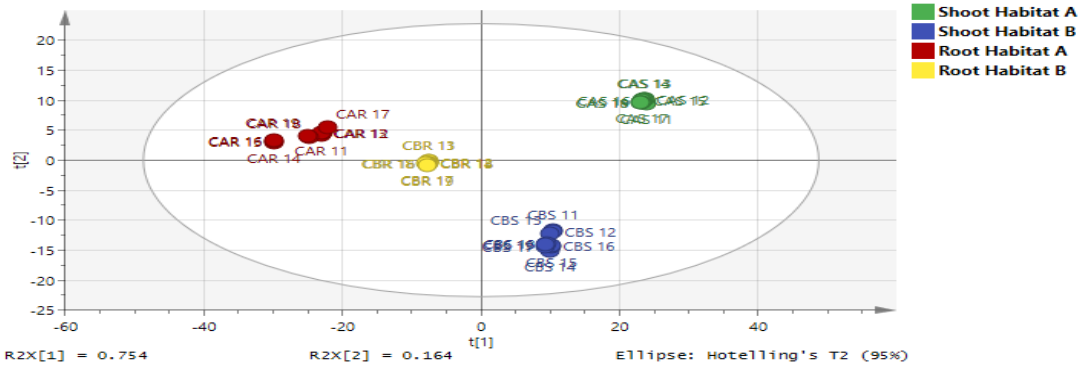


Fig. 2A: PCA score scatter plot of NIR data

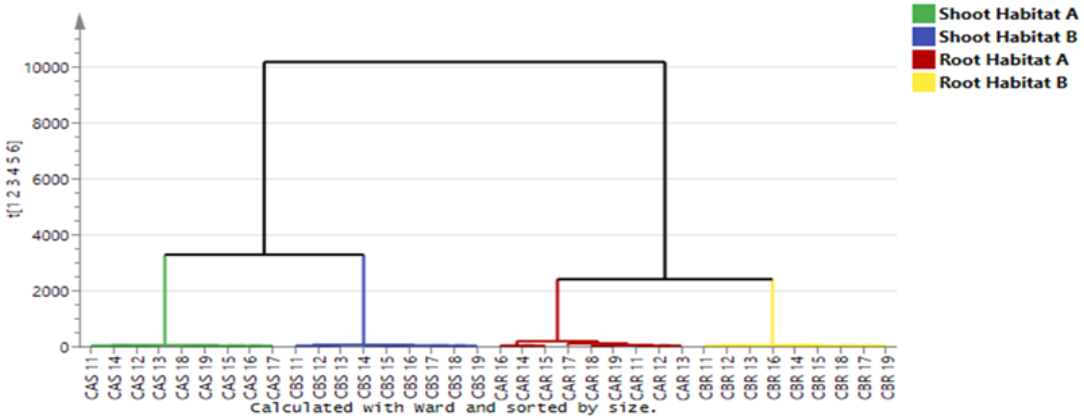


Fig. 2 B: HCA unsupervised pattern recognition of NIR data.

3.3. Investigating the clustering pattern of *E. campestre* samples using Orthogonal projection to latent structures-Discriminant analysis (OPLS-DA) of NIR data

Discriminant analysis focuses on mutual differences among different samples where models are constructed obeying Bayes' rule (Bylesjö et al., 2006). In our study, OPLS-DA was implemented to discriminate samples of different organs and habitats. The constructed OPLS-DA model (Fig 3 A) discriminated examined samples into four clusters representing different organs and geographical habitats. These results matched and were more clearly represented in the HCA dendrogram (Fig 3 B) based on the OPLS-DA model which discriminated examined samples into two distinct classes representing different organs, shoots and roots, each again subdivided into two subclusters representing different geographical habitats.

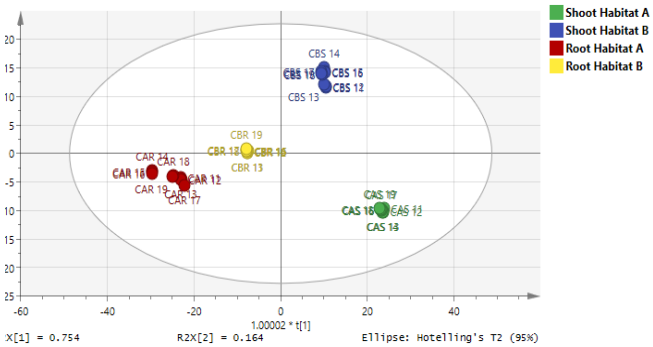


Fig. 3 A: OPLS-DA score scatter plot

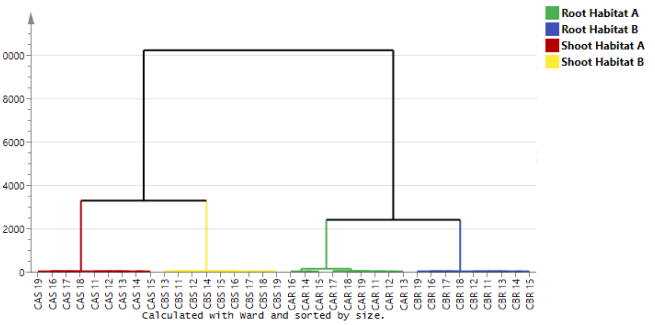


Fig. 3 B: HCA model of OPLS-DA

3.4. Untargeted analysis using HPTLC-image analysis and unsupervised pattern recognition (PCA and HCA)

The chemical profiles of the diverse extracts varied, as shown by the HPTLC profiles for each sample, produced under white light following post-chromatographic derivatization with anisaldehyde/H2SO4 (Fig 4). The described method was used to analyze the images of the HPTLC profiles of the various samples. To create plots with clear peak borders, the pictures were denoised using a two-pixel median filter. To rectify the inter- and intra-plate peak shift, the image warp process roughly adjusted the shift of the RF values of the various component bands along the development direction (Ristivojević et al., 2014). The chosen target image was chosen at random. To perform solely unidirectional registration, that is, from source picture to destination image, the registration mode "Mono" was chosen. When the pictures were warped, an HPTLC data set was obtained that included 189 pixel length profiles for each sample.

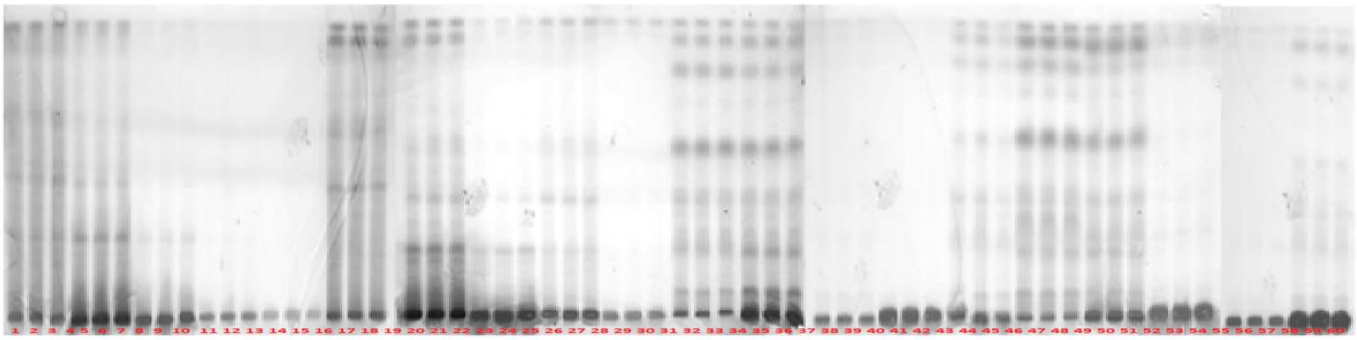
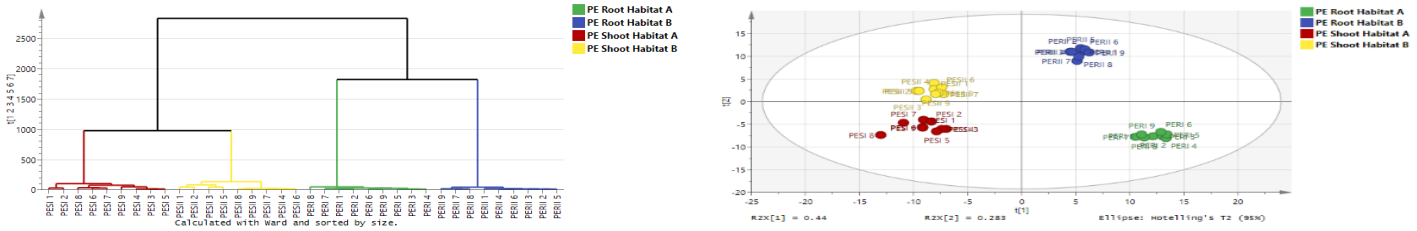


Fig. 4: Developed TLC plates of tested *E. campestre* samples (tracks 1 to 3: Petroleum ether extract of shoots habitat 1, tracks 4 to 6: chloroform extract of shoots habitat 1, tracks 7 to 9: ethyl acetate extract of shoots habitat 1, tracks 10 to 12: butanol extract of shoots habitat 1 tracks, 13 to 15: ethanol extract of shoots habitat 1, tracks 16 to 18: Petroleum ether extract of shoots habitat 2, tracks 19 to 21: chloroform extract of shoots habitat 2, tracks 22 to 24: ethyl acetate extract of shoots habitat 2, tracks 25 to 27: butanol extract of shoots habitat 2, tracks, 28 to 30: ethanol extract of shoots habitat 2, tracks 31 to 33: Petroleum ether extract of roots habitat 1, tracks 34 to 36: chloroform extract of roots habitat 1, tracks 37 to 39: ethyl acetate extract of roots habitat 1, tracks 40 to 42: butanol extract of roots habitat 1 tracks, 43 to 45: ethanol extract of roots habitat 1, tracks 46 to 48: Petroleum ether extract of roots habitat 2, tracks 49 to 51: chloroform extract of roots habitat 2, tracks 52 to 54: ethyl acetate extract of roots habitat 2, tracks 55 to 57: butanol extract of roots habitat 2, tracks, 58 to 60: ethanol extract of roots habitat 2)

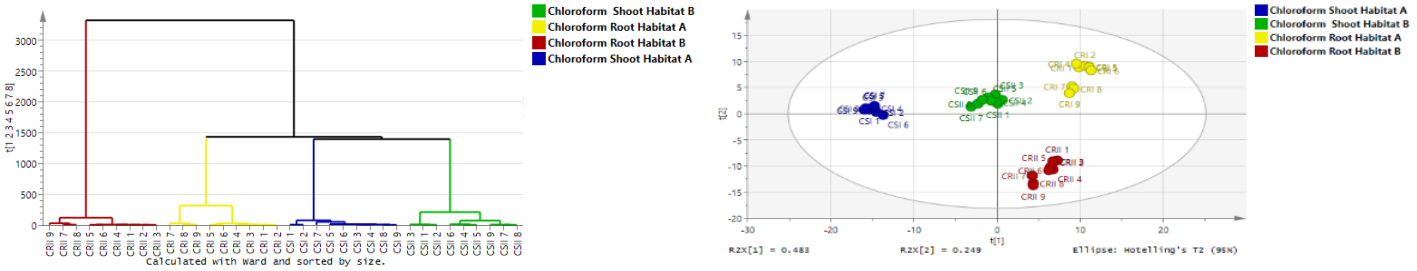
The matrix created by digitizing the chromatograms underwent principal component analysis (PCA). According to the plant part extracted and the matching geographic origin, all samples of each extractive solvent were clearly divided. Those findings were identical to those of HCA dendrograms (Fig 5).
classes, which successfully classified samples of different organs and habitats into variant four subclusters, when looking at samples of each extracting solvent independently .

3.5. Untargeted analysis using HPTLC-image analysis and supervised pattern recognition (OPLS-DA and HCA)

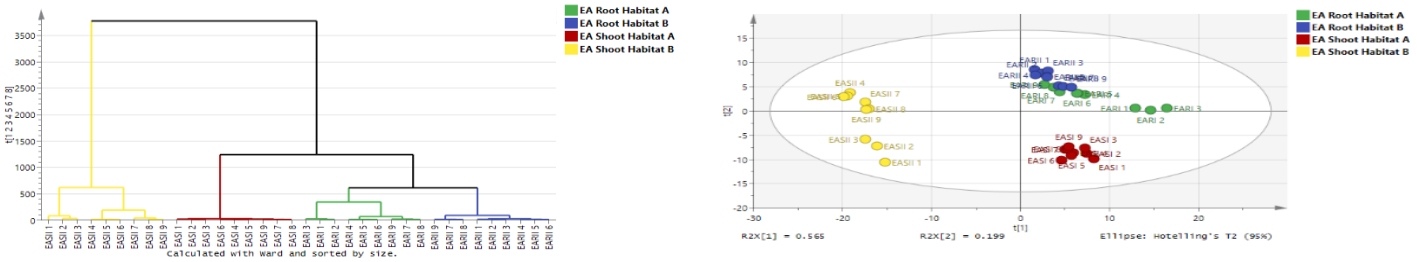
Hereby, OPLS-DA was repeatedly applied to distinguish samples of different organs and habitats extracted with different solvents. The constructed OPLS-DA model discriminated examined samples of each extracting solvent separately into four distinct Those classes represented samples of different organs and geographical habitats, reflecting varying metabolomic profiles. These results were obviously spotted in the dendrograms based on the OPLS-DA model where four sample subclasses for each solvent were framed out representing different plant parts and geographical origins.



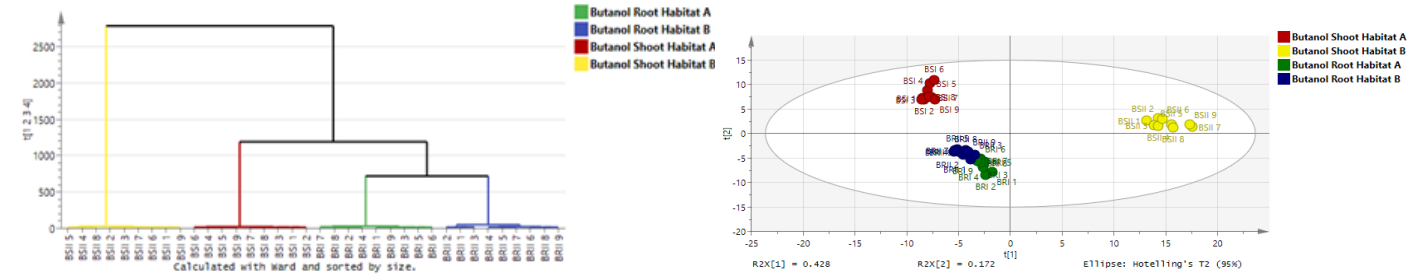
A



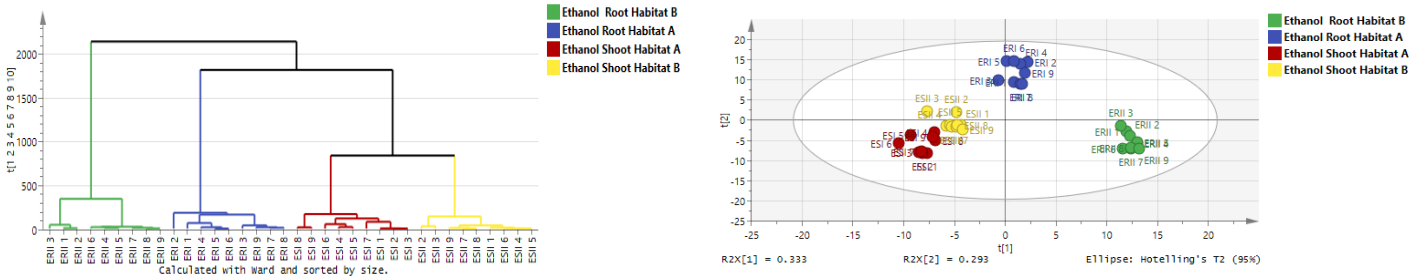
B



C

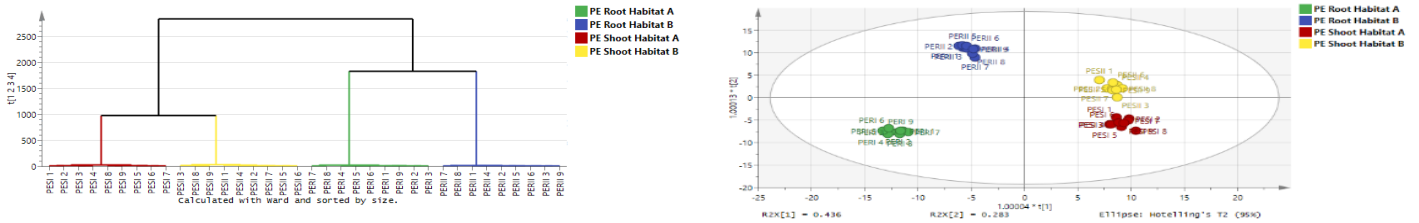


D

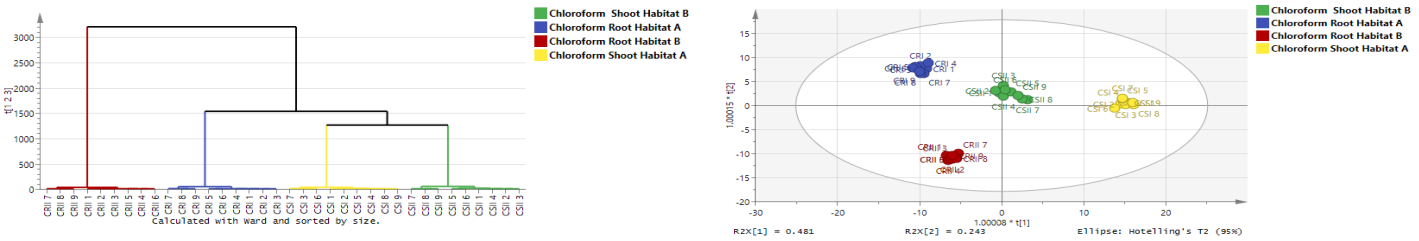


E

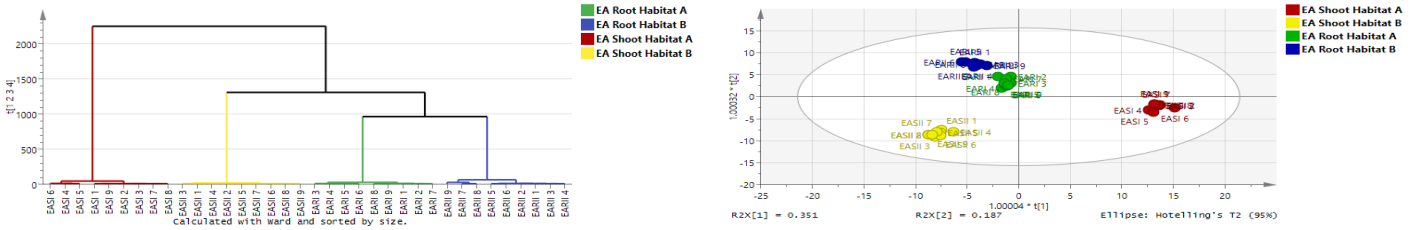
Fig. 9: Unsupervised pattern recognition (PCA, HCA) of *E. campstre* samples of extracted with petroleum ether A, chloroform B, ehtyl acetate C, butanol D and ethanol E



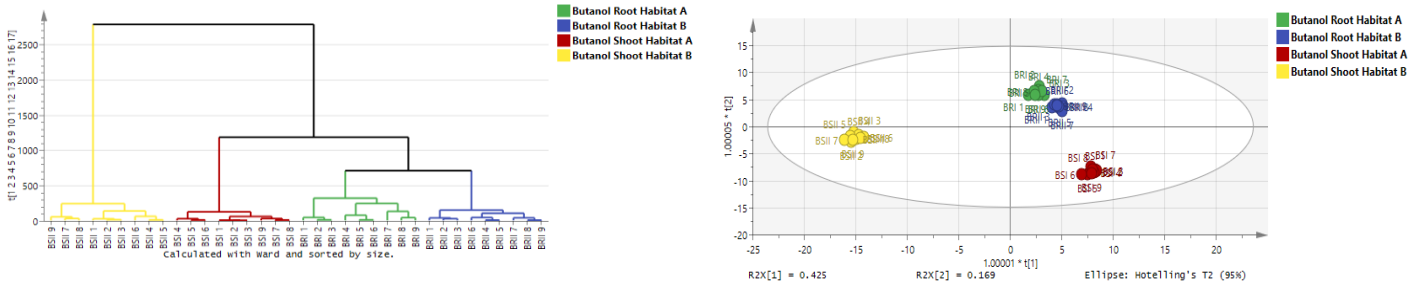
A



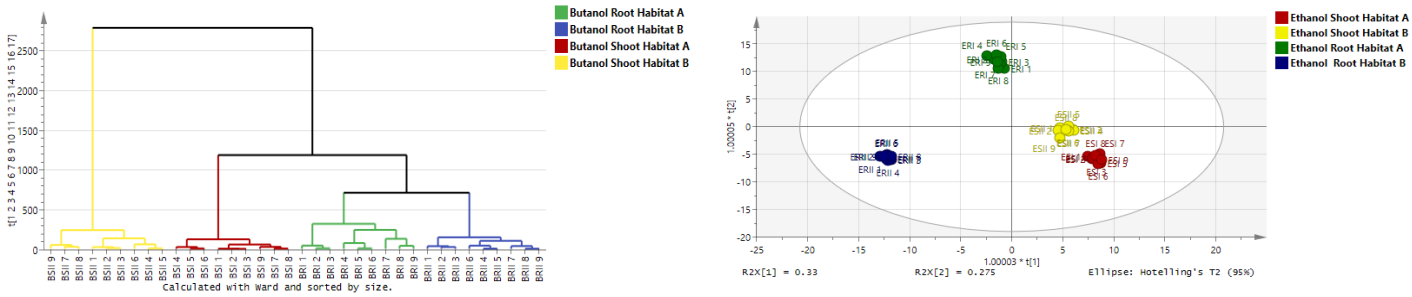
B



C



D



E

Fig. 10: Unsupervised pattern recognition (OPLS-DA, HCA) of *E. campstre* samples of extracted with petroleum ether A, chloroform B, ethyl acetate C, butanol D and ethanol E

3.6. Antibacterial activity testing

In testing the effect of plant species, habitat, plant part used, fractionation solvent, and bacterial species as factors and their interactions on bioactivity of bacterial growth inhibition, results showed highly significant effect, except when interaction didn't include bacterial species and included all other factors. This is expected, as the test is based on the effect on bacterial growth inhibition.

Chloroform and butanol solvents extracted fractions from *E. campestre* crude extract, which inhibited the growth of all the six tested pathogenic bacterial species and strains included multidrug resistant strains of K. pneumoniae and S. aureus. This means that such fractions could be of a general antibiotic type. while other fractions extracted using petroleum ether, ethyl acetate, and ethanol showed specificity. Future studies are strongly encouraged to furtherly correlate the influence of the found variation in the discriminated chemical profiles of samples of different extracts, based on their organs and habitats, on their biological matrices**.**

4. Conclusion

Working on the phytochemistry and bioactivity of the *campestre* species belonging to genus *Eryngium*, which grows in the north western desert of Egypt, the following pieces of information were concluded based on the obtained results.

- Egyptian *Eryngium campestre* contains potent antibacterial active compounds.
- Phytochemical fractions extracted using butanol and chloroform were the most effective antibacterial growth inhibitors.
- Species, habitat, plant part, and crude extract fractionation solvents are factors causing highly significant variation in the phytochemistry of the plant manifested in fractions yield and specificity of pathogenic bacterial growth inhibition.
- Supervised and unsupervised pattern recognition using near infrared (NIR) technique differentiated phytochemically the four samples of *Eryngium campestre* of the two habitats A and B, and the shoot and root systems.
- Supervised and unsupervised pattern recognition using thin layer chromatography (TLC) technique differentiated phytochemically the five fractions extracted using petroleum ether, chloroform, ethyl acetate, butanol, and ethanol from each of the four samples of *Eryngium campestre*, the two habitats A and B, and the shoot and root systems.
- In comparing the two species with respect to their effectiveness in inhibiting bacterial growth, the difference was highly significant, significant, and insignificant depending on habitat, fractionation solvent, and test bacterial species.

Hereby, supervised and unsupervised pattern recognition techniques were unable to differentiate samples patterns as TLC technique only could detect limited range of compounds which could be visualized for the used mobile phase and derivatizing agent applied in the present study. Meanwhile, recognition pattern is very discriminative on individual samples.

It is clear that the five fractions of petroleum ether, chloroform, ethyl acetate, butanol, and ethanol are distributed in five separate clusters, showing that the nature of metabolomics vary according to the extracting solvent reflecting their difference in polarity which in-turn was reflected on their biological activities.

Table 1: Antibacterial activity (inhibition zone diameter, cm) of *E. campestre* extracted fractions using five different solvents against six pathogenic bacterial species. Plant samples were collected from two different habitats A & B, extracts of roots and shoots were tested separately.

Plant species	Habitat	Part used	Tested bacterial species	Fractionation solvent				
				Petroleum Ether	Chloroform	Ethyl acetate	Butanol	Ethanol
				•• Average of growth inhibition zone diameter (cm)				
E. campestre	A	Shoot	*S. epidermidis*	1.50	1.90	1.60	1.70	1.40
		Root		1.30	1.60	1.60	1.65	1.10
	B	Shoot		1.40	1.50	1.60	1.50	1.40
		Root		1.80	1.90	1.40	1.60	1.30
	A	Shoot	*K. pneumoniae*	1.00	1.40	1.50	1.50	1.50
		Root		1.00	1.70	1.40	1.55	1.00
	B	Shoot		1.00	1.40	1.00	1.75	1.00
		Root		1.90	2.00	1.70	1.50	2.00
	A	Shoot	*K. pneumoniae* MDR	1.50	2.00	2.00	1.80	1.70
		Root		1.30	2.40	3.00	1.80	1.00
	B	Shoot		1.35	1.50	1.75	2.30	1.60
		Root		2.10	2.75	3.00	2.50	1.50
	A	Shoot	*P. aeruginosa*	2.00	2.00	2.00	1.50	1.70
		Root		1.50	2.65	3.00	1.80	4.50
	B	Shoot		1.00	2.55	3.00	1.35	1.00
		Root		1.35	1.60	1.00	1.40	1.60
	A	Shoot	*S. aureus*	1.00	1.65	1.70	1.40	1.50
		Root		1.00	1.50	1.20	1.50	1.00
	B	Shoot		1.00	1.70	1.20	1.20	1.00
		Root		1.00	1.20	1.75	1.20	1.00
	A	Shoot	*S. aureus* MDR	1.00	1.90	1.50	1.40	1.30
		Root		1.25	1.40	1.00	1.15	1.00
	B	Shoot		1.00	1.30	1.15	1.10	1.00
		Root		1.00	1.60	1.45	1.30	1.00

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