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SYSTEMATIC REVISION OF *CAPPARIS SPINOSA* L. VAR. (*CANESCENS*, *DESERTI*, *INERMIS*), THE ENDEMIC VARIETIES AMONG EGYPTIAN FLORA BASED ON MOLECULAR AND CHEMO-TAXONOMY

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Capparis spinosa is a xerophytic shrub that has amazing capacity for surviving in challenging circumstances. Therapeutic and pharmacological benefits of this plant species, and its culinary applications, are of significant interest. Various bioactive constituents discovered throughout several organs contribute to its phytochemical relevance, and its cultivation has significant potential commercial benefit. Furthermore, the broad heterogeneity of *C. spinosa* has made taxonomic classification challenging, and paucity of genetic investigations has led to uncertainty among numerous authors. Recent study epitomizes dataset regarding *C. spinosa* encompasses taxonomical, phytochemical and molecular investigation. Three Egyptian endemic varieties of *C. spinosa*; *canescens*, *deserti*, *inermis* were genetically identified using two marker approaches including inter simple sequence repeat (ISSR) and start codon targeted polymorphism (SCoT). Five primers for each approach totaled ten in total for the analysis. ISSR and SCoT primers generated 28 and 40 bands overall, respectively. Polymorphism levels ranged between 64.28 to 67.50% with ISSR and SCoT examination, respectively. Furthermore, chemo-taxonomic clustering employing UV-spectroscopic analysis besides phenolic content was coupled with unsupervised chemometric tools of Principal Components Analysis (PCA) and Hierarchical Cluster Analysis (HCA). Through molecular and phytochemical investigations, interspecific relationships among *canescens* and *deserti* varieties have been verified. Additionally, *inermis* variety was identified as a monotypic variety. Flavonoid content coincided with the conclusion, which was proved through different expression of phenylpropanoid biosynthetic genes. This research suggests raising var. *inermis* to the species level, namely *Capparis orientalis,* demonstrating that

chemotaxonomic information can mirror evolutionary connections between caper varieties and sheds light on the need of combining molecular and chemo-taxonomy's advantages to clarify the evolutionary connections within this species.

Keywords: Caper, biosystematic revision, ISSR, SCoT, gene expression, flavonoids

INTRODUCTION

Family Capparaceae is a mid-sized family with forty and fifty genera and around seven hundred species. Only a few of its members are economically or horticulturally significant. The family lives mostly in the tropics and subtropics within the two hemispheres, as well as in the Mediterranean region. It is extensively represented by roughly 15 genera in Africa, where it is a prominent component of the flora of arid areas (El Karemy, 2001). The Capparaceae are thought to be related to the Cruciferae and to have originated from a common ancestor. Heywood (1978) mentioned some of this family's relationships with the Papaveraceae and Resedaceae. Cleomoideae and Capparoideae, the two large subfamilies of Capparaceae, are highly different and have even been raised to family category by certain researchers (Airy Shaw, 1965 and Hutchinson, 1967). Pax and Hoffmann (1936) split the larger Capparoideae into 4 tribes, while Hutchinson (1967) divides it into 3 tribes (Capparaceae). Despite these taxonomic systems agree on certain points, most elements of Capparaceae connections remain unsolved. The Egyptian Capparaceae taxa are found in xerophytic habitats (Zahran and Willis, 1992 and Abd El-Ghani and Marei 2006). Previous researches on Capparaceae in Egypt relied mostly on leaf anatomy (Al-Gohary, 1982), seed shape (Al-Gohary, 1997), and pollen architecture (Khafagi and Al-Gohary, 1998).

Capparis spinosa (*n*=19) is a Mediterranean-endemic perennial xerophytic shrub that can thrive in a wide variety of climatic conditions, from semi-arid beach habitats to colder highland elevations. Caper shrubs play a vital role in ecosystem services because of their ability to grow in dry, adverse environments including the rocky cliffs, crevices, and stone walls. Furthermore, caper cultivation might offer an important solution for avoiding the effects of desertification and soil erosion in vulnerable ecosystems (Pegiou et al., 2023). Furthermore, the gradual growth of its fire-resistant vegetative portions suggests caper plantings as a viable choice for wildfire prevention and suppression in shrub-lands and pine forests, which are becoming more common and destructive in the Mediterranean region because of climate change (Pegiou et al., 2023).

Caper is a medicinal plant whose pharmacological efficacy is based on its abundance of various bioactive constituents (Shahrajabian et al., 2021). Caper's major edible source is flower buds, which are widely utilized in the

Mediterranean diet (Kdimy et al., 2022). Caper cultivation is widespread throughout the Mediterranean, particularly in Egypt, Italy, Spain, Morocco, Greece, and Turkey, along with the Near East, however statistics on crop areas are still lacking (Grimalt et al., 2022 and Mahmodi et al., 2022). Despite its widespread distribution, the species is presently considered at risk of genetic erosion, mostly because of overgrazing and overharvesting through bud collecting from the wild for domestic and commercial purposes, which has a negative influence on the species' variety, conservation, and evolution.

Previous studies revealed dispute and uncertainty over the species and varieties of *C. spinosa*. Recently, Boulos claimed that *C. spinosa* species encompasses three varieties, including var. *canescens*, *deserti* and *inermis* (Boulos, 1974). The systematic classification and evolutionary affiliations of Egyptian *Capparis* species remain unknown, and unresolved concerns surrounding their biology and classification require more evidence and validation (Kamel et al., 2009). *Capparis* is a vast and polymorphic genus with a global allocation in the tropics and subtropics. Several researchers suggested infrageneric treatments for it (De Candolle 1824; Grisebash, 1859-64; Eichler, 1865 and Pax and Hoffmann 1936), and while Jacobs (1965) presented a revision of the South Asian and Pacific taxa, no recent universal categorization is available other than Hutchinson (1967), who divided Capparis into numerous tiny genera that few other than himself agreed to.

DNA fingerprinting technologies rely mostly on polymerase chain reaction (PCR) to identify a specific DNA profile of a particular genotype, which is as distinctive as a fingerprint. To indicate biodiversity among species as well as investigate phylogenetic relationships of plants from various families, genera, species, and even varieties, extremely distinct dominant markers that expand to a considerable proportion along the entire genome randomly recognizing either non-coding neither coding areas are used (Mueller and Wolfenbarger, 1999). Due to the ease of use and the relatively large amount of DNA markers that could be produced per sample, random amplified polymorphic DNA (RAPD) markers (Welsh and McClelland, 1990), inter simple sequence repeat (ISSR) markers (Blair et al., 1999), and amplified fragment length polymorphism (AFLP) markers (Vos et al., 1995) have been widely utilized. The fast advancement of genomics studies requires the use of gene-targeted rather than random DNA markers (Andersen and Lübberstedt, 2003). Start codon targeted polymorphism (SCoT), a potential marker technique, has been used in this context to provide more precise genetic analyses.

The ISSR approach amplifies genomic DNA using a single random oligonucleotide primer. This results in many amplification loci and makes usage of microsatellite sequences that are widely dispersed along the genome, allowing it to be employed as a dominant multi-locus marker platform for genetic variation study. ISSR markers are relatively fast, prolific, reproducible, and capable of distinguishing many polymorphisms in a given

population (Reddy et al., 2002). ISSR marker production is more reproducible than RAPD (Kuras and Korbin, 2004). ISSR was frequently utilized in the genetic diversity research of medicinal plant species such as *Memecylon* (Ramasetty et al., 2016), *Ocimum* species (Patel et al., 2015) and *Albizia* (El Khodary et al., 2021).

SCoT is an unpretentious and innovative DNA marker approach that relies on a small, conserved space in plant genes bordering the well-identified ATG translation start codon. SCoT outperforms RAPD in terms of polymorphism, efficiency, and reproducibility. Furthermore, in species that are very similar, SCoT polymorphism is linked to functional genes and their accompanying properties, including distinct metabolites (Collard and Mackill, 2008). The SCoT polymorphism marker approach has been used to effectively identify and analyze *Echinacea* (Jedrzejczyk, 2020), *Nigella* (Golkar and Nourbakhsh, 2019), rice, peanut, mango, citrus, grapes, potato, and orchids (Rajesh et al., 2015).

Phenolics comprise basic phenols, phenolic acids (cinnamic and benzoic acid derivatives), flavonoids, coumarins, stilbenes, hydrolysable and condensed tannins, lignans, and lignins (Gottlieb and Borin, 2000). Because they are widespread and easily detectable using relatively basic techniques of investigation, phenolic chemicals, particularly flavonoids, have garnered far more attention as chemotaxonomic indicators than any other family of secondary metabolites. Variations in substitution patterns have a strong association with taxonomy "advancement." Furthermore, because of the evolutionary process, replacement patterns have become simpler. Harborne (1966) presented a set of primitive and advanced flavonoid characteristics that may be utilized to determine a taxon's phylogenetic status. The Capparaceae have also been mentioned as a family having an intriguing flavonoid distribution (Wink et al., 2018). A wide range of phenolic compounds have been identified from several *Albizia* species. For example, concerning species under investigation; tri-O-glycoside flavonols, kaempferol have been reported in the leaves of *C. spinosa* var. *deserti* (Abo El-Fadl et al., 2021).

UV spectroscopy provides a straightforward analytical method for determining the absorbance of linked phenolic compounds. UV spectroscopy, when combined with chemometric methods, can give selective studies between distinct species in terms of total phenolic content. This permit enabling the construction of an accurate taxonomy categorization based on chemical profile diversity, which will help to improve the phylogenetic relationships discovered by genetic research (Jolayemi et al., 2018). Principal Components Analysis (PCA) and Hierarchical Cluster Analysis (HCA) are frequently employed as "unsupervised identification" approaches to clarify relations between bioactive constituents based on resemblance and hidden patterns between specimens when the raw data and categorizing relationships are unknown (Gad et al., 2013a; Ibrahim and Mouss, 2020 and 2021). Several

recent investigations have used UV spectroscopy and chemometrics to differentiate taxa (Gad et al., 2013b and Sima et al., 2015).

Real-time PCR (RT-PCR) was created in 1984 thanks to Kary Mullis' innovation of PCR technique. RT-PCR, or gene detection and study of gene(s) expression in real-time, has fundamentally altered biological science in the 21st century because of its wide range of applications in quantitative genotyping, genetic diversity within and across species, early illness diagnosis, and forensic, to mention a few. A thorough examination of several RT-PCR-related topics was provided, including technology advancements as well as applications in every branch of science, from medicine to environmental studies to plant taxonomy (Deepak et al., 2007).

The present research attempts to explain the significance of using modern molecular taxonomy techniques that include ISSR and SCoT assessment, as well as chemical categorization relying on the UV spectroscopic pattern regarding total phenolic content, to identify phylogenetic relationships between selected varieties of *C. spinosa*, including var. *canescens*, *deserti*, and *inermis*. This is the first study to combine the powers of molecular and phytochemical analysis to determine the evolutionary connection of the genus *Capparis*. Furthermore, a recent study revealed the variation in flavonoid content and its biosynthetic genes across selected varieties of *C. spinosa*.

MATERIALS AND METHODS

1. Plant Material, Geographical and Ecological Data

Fresh materials of three *C. spinosa* L. var. (*canescens*, *deserti*, *inermis*) were recorded and collected in summer 2019 from Saint Catherine Protectorate on distance of 1,586 meters (5,203 ft) above sea and the Western Desert regions, Egypt on distance of 150 meters (500 ft). In Saint Catherine, the summers are long, warm, arid, and clear and the winters are very cold, dry, and mostly clear. Over the course of the year, the temperature typically varies from 29°F to 80°F and is rarely below 23°F or above 84°F. On the other hand, Western Desert is one of the coldest regions in Egypt with an average daily high temperature of only 29 degrees. For 9 months, the average temperatures are over 25 degrees (Agrawala et al., 2004). All samples were then identified in the herbarium of Desert Research Center, Cairo, Egypt.

2. Samples Preparation, Molecular Amplification and Gene Detection

In 2019, fully ripe fruits were shade-dried and pulverized to achieve fine powder suited to extraction for the UV spectroscopic, total phenolic and flavonoid content assays. A rotary evaporator was used to evaporate a methanolic extract of dried fruits, which was subsequently lyophilized and kept at -18°C until needed. fruits were kept at -70°C for DNA and RNA extraction before being pulverized to a fine powder with liquid nitrogen.

DNA extraction and analysis was performed at the laboratories of Desert Research Center, Ministry of Agriculture and Land Reclamation, Cairo, Egypt and Molecular biology laboratories, Faculty of Pharmacy, Tanta University, Tanta, Gharbia, Egypt. The bulked DNA extraction was carried out utilizing a DNeasy plant Mini Kit (QIAGEN) and a DNeasy-like technique (Alexander et al., 2007). ISSR and SCoT markers were amplified using PCR using a 25 ng DNA template and the thermal cyclic profile shown below. The reaction solution contained 1X TBE buffer, 25 ng DNA template in the total reaction volume of 25 µl, 2.5 mM $MgCl_2$, 10 pmol primer, 1 U Taq DNA polymerase (Promega®) and 0.25 mM dNTPs. The DNA amplifications were carried out in a computerized thermal cycle model (Techne 512, Bibby Scientific™, UK.) designed for one denaturation cycle at 94°C for 4 minutes, subsequent to 45 cycles of 1 minute at 94°C, an annealing cycle for 1 minute at 57°C, and an elongation cycle for 2 minutes at 72°C. subsequently the primer extension segment was extended to 10 minutes. Electrophoresis employing agarose (1.5% w/v in 1 TBE buffer) combined with Ethidium bromide (0.5 g/ml) was used to identify the amplified products. In each well, 15 µl of DNA amplified product was added, and a DNA ladder (100 bp) mix was employed as a standard. The run lasted around 30 minutes at 80 V in a tiny submarine gel Bio-Rad. 10 primers were acquired from Metabion International AG (Germany), 5 primers were selected for ISSR assessment (Table 1), and 5 primers were selected for SCoT examination (Table 2).

3. ISSR and SCoT Analysis

PCR amplification products were electrophoretically separated, optically identified, and then analyzed either the existence (1) or missing (0) of DNA bands. The gels were lighted with a UV transilluminator (Vilber LuormatTM, France). The similarity matrix was calculated employing a cluster assessment of data using the unweighted pair cluster technique via arithmetic average (UPGMA) (Sneath and Sokal, 1973). Similarity matrices were made with Gel works ID sophisticated program UVP-England Program. The SPSS windows (Version 24) program was used to analyze the relationships between genotypes shown by dendrograms. The DICE computer software was utilized to build the pairwise difference matrix and produce the phenogram between the examined varieties (Yang and Quiros, 1993). Dice similarity coefficient (DSC) is a basic spatial overlap indicator and a tool for validating repeatability. A DSC score ranges from 0 to 1, suggesting that there is no geographic overlap among the 2 sets of binary segmentation results (Dice, 1945).

Table (1). List of the ISSR primer names, their nucleotide sequence, banding pattern and degree of polymorphism for the genetic relationship between studied *Capparis spinosa* varieties.

No	Name	Sequence 5′–3′	Total bands	Mono-morphic bands	Poly-morphic bands	Unique bands	Polymorphism %	Genetic similarity %
1	44-B	CTCTCTCTCTCTCTCTGC	5	1	4	1	80.00	20.00
2	HB-9	GTGTGTGTGTGTGC	6	3	3	1	50.00	50.00
3	HB-10	GAGAGAGAGAGACC	6	2	4	1	66.66	33.33
4	HB-12	CACCACCACGC	6	2	4	1	66.66	33.33
5	HB-15	GTGGTGGTGGC	5	2	3	2	60.00	40.00
Total no. of bands for ISSR Technique			28	10	18	6	64.28	35.71

Table (2). List of the SCoT primer names, their nucleotide sequence, banding pattern and degree of polymorphism for the genetic relationship between studied *Capparis spinosa* varieties.

No	Name	Sequence 5′–3′	Total bands	Mono-morphic bands	Poly-morphic bands	Unique bands	Polymorphism %	Genetic similarity %
1	SCoT-1	ACGACATGGCGACCACGC	7	3	4	-	57.14	42.85
2	SCoT-2	ACCATGGCTACCACCGGC	8	3	5	3	62.50	37.50
3	SCoT-3	ACGACATGGCGACCCACA	10	2	8	2	80.00	20.00
4	SCoT-4	ACCATGGCTACCACCGCA	9	3	6	-	66.66	33.33
5	SCoT-6	CAATGGCTACCACTACAG	6	2	4	-	66.66	33.33
Total no. of bands for SCoT Technique			40	13	27	5	67.50	32.50

4. Expression Analyses of Flavonoid's Biosynthetic Genes

The first-strand complementary DNA (cDNA) was generated using 3 µg of total RNA and 200 U of M-MLV reverse transcriptase (from Invitrogen), following the manufacturer's instructions. Total RNA was isolated using Trizol solution (Invitrogen). Using first-strand cDNA as a template, RT-PCR was performed to amplify a 400 bp fragment of each flavonoid's biosynthetic gene with 31 cycles. As an additional internal control, the action was amplified for 24 cycles. Using the SYBR Premix Kit F-415 and an AB StepOnePlus PCR equipment from Applied Biosystems, RT-PCR was carried out on an optical 96-well plate (Thermo Scientific). A relative quantification method was used to determine relative gene expression. The list of all primers used in this investigation can be found in Table (3).

Table (3). List of primer names of the flavonoid biosynthetic genes that were used in qRT-PCR analysis, their nucleotide sequences, used to investigates expression levels in *Capparis spinosa* varieties.

Name	Sequence 5′–3′ Fw	Sequence 5′–3′ Rv
PAL	AACCTATCTCGTGGCTCTTT	TCTTTTTCGCTGAATCTTGC
CHS	TGGTCACCGTGGAGGAGTATC	GATCGTAGCTGGACCCTCTGC
DFR	GAGCATGAAGTTGGGCCAAT	TGGTGGGTTGGCCTCATTAA
ANS	GAACTAGCACTTGGCGTCGAA	TTGCAAGCCAGGCACCATA

5. Total Phenolic Content

The Folin-Ciocalteu reagent was utilized in a spectrophotometric technique to determine the total phenolic content in plant extracts (Schofield et al., 2001 and Faheem et al., 2020). To create a calibration curve, a standard solution of gallic acid (1 mg/ml) was made, then serially diluted to yield solutions of 40, 80, 120, 160, 200, 240, and 280 g/ml. One ml of each dilution was mixed with 10 ml of distilled H_2O and 1.5 ml of Folin Ciocalteu reagent. After five minutes, 4 ml of 20% sodium carbonate solution were added, followed by 25 ml of distilled H_2O and 30 minutes at room temperature in the dark. UV-Vis spectrophotometer (JASCO V-630, Shimadzu, Japan) was used to measure absorbance at 765 nm. Likewise, samples at a dosage of 1 mg dried extract/ml were produced and combined using the same reagent as described previously. After 30 minutes, the absorbance was measured at 765 nm. A blank was made in the same manner as the sample, but with 1 ml of H_2O rather than the sample. The mean (standard deviation, ± SD) findings of triplicate assays were represented as mg gallic acid equivalents per gram dry extract (mg GAE/g DE) using the gallic acid straight line equation ($y = 0.0041x + 0.0057$, R2 = 0.9996), in which y denotes determined absorbance and x denotes estimated concentration.

6. UV Spectroscopic Assay

Extracts of the three varieties were prepared using high performance liquid chromatography (HPLC) grade methanol (Fisher Scientific®). Initial analysis was conducted to determine the best extract concentration. The concentration was fixed at 0.1 mg dry extract/ml according to these trials. The absorbance of the extracts was measured using a spectrophotometer at a wavelength range of 250-400 nm. The wavelength range was chosen since the methanol spectra of flavones and flavonols show two prominent absorption peaks in the 240-400 nm range. These two peaks are known as Band I (typically 300-380 nm) and Band II (typically 240-280 nm) (Mabry et al., 1970). The studies were conducted in duplicate.

7. Multivariate Data Analysis

To differentiate between different varieties, Unscrambler X 10.4, CAMO program (Computer Aided Modelling, AS, Norway) was used to perform unsupervised chemometric methods such as PCA and HCA with the average linkage approach built on the Euclidean distance within varieties.

8. Extraction of Plant Material for Quantitative Estimation of Flavonoids

Using an IKA experimental mill (Staufen, Germany), dehydrated and powdered fruit material (1 g) was extracted three times with 10 ml of methanol: H_2O (80:20 v/v). At room temperature, samples were well mixed and shaken on an orbital shaker for 15 minutes (Niranjan, 2011 and Pandey et al., 2012). Filtrate was obtained in a transparent glass HPLC vial (Agilent Technologies, USA) and injected into an HPLC-MS-MS system.

9. Liquid Chromatography-Tandem Mass Spectroscopy and Data Analysis

All samples were examined utilizing an HPLC system (1260 infinite series, Agilent Technologies, Singapore) outfitted using a pump, autosampler, column compartment, and thermostat and employing an Agilent Zorbax Eclipse Plus column (4.6 100 mm, 3.5 m) kept at 40 °C. A specific chemical was also employed in the guard column. The mobile phase was an aqueous solution of 0.1% formic acid (mass spectrometry LC-MS grade; Solution A) and an acetonitrile solution of 0.1% formic acid (LC-MS grade; Solution B). The gradient was set as follows: 0-2 minutes, Solution B 2%; 2-22 minutes, Solution B 2-98%; 22.01-25 minutes, Solution B 98-2%. Another chromatographic setting was a steady flow rate of 0.6 ml/min, an injection amount of 5 µl, an autosampler temperature of 10°C, and a run duration of 25 minutes including equilibration. All samples were separated via 0.22 m needle membrane filters (Millipore, USA) before measurement. The analysis was carried out utilizing an HPLC system linked to a triple quadrupole system QTRAP 5500 (ABSciex, Singapore) via ESI probes in both modes of ionization. The voltage was fixed at 5.5 KV for positive ionization and 4.5 KV for negative ionization, the Gas1 and Gas2 values were set at 50, the curtain gas (CUR) was set at 30, collision aided dissociation (CAD) was set at moderate, and the temperature of the source (TEM) was set at 550°C. The mass spectrometer was set to monitor several reactions simultaneously (MRM). Analyzer program (version 1.5.2) and MultiQuant program (version 2.0.2) were used for recognizing and quantifying analyses.

RESULTS

1. Molecular Analysis

Three endemic Egyptian varieties of the Caper species (*C. spinosa* L.) were collected from their habitats as shown in (Fig. 1a and b) for genetic uniformity analysis employing ISSR and SCoT markers; *C. spinosa* var. *canescens*, *deserti* and *inermis* as shown in Tables (1 and 2). This analysis included ten primers, five for each approach (Fig. S1, Tables S1 and S2) that show the banding patterns generated by ISSR primers (HB-15, 44B, HB-9, HB-10, and HB-12), as well as those generated using SCoT primers (SCoT-1, SCoT- 2, SCoT-3, SCoT-4, and SCoT-6). Bands are formed because of amplification within the employed primer and a specific region on the sample genome indicating functional gene locations. Regardless of proportion, ISSR and SCoT bands were declared existent or missing. The ISSR and SCoT-PCR markers showed each of the primers were generated. Each DNA locus was regarded as a distinct feature. The entire number of bands was 68, including 40 bands using the SCoT approach and 28 using the ISSR technique (Tables 1 and 2). ISSR-based study produced 10 monomorphic and 18 polymorphic bands, resulting in a polymorphism rate of 64.28% and a genetic

similarity rate of 35.71%. Furthermore, 6 bands (Table 1) were shown to be distinct. Table (1) shows the variation in banding patterns generated with the 5 ISSR primers (HB9, HB10, HB12, HB15 and 44B). The bands that were created have molecular weights that ranged between 285 to 2340 bps. Since the HB-15 primer only generated distinct bands with *C. spinosa* var. *inermis* (3) at 1290 and 825 base pairs, it may be stated to be "variety specific". With a percentage of polymorphism reaching 67.50% and a percentage of genetic similarity of 32.5%, 13 monomorphic as well as 27 polymorphic bands were found employing SCoT-based screening. Additionally, 5 distinct bands were found (Table 2). Table (2) displays the banding arrangements generated with the 5 SCoT primers (SCoT-1, SCoT-2, SCoT-3, SCoT-4, and SCoT-6). The bands that were generated have molecular weights that ranged between 230 to 1780 bps. Since they only generated distinct bands with *C. spinosa* var. canescens (7) at 1370 base per and *C. spinosa* var. *deserti* (8) at 1280 base per, respectively, SCoT-2 and SCoT-3 can be regarded as "variety specific" primers. All primers exhibited polymorphic bands using ISSR markers. The primers HB10, HB12 and 44B exhibited the greatest polymorphism ratios, approximately 66.6, 66.6 and 80% respectively. Likewise, polymorphic bands were generated by all SCoT primers. The primers SCoT-3, SCoT-4, and SCoT-6 showed top polymorphism ratios, roughly 80, 66.6, and 66.6%, respectively. Primers SCoT-3, SCoT-4, and SCoT- 6 are favored for SCoT-based differentiation, while primers HB-10, HB-12 and 44B are favored for ISSR-based discriminating between verities and species by considering the polymorphism ratio. Dendrograms obtained from molecular evaluation were computerized diagrams employed in sampling and gene grouping. Cluster analysis of the three varieties revealed minor changes according to the approaches employed to assess genetic diversity. The three Caper verities were divided into two main clusters using an ISSR-obtained dendrogram (Fig. 2a), with Dice similarity scores fluctuating between 0 to 1 (Table S3). The first cluster (I) consisted of two sub-clusters including *C. spinosa* var. *canescens* and *deserti* while the second cluster (II) consisted of the remaining *C. spinosa* var. *inermis.*

The dendrogram constructed using SCoT information also divided the three Caper varieties into two main groups (Fig. 2b), resulting in a Dice-similarity score that fluctuated between 0 to 1 (Table S4). The first cluster (I) contained *C. spinosa* var. *inermis* as a monotypic variety, while the second cluster (II) consisted of the remaining two Caper varieties. Cluster II had two sub-clusters, including *C. spinosa* var. *canescens* and *deserti*.

Dendrogram generated from integrated ISSR and SCoT information divided the three Caper varieties into two main clusters (Fig. 2c), with Dice-similarity scores fluctuating between 0 to 1 (Table S5). The first cluster (I) had just *C. spinosa* var. *inermis* as a monotypic variety, whereas the second cluster (II) contained the remaining two varieties individually. Cluster (II) has two sub-clusters, which include *C. spinosa* var. canescens and *deserti*.

Fig. (1). Phenotypic description (**a**), and Geographic distribution (**b**) of three endemic Egyptian varieties of the Caper species (*Capparis spinosa* L.) including *C. spinosa* var. *canescens*, *deserti* and *inermis*.

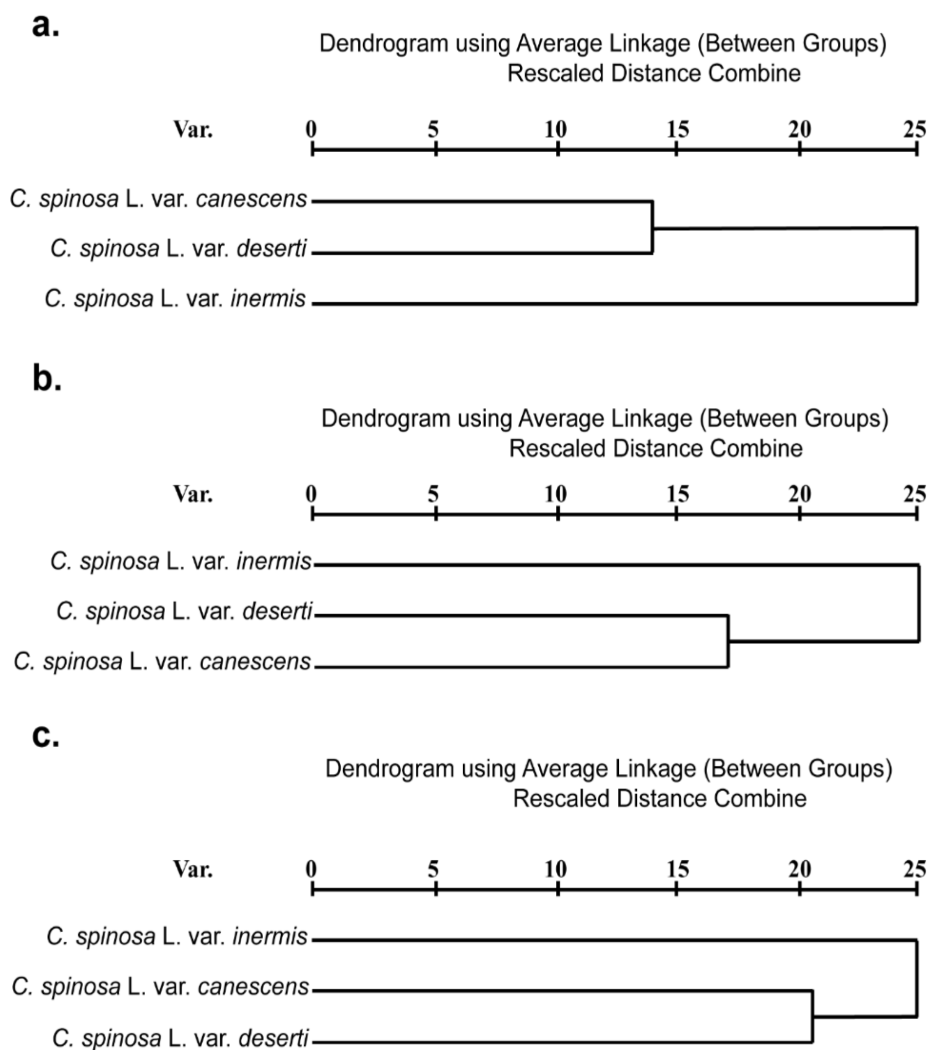

Fig. (2). UPGMA dendrograms showing the clustering of the three *Capparis* varieties based on ISSR analysis (**a**), SCoT analysis (**b**) and combined ISSR and SCoT analysis (**c**).

2. Total Phenolic Content

Caper fruit is broadly famed by its high nutritional value (Abo El-Fadl et al., 2021). To evaluate the effect of phytochemical compounds variation on the genetic diversity between three *C. spinosa* varieties including *canescens, deserti* and *inermis* the corresponding phytochemical analyses were performed in Caper fruits at the ripening stage. The amount of total phenolic content per gramme of dry extract was measured in ml of gallic acid equivalent (GAE). The total phenolic content of fruit extracts from several *C.*

spinosa varieties ranged from 48.55 ± 0.68 to 273.32 ± 0.14 mg of GAE/g dry extract. The highest phenolic content was found in *C. spinosa* var. *canescens* followed by *C. spinosa* var. *deserti* with values of 273.32 ± 0.14 and 226.49 ± 0.14 mg of GAE/g of dry extract, respectively, indicating substantial possibilities for medicinal application. Contrarily, *C. spinosa* var. *inermis* exhibited the lowest level of phenolic content among all varieties of the present study, with 48.55 ± 0.68 mg of GAE/g of dry extract (Fig. 3).

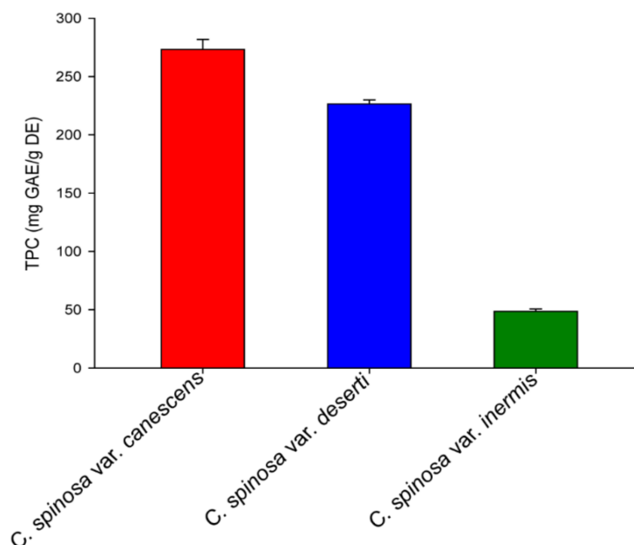

Fig. (3). Total phenolic content expressed as mg of gallic acid equivalent (GAE) per g of dried extract (DE) ± standard deviation *Capparis spinosa* varieties.

3. UV Spectroscopy in Conjunction to Multivariate Data Analysis

To validate the classification pattern discovered through genetic analysis, comparative UV spectroscopic profiling of the three Caper varieties was performed (Fig. 4). PCA was performed to identify variations in UV absorbance patterns across *C. spinosa* varieties. PC1 and PC2 simultaneously accounted for 98% of the divergence within varieties. The PCA score plot (Fig. 5) revealed groupings of extremely similar varieties. Based on PC1 (which equates to 95% of variations within varieties), *C. spinosa* var. *canescens,* and *C. spinosa* var. *deserti* was seen at the positive side while *C. spinosa* var. *inermis* was seen at the negative side. Both *C. spinosa* var. *canescens* and var. *deserti* were classified as "outliers", and were observed in the positive side, this inference may be articulated depend on by their total phenolic content, as these two varieties exhibited the greatest amounts (Fig. 5). This was corroborated via (Fig. 6), which demonstrated strong grouping amongst phylogenetically related varieties. The three varieties were divided into two main clusters based on the HCA dendrogram: the first cluster (I)

contained *C. spinosa* var. *inermis* alone as a monotypic variety, whereas the second cluster (II) contained the other two varieties *C. spinosa* var. *canescens* and var. *deserti* individually under two sub-clusters.

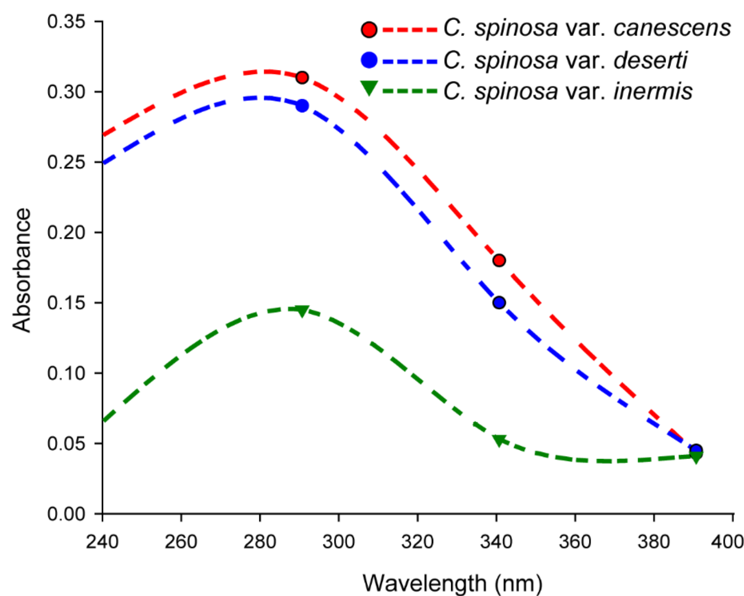

Fig. (4). UV spectroscopic profiling coupled to unsupervised chemometric techniques. UV spectra of the studied *Capparis spinosa* varieties.

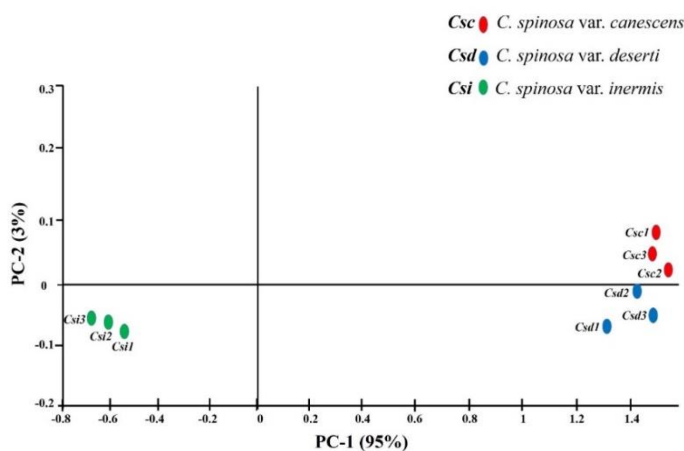

Fig. (5). UV spectroscopic profiling coupled to unsupervised chemometric techniques. Principal Components Analysis (PCA) score plot of the studied *Capparis spinosa* varieties.

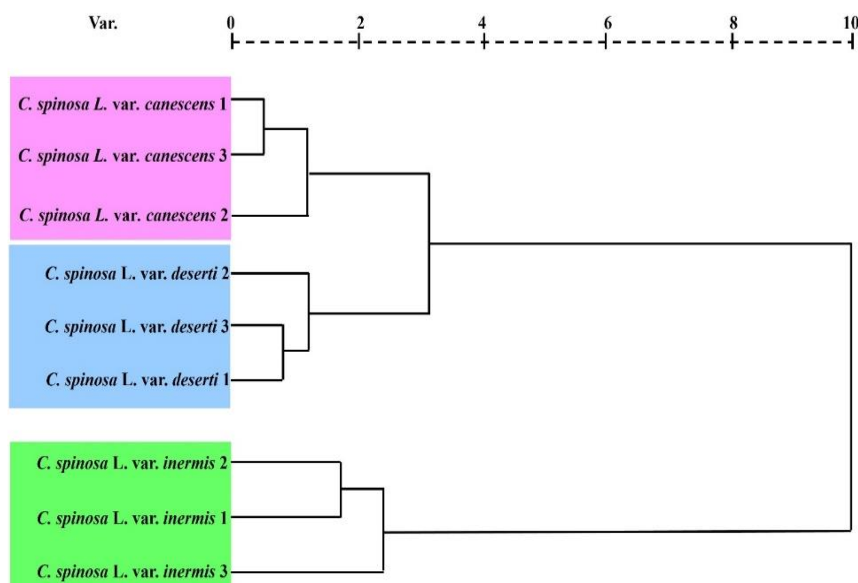

Fig. (6). UV spectroscopic profiling coupled to unsupervised chemometric techniques. Hierarchical Cluster Analysis (HCA) score plot of the studied *Capparis spinosa* varieties.

4. The Effect of Genetic Diversity Between Caper Varieties on Flavonoid Content

The edible medicinal plant *C. spinosa* has been recognized as one of the best genetic sources of flavonoids (Abo El-Fadl et al., 2021). To figure out if genetic variation affects flavonoid production, total flavonoid concentration in methanolic extracts of Caper varieties fruits was quantified using quercetin equivalent. As expected, the total phenolic content was reflected on the total flavonoids accumulation rate in Caper fruits. Total flavonoid content in *C. spinosa* var. *canescens* fruits was higher than in other varieties, reaching (0.45 mg g^{-1} FW), followed by *C. spinosa* var. *deserti* fruits around (0.32 mg g^{-1} FW). In contrast, total flavonoids were found to be significantly lower in *C. spinosa* var. *inermis* fruits (0.07 mg g^{-1} FW) when compared to those of var. *canescens* and *deserti* fruits (Fig. 7).

Consistently, analysis of the different flavonoid species revealed elevated levels of major flavonoids including rutin, kaempferol, and quercetin in *C. spinosa* var. *canescens* fruits, reaching (2.7, 1.2, and 1.3 mg/g FW) respectively, followed by those of *C. spinosa* var. *deserti* up to (2.1, 0.98, and 1.11 mg/g FW). Conversely, a marked decrease was recorded in the major flavonoids including rutin, kaempferol, and quercetin in *C. spinosa* var. *inermis* fruits up to (0.034, 0.0078 and 0.0089 mg/g FW). Surprisingly, the naringenin content in *C. spinosa* var. *inermis* fruits has been significantly

increased (1.53 mg/g FW) compared to those of *C. spinosa* var. *canescens* and *deserti* fruits (0.14, and 0.16 mg/g FW), respectively (Fig. 8).

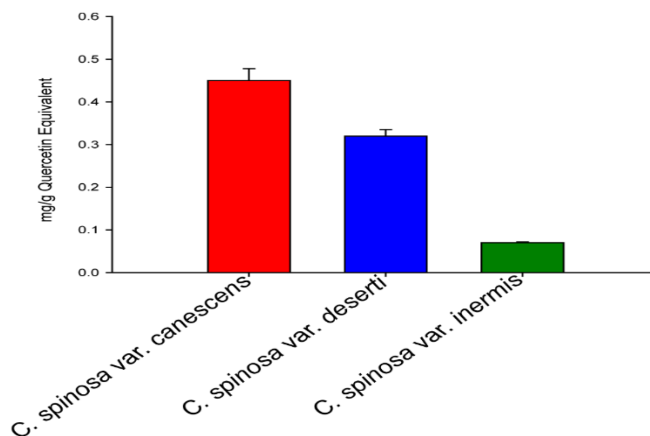

Fig. (7). Quantification of total flavonoid content in methanolic extracts of *Capparis spinosa* varieties fruits using quercetin equivalent. The data shown are the mean ± SE (n = 3).

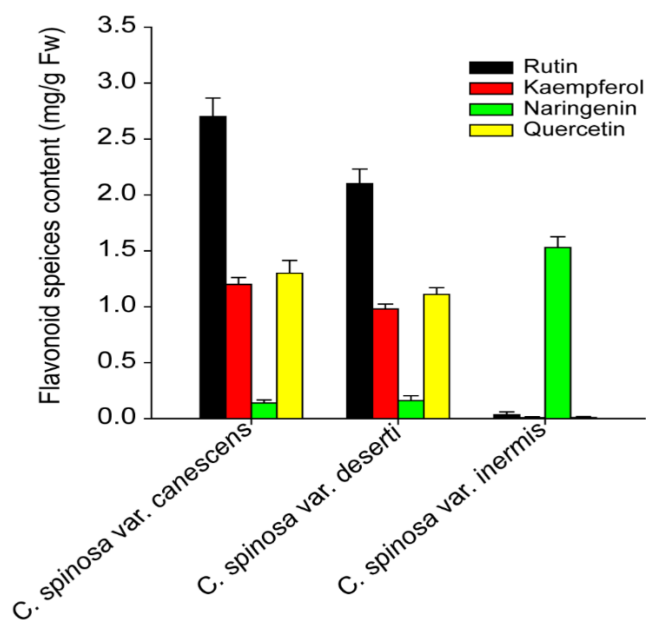

Fig. (8). Quantification of flavonoid species content (mg/g Fw) in the fruits methanolic extracts of *Capparis spinosa* varieties. The data shown are the mean ± SE (*n* = 3).

5. Validation of Flavonoid Content Variation Within Caper Varieties via Quantitative Real-Time PCR

Among 14 key genes involved in the phenylpropanoid synthesis pathway, only four biosynthetic genes have same primer sequences were present in the genomes of the three Caper species. A quantitative real-time PCR (qRT-PCR) was used to analyze the transcription level of the genes encoding Phenylalanine amonia lyase *(PAL)*, Chalcone synthase *(CHS)*, Dihydroflavonol 4-reductase *(DFR)*, and Anthocyanidine synthase *(ANS)* in caper fruits at the ripening stage. Interestingly, the expression level of *PAL*, *CHS*, *DFR*, and *ANS* genes was elevated significantly up to 8.8, 6.5, 4.6 and 17.2, respectively in *C. spinosa* var. *canescens* fruits (Fig. 9), followed by those of *C. spinosa* var. *deserti* fruits reaching 7.2, 6.1, 3.3 and 12.9, respectively (Fig. 9). In contrast, the transcription level of *PAL*, *CHS*, *DFR*, and *ANS* genes was dramatically reduced in *C. spinosa* var. *inermis* fruits approximately 1.8, 0.88, 1.5 and 2.7, respectively (Fig. 9). These findings support the accuracy of the findings on the total phenol content presented above.

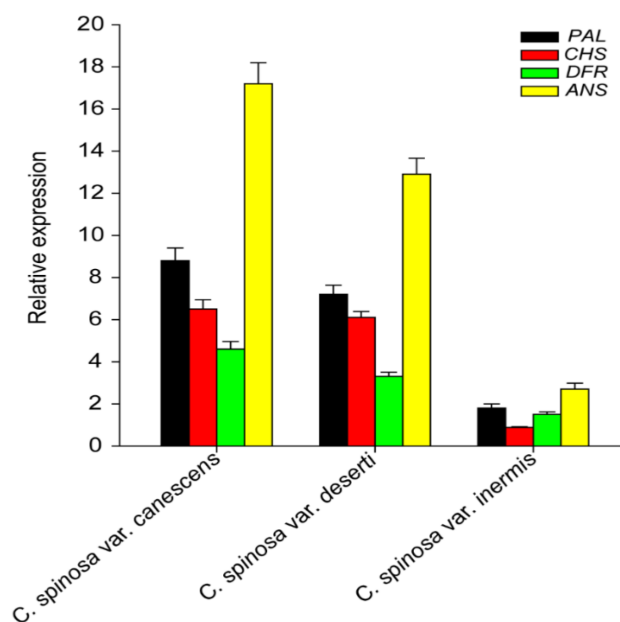

Fig. (9). Quantitative expression analysis of the structural genes of phenylpropanoid pathway /flavonoid pathway in fruit of *Capparis spinosa* varieties at ripening stage. *PAL*, Phenylalanine amonia lyase; *CHS*, Chalcone synthase; *DFR*, Dihydroflavonol 4-reductase; *ANS*, Anthocyanidine synthase. The data shown are the mean ± SE (n = 3).

DISCUSSION

The development of future breeding strategies and improved therapeutic utilization of the genus *Capparis* depend heavily on an in-depth knowledge of the genetic and phytochemical heterogeneity in this species. In the current study, three *C. spinosa* varieties including were genetically identified using two marker approaches, ISSR and SCoT, together with an examination of four phytochemical characteristics, including the UV spectroscopic profile, phenolic and flavonoid content, along with expression profiling of phenylpropanoid biosynthetic genes. This is the first publication which employs phytochemical profiling with ISSR and SCoT markers to further the taxonomic categorization of the genus *Capparis*.

Establishing an accurate chemo-taxonomic categorization of the reviewed varieties can be aided by integrating those results of ISSR, SCoT, total phenolic content (TPC), and UV spectroscopic examinations with earlier findings on the genus *Capparis*. *C. spinosa* L. var. *inermis* established a separate cluster as a monotypic variety, as shown by the integrated ISSR and SCoT-based dendrogram, which was validated further by the UV-derived HCA dendrogram. This is consistent with other studies on DNA barcoding based on study of the *rbcL* and *matK* genes (Mohamed, 2016 and Pegiou et al., 2023).

A relationship may be deduced between *C. spinosa* var. *inermis* and the remaining varieties depending on their shared sub-clustering in the merged ISSR and SCoT-based dendrogram and their clustering at the negative side of PC1 (which correspond to 95% of differences between varieties) of the PCA score plot. This supports the conclusions reached by Elzayat et al. (2020) when they utilized SRAP and ISTR analysis in conjunction with morphological description to classify the species of *Capparis* at the genetic level (Kamel et al., 2009 and Elzayat et al., 2020). A close relationship between *C. spinosa* var. *canescens* and *deserti* was also demonstrated by the fact that they shared a subcluster in the integrated ISSR and SCoT based dendrogram, which reflected their genetic similarity, and that they grouped at the positive side of PC1 of the PCA score plot, which expressed their chemical resemblance. This could be seen in the UV-derived HCA, where both varieties were found together in the second cluster's subcluster. Several previous studies have agreed with the same results as the current study, depending on different genetic techniques, for example, DNA and various molecular markers (Shinwari et al., 2014 and Elzayat et al., 2020).

The total phenolic content (TPC), which indicated that *C. spinosa* var. *canescens* and *C. spinosa* var. *deserti* had the greatest TPC and *C. spinosa* var. *inermis* had the lowest TPC, supported this conclusion. The harmony between molecular and phytochemical markers for these three varieties supports further thorough chemical identification. The majority of naturally occurring phenolic chemicals, known as flavonoids, may be found in diverse plant

sections both in their free form and as glycosides (Sulaiman and Balachandran, 2012). According to Deng and Lu (2017), flavonoids are essential for plant defense, floral pigments, survival, and structural support. Additionally, flavonoids have been found to have anti-inflammatory, anti-osteoporosis, and anticancer properties (Pandey et al., 2015; García-Pérez et al. 2018 and 2019). The edible medicinal plant *C. spinosa* has been recognized as one of the best genetic sources of flavonoids (Abo El-Fadl et al., 2021 and Ewas et al., 2022). Results of the current study exhibited a marked increase of both total and individual flavonoid contents in the leaves of *C. spinosa* var. *canescens* and *C. spinosa* var. *deserti*. Conversely, considerable decrease of total and individual flavonoid contents was recorded in the leaves of *C. spinosa* var. *inermis.* These results could be explained by the fact that the alteration in phenolic compounds content is associated with an increase or decrease in the synthesis of flavonoid compounds, which are phenolic derivatives (Ewas et al., 2022). The results of qRT-PCR are in line with the results of the phytochemical analyses. Expression level of *PAL*, *CHS*, *DFR*, and *ANS* genes was elevated significantly in *C. spinosa* var. *canescens* fruits, followed by those of *C. spinosa* var. *deserti* fruits. In contrast, the transcription level of *PAL*, *CHS*, *DFR*, and *ANS* genes was dramatically reduced in *C. spinosa* var. *inermis* fruits. These findings support the accuracy of the findings on the total phenol content presented above. At molecular level, the elevation of phenylpropanoids resulted in significant increase of flavonoid compounds accumulation (Baldwin et al., 2011; Pandey et al., 2015; Shoeva et al., 2016 and Ewas et al., 2022). Even though the data have led to a broad perspective of taxa inside the general orbit of *C. spinosa*. This study disputed Zohary and Tackholm's approach, in which *C. spinosa* taxa were considered as three varieties (*canescens, deserti, inermis*), and only recognized two varieties, namely *C. spinosa* var. *canescens* and var. *deserti*. Based on the present genetic and chemical investigation, it is suggested to raise var. *inermis* to the species level, namely *Capparis orientalis*, as indicated by Täckholm (1974), Kamel et al. (2009), and Inocencio et al. (2005) and contradicted by Boulos (1999). The genetic and phytochemical diversity discovered in *C. spinosa* varieties could enable us to evaluate the systematics and taxonomy of the various genotypes and may serve as a starting point when selecting the best varieties to employ in various phytotherapeutic applications and, in addition, for breeding programa.

CONCLUSION

The genus *Capparis* is regarded as a significant source for various secondary metabolite categories with a broad spectrum of pharmacological effects. *Capparis*, however, had previously been associated with frequent taxonomic changes. To improve the therapeutic use of this genus, a trustworthy evaluation technique of varieties diversity and closeness must be

established. In the present research, 10 primer pairs—5 for each approach—were used to genetically classify three endemic *Capparis* varieties grown in Egypt employing ISSR and SCoT markers. Selected varieties underwent thorough phylogenetic analyses, which were then translated into phylogeny-based classifications. Phytochemical features based on the total phenolic content and UV spectroscopic pattern were assessed against the results of the molecular evaluation. Incorporating the findings with unassisted chemometric approaches like PCA and HCA, agreement between the two approaches was found, indicating that chemotaxonomic results could indicate phylogenetic relationships within the tested *Capparis* varieties. The selection of relevant varieties for prospective preservation and breeding programs can be aided via this study. In terms of phylogenetic identification within the Capparaceae family, prospective approaches could be employed to develop and enrich taxonomic categorization, such as increasing varieties-level taxon sampling, collecting more accurate morphological datasets within the family, and integrating with molecular information sets.

HPLC coupled to LC/MS was used to create a metabolomic profile suitable for determining chemical differences and similarities within varieties for this research's complete chemotaxonomic analysis. A wider phytochemical profile was provided, covering other significant groups of secondary metabolites in the genus *Capparis*, such as flavonoid species, which are not easy to evaluate via UV spectroscopic assessment. Even though the data have led to a broad perspective of taxa inside the general orbit of *C. spinosa*. This study disputed Zohary and Täckholm's approach, in which *C. spinosa* taxa were considered as three varieties (*canescens, deserti, inermis*), and only recognized two varieties, namely *C. spinosa* var. *canescens* and var. *deserti*. Based on the present genetic and chemical investigation, it is suggested to raise var. *inermis* to the species level, namely *Capparis orientalis*, as indicated by Täckholm (1974), Kamel et al. (2009), and Inocencio et al. (2005) and contradicted by Boulos (1999). It is advisable to combine multiple DNA or RNA-based approaches for more precise and trustworthy findings. "Next generation sequencing" (NGS) can be employed in conjunction with standard DNA fingerprinting or RNA sequencing approaches. That is a promising technique which may swiftly and economically sequence complete genomes. NGS techniques are now capable of addressing even the huge plant nuclear genomes.

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مراجعة منهجية لأصناف القبار *Capparis spinosa* **L.** (*canescens*, *deserti*, *inermis*) المتوطنة ضمن الفلورا المصرية بناء على التصنيف الجزيئي والكيميائي

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القبار .*Capparis spinosa* L هي شجيرة نباتية صحراوية تتمتع بقدرة مذهلة على البقاء في الظروف البيئية الصعبة. كما أن الفوائد العلاجية والدوائية لهذا النوع من النباتات، وتطبيقات الطهي لها أهمية كبيرة. العديد من المكونات النشطة بيولوجيًا المكتشفة في العديد من أجزائه النباتية لها أهميتها الكيميائية النباتية الكبيرة، كما أن زراعتها لها فائدة تجارية محتملة كبيرة. علاوة على ذلك، فإن عدم التجانس الواسع للقبار جعل التقسيم التصنيفي أمرًا صعبًا، وأدت ندرة التحقيقات الجينية إلى حالة من عدم اليقين بين العديد من الباحثين. تلخص الدراسة الحالية مجموعة البيانات المتعلقة بـالقبار والتي تشمل التحقيقات التصنيفية والكيميائية والجزيئية. كما تم التعريف الوراثي لثلاثة أصناف مستوطنة مصرية من *Capparis spinosa*؛ وهي *canescens*، *deserti*، *inermis* باستخدام تقنيتين للواسمات الجزيئية بما في ذلك هما ISSR وSCoT. باستخدام خمس بادئات لكل تقنية وبإجمالي عشرة بادئات للتحليل. بشكل عام؛ لوحظ ٢٨ و ٤٠ نطاقًا باستخدام بادئات ISSR وSCoT، على التوالي. تراوحت مستويات التعدد المظهري بين ٦٤.٢٨ و ٦٧.٥٠٪ مع فحص ISSR وSCoT على التوالي. علاوة على ذلك، اقترن التقسيم التصنيفي الكيميائي الذي يستخدم التحليل الطيفي للأشعة فوق البنفسجية إلى جانب المحتوى الفينولي بأدوات القياس الكيميائي غير الخاضعة للرقابة PCA وHCA. من خلال التحقيقات الجزيئية والكيميائية النباتية، تم التحقق من العلاقات الداخلية المتخصصة بين كلا الصنفين *canescens* وdeserti. بالإضافة إلى ذلك، فقد تم تعريف الصنف *inermis* على أنه صنف فريد monotypic. كما تزامن محتوى الفلافونويدات مع هذا الاستنتاج، والذي تم إثباته من خلال التعبير المختلف عن جينات التخليق الحيوي للفينيل بروبانويد. يقترح هذا البحث رفع الصنف *inermis* إلى مستوى النوع تحت الاسم العلمي الجديد *Capparis orientalis*، مما يدل على أن معلومات التحليل الكيميائي يمكن أن تعكس الروابط التطورية بين أصناف القبار ويلقي الضوء على الحاجة إلى الجمع بين مزايا التصنيف الجزيئي والكيميائي لتوضيح الروابط التطورية داخل هذا النوع.