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# Identification and Assessment of Therapeutic Phytoconstituents of *Catharanthus roseus* through GC-MS Analysis

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**Abstract:** The leaves of *Catharanthus roseus* (L.) G. Don contain a large number of diverse secondary metabolites, making them comparably complex. The *Catharanthus* genus has received increased interest from scientists in recent years due to its extensive applications in several domains, including the pharmaceutical sector, where precise characterization of its characteristics is required. An effective inquiry technique is needed for chemo-profiling to identify the metabolites in plant samples. The main goal of this research is to provide supplementary data on the chemical composition of the leaves of twenty-five different accessions of *C. roseus* through the application of gas chromatography-mass spectrometry (GC-MS). The study's findings reveal the existence of a vast number of phytochemicals, allowing for a comparison of the different accessions. Furthermore, a meticulous statistical analysis of this data using principal components analysis (PCA) and a heatmap, and hierarchical cluster analysis (HCA) may aid in providing more relevant information on *C. roseus* leaves for possible investigation of their metabolites in further scientific studies.

**Keywords:** *Catharanthus roseus*; gas chromatography-mass spectrophotometry; phytochemicals; principal component analysis; hierarchical cluster analysis (HCA)



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## 1. Introduction

Medicinal plants possess various secondary bioactive metabolites that help plants produce countless valuable drugs for the cure and prevention of animal and human diseases in different cultures [1]. Plant-based bioactive phytochemicals, either alone, in combination with other compounds, or synergistically with other compounds, are used to cure a variety of maladies and support immune responses [2,3]. In the pharmaceutical industry, phytochemicals are indispensable for the preparation of new drugs and therapeutic agents [4]. Identifying vigorous and dynamic ingredients from natural resources is the initial step in new drug discovery. Screening plant extracts is an innovative approach for determining remedially effective constituents in various plant species [1,5].

Alkaloids, flavonoids, phenols, terpenoids, quercetin, and saponins have anti-ulcer, anti-inflammatory, anticancer, antimicrobial, and antidiarrheal effects that can significantly improve overall health. They also act as antioxidants and help to protect the body from free radical damage [6]. Researchers' motivations are to investigate the pharmacological properties of herbs and assertions that they are effective as conventional herbal remedies. Scientific research is currently being conducted on the potential therapeutic value of biologically active medicinal plants and their components. It is likely that in the future, safe and effective medications to treat a variety of deteriorating conditions will be developed from medicinal plants [7].

*Catharanthus roseus* (L.) G. Don., an important medicinal plant with wide distribution in Madagascar and tropical Africa, is a member of the Apocynaceae family. *C. roseus* is a dicotyledonous angiosperm that produces two major anticancer indole alkaloids: vinblastine and vincristine [8,9]. There are eight varieties in the genus *Catharanthus*, and they are notable for several bioactive substances such as phytols, phenolic acids, flavonoids, etc. [10]. Folklore supports the use of decoctions of *C. roseus* for the treatment of malaria, dengue fever, diabetes, diarrhoea, dysentery, dyspepsia, insect bites, skin infections, leukaemia, eye irritation, toothache, sore tongue, and lung congestion. The plant's root has calming, hypotensive, and tranquillizing effects. It is primarily used to manage diabetes in Ayurveda [11].

All parts of the *C. roseus* plant contain different bioactive phytoconstituents, including, phenols, flavonoids, aldehydes, fatty acids, ketones and indole alkaloids. Some of these compounds show distinct pharmaceutical properties. In addition, the active phytochemical concentration has been reported to be highest in plant parts during their flowering stage [5,8]. Therefore, in the present study we procured the leaves of 25 *C. roseus* accessions and chemically investigated their bioactive phytoconstituents. These molecules provide the plant with defence against herbivory as well as therapeutic benefits to animals. The commonly used techniques in phytochemistry include extraction, isolation, structural interpretation of natural products, and various chromatography techniques such as HPLC, MPLC, LC-MS, and GC-MS. GC-MS is a common analytical technique used to identify compounds in plant samples which involves separating volatile components and examining them in detail [12,13]. We used GC-MS technology to obtain a thorough picture of the chemical composition of the plants. The data were used to conduct a comparative analysis using sophisticated statistical analytic tools to further highlight qualitative and quantitative variations in the phytoconstituents' chemical profiles.

## 2. Materials and Methods

### *2.1. Sample Collection and Identification*

Fresh leaves of 25 different accessions of *C. roseus* were collected from various localities in the northern Indian states of Chandigarh, Delhi, Haryana, and Punjab (see Supplementary Figures S1 and S2) depending upon morphological variations. Among the accessions,14were collected from various districts of Haryana, viz., Rhotak, Bhiwani, Jind, Panchkula, Kaithal, Rewari and Hisar, 8 from different regions of Punjab, such as Samana, Patra, Rajpura, Patiala and Devigrah, 2 from Chandigarh and 1 from Delhi. The botanical identities of the plants were authenticated and verified by plant taxonomists and horticulturists based on their taxonomic characters using multiple sources of information, viz., Herbaria, Botanical Survey of India, eFlora of India, iNaturalist, PlantNet, and Vascular Flora of Punjab and Chandigarh [14,15]. Voucher specimens of the identified plant materials were collected and deposited in the Herbarium (PUN) of the Department of Botany, Punjabi University, Patiala, (Punjab), India. They can be identified using the voucher numbers provided in Table 1 and Figure 1.

**Table 1.** Data on latitude, longitude and altitude of various accessions of *Catharanthus roseus* (L.) G. Don collected from different locations.

| Sr. No. | Accession No. | PUN No. | District | Latitude | Longitude | Altitude (m) |
|---|---|---|---|---|---|---|
| 1 | Cr00PFRE | 62713 | Bhiwani, Haryana | 30.30° N | 74.60° E | 219 m |
| 2 | Cr00LPFLPE | 62714 | Patiala, Punjab | 30.36° N | 76.45° E | 250 m |
| 3 | Cr00DPF | 62715 | Narwana, Haryana | 29.60° N | 76.11° E | 232 m |
| 4 | Cr00WFYE | 62716 | Hissar, Haryana | 29.15° N | 75.72° E | 216 m |
| 5 | Cr00WFRE | 62717 | Jind, Haryana | 29.32° N | 76.30° E | 219 m |
| 6 | Cr00WFRE2 | 62718 | Rohtak, Haryana | 28.89° N | 76.59° E | 220 m |

**Table 1.** *Cont.*

| Sr. No. | Accession No. | PUN No. | District | Latitude | Longitude | Altitude (m) |
|---|---|---|---|---|---|---|
| 7 | Cr00WFSRE | 62719 | Delhi | 28.64° N | 77.09° E | 216 m |
| 8 | Cr00WFYE2 | 62720 | Patra, Punjab | 29.20° N | 76.19° E | 250 m |
| 9 | Cr00DP | 62721 | Rewari, Haryana | 28.20° N | 76.62° E | 244 m |
| 10 | Cr00LP | 62722 | Bhiwani, Haryana | 30.30° N | 74.60° E | 219 m |
| 11 | Cr00BPF | 62723 | Panchkula, Haryana | 30.69° N | 76.85° E | 217 m |
| 12 | Cr00SFP | 62724 | Bhiwani, Haryana | 30.30° N | 74.60° E | 219 m |
| 13 | Cr00CAF | 62725 | Chandigarh | 30.71° N | 76.78° E | 321 m |
| 14 | Cr00LPNF | 62726 | Kaithal, Haryana | 29.79° N | 76.41° E | 248 m |
| 15 | Cr00SBRF | 62727 | Devigarh, Punjab | 24.77° N | 73.74° E | 251 m |
| 16 | Cr00PLMF | 62728 | Patiala, Punjab | 30.36° N | 76.45° E | 250 m |
| 17 | Cr00CHEF | 62729 | Samana, Punjab | 30.15° N | 76.19° E | 250 m |
| 18 | Cr00SNFF | 62730 | Bhiwani, Haryana | 30.30° N | 74.60° E | 219 m |
| 19 | Cr00CLF | 62731 | Chandigarh | 30.71° N | 76.78° E | 321 m |
| 20 | Cr00SAF | 62732 | Hansi, Haryana | 29.10° N | 75.96° E | 219 m |
| 21 | Cr00TDRF | 62733 | Rajpura, Punjab | 30.47° N | 76.59° E | 250 m |
| 22 | Cr00DRYE | 62734 | Jind, Haryana | 29.32° N | 76.30° E | 219 m |
| 23 | Cr00FRFF | 62735 | Tosham, Haryana | 28.87° N | 75.89° E | 219 m |
| 24 | Cr00PFWE | 62736 | Patiala, Punjab | 30.36° N | 76.45° E | 250 m |
| 25 | Cr00PFYE | 62737 | Patiala, Punjab | 30.36° N | 76.45° E | 250 m |

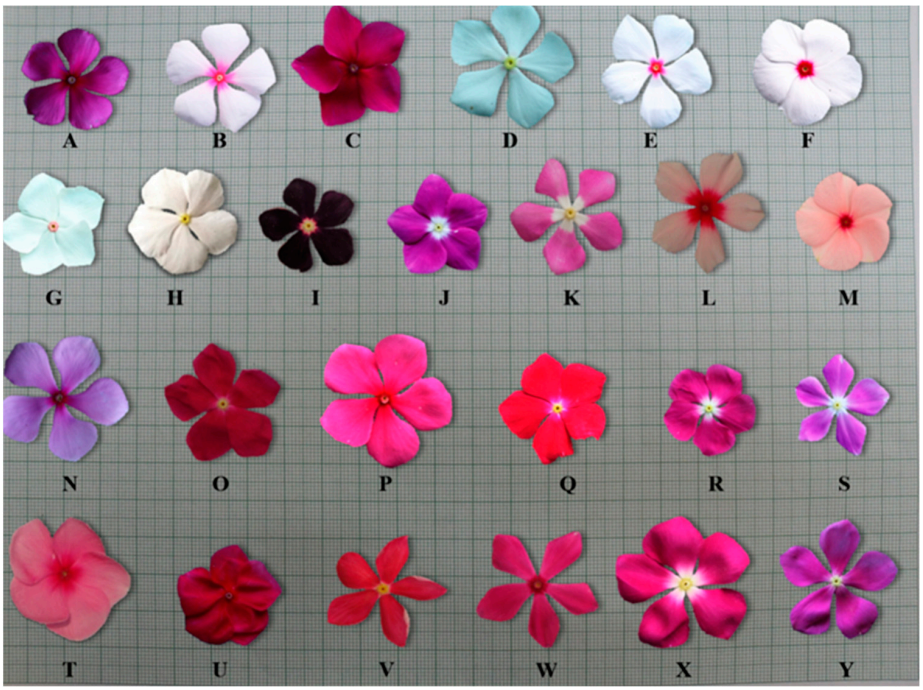


**Figure 1.** Flower characteristics (upper view) of 25 different accessions of *C. roseus*. (**A**) Cr00PFRE, (**B**) Cr00LPFLPE, (**C**) Cr00DPF, (**D**) Cr00WFYE, (**E**) Cr00WFRE, (**F**) Cr00WFRE2, (**G**) Cr00WFSRE, (**H**) Cr00WFYE2, (**I**) Cr00DP, (**J**) Cr00LP, (**K**) Cr00BPF, (**L**) Cr00SFP, (**M**) Cr00CAF, (**N**) Cr00LPNF, (**O**) Cr00SBRF, (**P**) Cr00PLMF, (**Q**) Cr00CHEF, (**R**) Cr00SNFF, (**S**) Cr00CLF, (**T**) Cr00SAF, (**U**) Cr00TDRF, (**V**) Cr00 DRYE, (**W**) Cr00FRFF, (**X**) Cr00PFWE, and (**Y**) Cr00PFYE.

### 2.2. Preparation of Extract from Plant Leaves

The freshly gathered *C. roseus* leaves were cleaned in tap water before being rinsed with distilled water. The leaves were then allowed to air dry at room temperature for 6–7 days before being crushed into a fine powder and stored in an airtight bottle for further analysis. The procedure provided by Kupchan and Tsou [16] and the updated method of Wagenen et al. [17] were used to perform the solvent extraction with methanol [18]. The extract was prepared by macerating 1300 g of powder in 80% methanol for 2 days at room temperature. The filtrate was passed through Whatman No. 1 filter paper to remove any impurities or solids. It was then transferred to a rotary evaporator, which removed the solvent via evaporation. For fractionation, n-hexane, ethyl acetate, and 20% methanol ($v/v$) were utilized. To create a stock solution, 20 g of crude extract was dissolved in 250 mL of 80% aqueous methanol. The solution was combined with 250 mL of n-hexane in a separate funnel. The mixture was allowed to stand for 20 min for proper separation, and the upper part was collected in a beaker. The aqueous methanol part was washed repeatedly with n-hexane and different n-hexane fractions were collected. The above procedure was repeated using ethyl acetate. At the end, ethylacetate fractions were collected and concentrated [19]. The aqueous methanol fraction was used for further studies after subjecting the different fractions to a preliminary study.

### 2.3. GC-MS Analysis

The Agilent 19091-433HPGC-MS system was used to analyze the samples. The column was an HP-5MS fused silica column with 5% phenyl methyl siloxane content, a size of 30.0 m × 250 µm and a film thickness of 0.25 µm. Helium gas at a constant flow rate of 1 mL/min was used as the carrier gas. The sample was then thermally desorbed, and the obtained ionized molecules were separated and quantified using the system. Several additional parameters were set as follows: interface temperature, 280 °C; pressure, 16.2 psi; ion source temperature, 250 °C; out time, 1.8 mm; and a 1 µL injector with a split ratio of 10:1 at 250 °C injector temperature. The split ratio was chosen to ensure accurate sample analysis while preserving sample integrity. A high temperature was maintained to sufficiently vaporize the sample compounds but was not so high that it resulted in sample degradation. The 1 µL sample size was chosen to ensure peak shape while reducing the total sample size and providing the most accurate data. The initial temperature of the column was 36 °C, which was maintained for 5 min. After that, the column temperature was raised at a rate of 4 °C/min to reach 250 °C. The overall elution time was 76 min [20]. The separated compounds were eluted from the column and examined by a detector to generate a signal. The interval from the time of the initial injection until the elution occurs is known as the retention time (RT). The graph that a computer creates after the instrument has run is known as a chromatogram. Each of the peaks in the chromatogram represents the signal created when a compound elutes from the gas chromatography column into the detector. To quantify the component in the sample, the y-axis indicates the signal intensity, while the x-axis represents the retention time. Compounds that were separately eluted are placed in an electron ionization detector, where they are bombarded by electrons, causing them to fragment into smaller pieces. These smaller pieces are charged ions with a certain mass [21]. The obtained graph, also referred to as the mass spectrum graph, is used to calibrate the mass and charge ratio. The relative percentage amount of each phytocomponent was calculated by comparing its average peak area to the total area.

### 2.4. Identification of Compounds

The NIST database provides accurate mass spectra and retention values for a wide range of compounds. This data can be used to identify components, such as organic acids and proteins, by their unique retention values. This allows scientists to quickly and accurately detect trace components in a sample and interpret mass spectra, and over 62,000 well-known compounds have been included in the NISTII archive. The NIST library was used to compare the mass spectra of the unidentified components of the *C. roseus*

fraction to their known counterparts. The analysis yielded results in the form of similarity scores, which helped in identifying unknown molecules [22].

### *2.5. Principle Component Analysis (PCA)*

A total of 29 metabolites were found in the various accessions of *C. roseus* based on the GC-MS analysis. A data set consisting of a total of 29 different phytocompounds was considered in this study. There are several multivariate exploratory methods, but we utilized principal component analysis (PCA)and hierarchical cluster analysis (HCA) with the help of XLSTAT statistical software (version 2016; Addinsoft; New York, NY, USA). Principle component analysis (PCA) was used to group the accessions by analyzing the major contributory factors and summarize the correlation and variation between the 25 *C. roseus* accessions based on the identified biochemical components. Principal components were computed using a correlation matrix to examine the percentage contribution of each phytoconstituent. Additionally, a heatmap was plotted to visualize the variations in potential markers to separate samples with different processing times into different groups.

## 3. Results

### *3.1. Gas Chromatography-Mass Spectrophotometry (GC-MS) Analysis*

The results of the GC-MS analysis revealed that different accessions of *C. roseus* had various bioactive compounds. The findings of the aqueous methanolic extract are presented in Table 2 and Supplementary Figure S3. A total of 29 different bioactive components were found in all studied accessions. With 22 identified components, the Cr00DP accession contained the highest number of components (Figure 2), followed by the Cr00PFRF accession, which contained 21 components (Figure 3). Three accessions (Cr00CAF, Cr00SAF, and Cr00LPNF) contained only five components, which was the lowest number. The first compound identified at a retention time of 5.75 min was 4H-pyran-4-one, whereas phthalic acid, di(oct-3-yl) ester, was the last with the maximum retention time (22.97 min). There was extensive variation in the compositions of phytochemicals in the 25 *C. roseus* accessions, as shown in Supplementary Table S1. The recognized phytocompounds in 25 accessions were divided into various groups and are illustrated in Figure 4. The 29identified compounds were categorized as fatty acids (5), ketones (2), alkaloids (3), terpenoids (2), sugar moieties (7), and one heterocyclic compound, phenolic compound, acetate, aldehyde, polyhdroxyl compound, nitrogen compound, quinic acid, and plasticizer compound, as shown in Table 3.

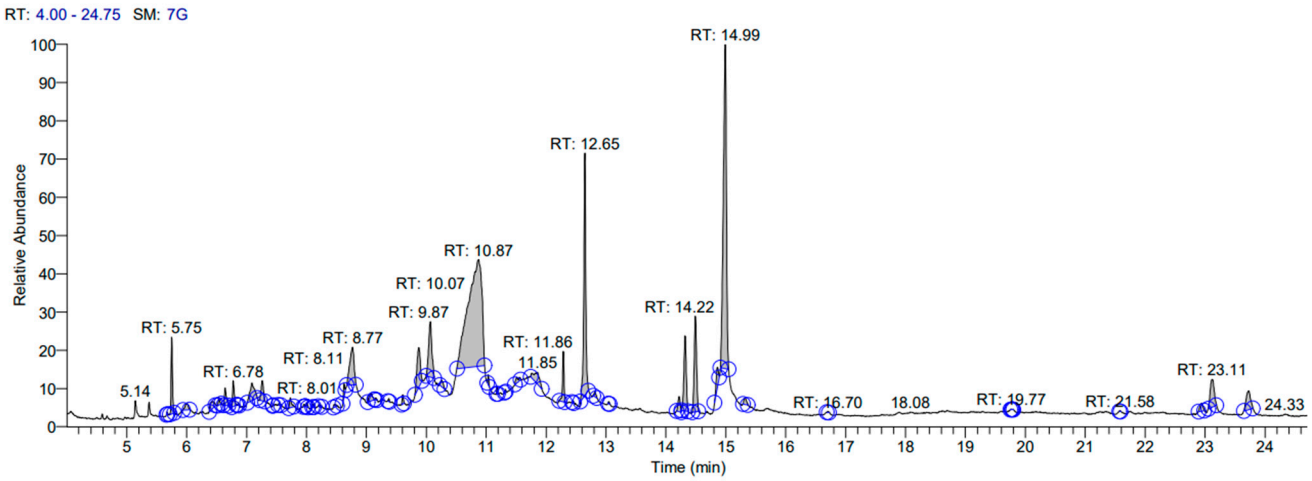


**Figure 2.** GC–MS chromatogram of methanolic leaf extracts of Cr00DP accession.

**Table 2.** Results of the GC–MS analysis from the leaves of *Catharanthus roseus*.

| Sr. No. | RT | Name of the Compound | Molecular Formula | MW | Peak Area (%) | Pharmacological Actions | Relative % Amount of Compounds |
|---|---|---|---|---|---|---|---|
| 1 | 5.75 | 4H-Pyran-4-one | $C_6H_8O_4$ | 144 | 0.63 | Antioxidant, Antimicrobial, Anti-inflammatory [23] | 0.90 |
| 2 | 6.39 | Catechol/Resorcinol | $C_6H_6O_2$ | 110.1 | 0.06 | Dermatological/Acne treatment [24] | 0.08 |
| 3 | 6.53 | Benzofuran, 2,3-dihydro | $C_8H_8O$ | 120.1 | 0.61 | Antioxidant, Analgesic, Antimutagenic [25] | 0.87 |
| 4 | 6.67 | 5Hydroxymethylfurfural | $C_3H_6O_3$ | 126 | 30.86 | Antioxidant, Antiproliferative activity [26] | 1.2 |
| 5 | 6.77 | 1,2,3-Propanetriol, 1-acetate/acetin | $C_5H_{10}O_4$ | 134.1 | 0.84 | Antimicrobial [27] | 0.01 |
| 6 | 7.03 | L-Glucose | $C_6H_{12}O_6$ | 180.1 | 1.17 | Sweetening agents [28] | 0.20 |
| 7 | 7.44 | Ascaridole epoxide | $C_{10}H_{16}O_3$ | 184 | 0.14 | Anticarcinogenic [29] | 0.0 |
| 8 | 8.01 | Deoxyspergualin | $C_{17}H_{37}N_7O_3$ | 387.5 | 0.11 | Treatment of autoimmune disease [30] | 0.24 |
| 9 | 8.73 | Sucrose | $C_{12}H_{22}O_{11}$ | 342.2 | 3.14 | Hypercholesterolemic, Preservative [31] | 4.52 |
| 10 | 8.77 | D-fructose | $C_6H_{12}O_6$ | 180.156 | 3.36 | Hypercholesterolemic, Preservative [32] | 0.093 |
| 11 | 9.02 | D-allose | $C_6H_{12}O_6$ | 180.156 | 0.86 | Antioxidant [33] | 0.24 |
| 12 | 9.07 | Desulphosinigrin | $C_{10}H_{17}NO_6S$ | 279.3 | 0.06 | Antioxidant [34] | 0.07 |
| 13 | 9.38 | 3′,5′-Dimethoxyacetophenone | $C_{10}H_{12}O_3$ | 180.2 | 0.17 | Antioxidant [35] | 7.37 |
| 14 | 9.83 | α-D-Glucopyranoside, O-α-Dglucopyranosyl-(1.fwdarw.3)-ß-d-fru | $C_{18}H_{32}O_{16}$ | 504 | 0.06 | Antidiabetic and antitumour activity [31] | 0.07 |
| 15 | 10.05 | 1,2,3,5-Cyclohexanetetrol+ | $C_6H_{12}O_4$ | 148.1 | 5.12 | Poly-Hydroxy compound, Analgesic, Anesthetic, Antioxidant, Antiseptic, Antibacterial, Antiviral, Cancer preventive [36] | 9.24 |
| 16 | 10.07 | Quinic acid | $C_7H_{12}O_6$ | 192.167 | 3.73 | Antioxidant [37] | 43.88 |
| 17 | 10.94 | Myo-Inositol, 4-C-methyl- | $C_7H_{14}O_6$ | 194.1 | 30.45 | Antioxidant and Antimicrobial [33] | 3.43 |
| 18 | 11.90 | Muco-Inositol | $C_6H_{12}O_6$ | 180.1 | 2.38 | Chemopreventive [33] | 1.40 |
| 19 | 12.29 | Hexadecanoic acid, methyl ester | $C_{17}H_{34}O_2$ | 270.4 | 0.97 | Palmitic acid methyl ester Antioxidant, Hypocholesterolemic, Nematicide, Pesticide, Antiandrogenic flavor, Hemolytic,5-Alpha reductase inhibitor [38] | 15.00 |
| 20 | 12.64 | Pentadecanoic acid | $C_{15}H_{30}O_2$ | 242.4 | 10.41 | Antimicrobial, Antioxidant, Anticancer | 3.47 |
| 21 | 13.38 | Phytol | $C_{20}H_{40}O$ | 296 | 17.17 | Hypocholesterolemic, Antimicrobial, Anticancer, Diuretic, Anti-inflammatory [39] | 3.89 |
| 22 | 14.32 | 9,12,15-Octadecatrienoic acid, methyl ester | $C_{19}H_{32}O_2$ | 292.2 | 2.70 | Anticancer, Nematicides, Antocoronary [38] | 1.75 |
| 23 | 16.08 | 2,20-Cycloaspidospermidine-3-carboxylic acid, Vindolinine | $C_{21}H_{24}N_2O_2$ | 336 | 4.95 | Anticancer [38] | 0.05 |
| 24 | 16.73 | 9,12,15-Octadecatrienoic acid, | $C_{27}H_{52}O_4Si_2$ | 496.2 | 0.04 | Anti-inflammation [38] | 0.24 |
| 25 | 19.77 | Octadecane, 3-ethyl-5-(2-ethylbutyl)- | $C_{26}H_{54}$ | 366 | 0.01 | Antimicrobial and antifungal [40,41] | 0.41 |
| 26 | 20.57 | Aspidospermidine-3-carboxylic acidvindoline | $C_{22}H_{28}N_2O_5$ | 400 | 12.09 | Alkaloids [41] Anticancer | 0.15 |
| 27 | 22.69 | Condyfolan, 14,19-didehydro-12-methoxy-, (14E)- | $C_{19}H_{24}N_2O$ | 322.4 | 0.29 | Antimicrobial [42] | 0.78 |
| 28 | 22.76 | Aspidospermidine-3-carboxylic acid, | $C_{22}H_{28}N_8O_5$ | 400.4 | 0.11 | Anticancer [43] | 0.19 |
| 29 | 22.97 | Phthalic acid, di(oct-3-yl) ester | $C_{24}H_{38}O_4$ | 390.5 | 0.54 | Antimicrobial and Antifouling [44] | 0.05 |

Abbreviations: **RT** = retention time, **MW** = molecular weight.

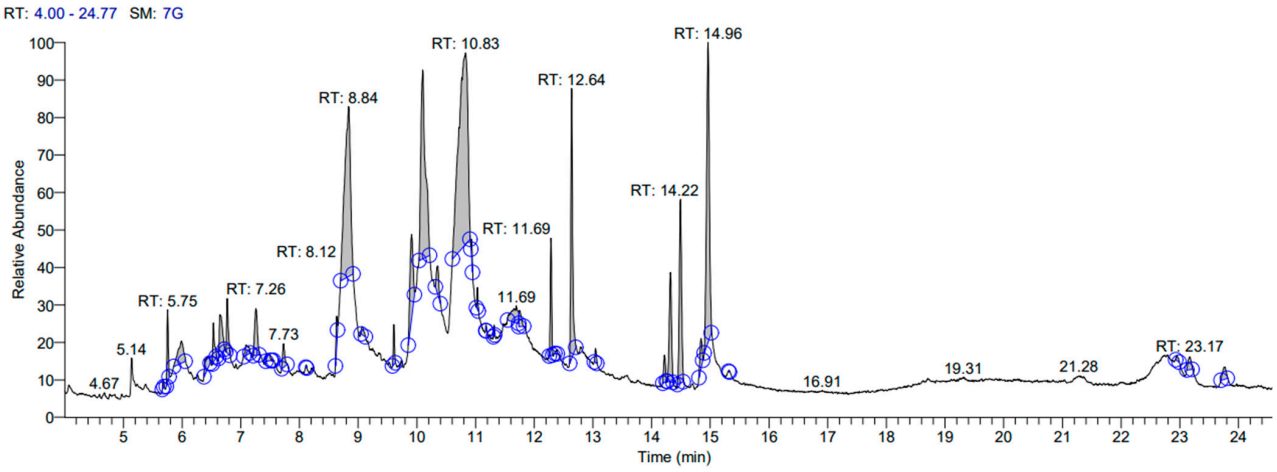


**Figure 3.** GC–MS chromatogram of methanolic leaf extracts of Cr00PFRE accession.

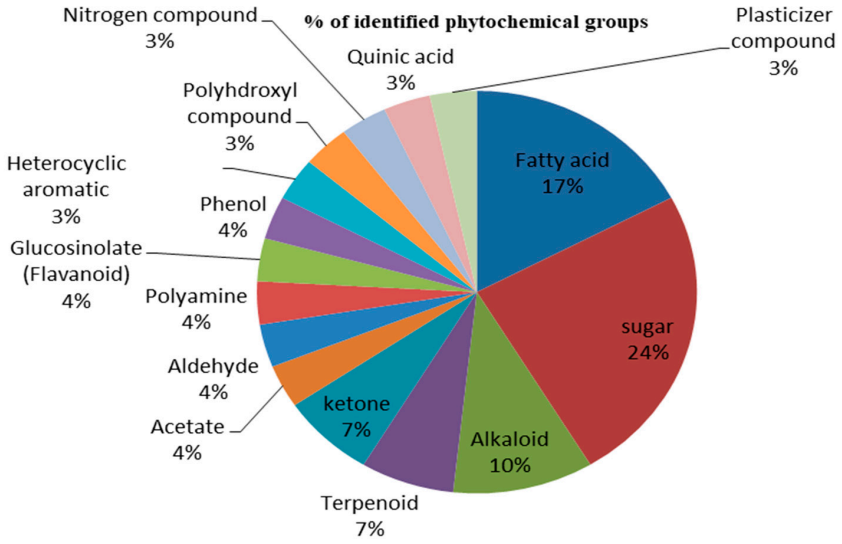


**Figure 4.** The percentage content of phytochemical groups identified in 25 accessions of *C. roseus*.

**Table 3.** List of phytocompounds and their identified nature in the methanolic extract of *C. roseus* by GC–MS.

| Sr. No. | Name of Compounds | Nature of Compounds |
|---|---|---|
| 1. | Hexadecanoic acid, methyl ester | Fatty acid |
| 2. | Pentadecanoic acid | |
| 3. | 9,12,15-Octadecatrienoic acid, | |
| 4. | Octadecane, 3-ethyl-5-(2-ethylbutyl)- | |
| 5. | 9,12,15-Octadecatrienoic acid, methyl ester | |
| 6. | L-Glucose | Sugar |
| 7. | Sucrose; D-Glucose-6-O (sugar), α-D-galactopyranosyl | |
| 8. | D-fructose | |
| 9. | D-allose | |
| 10. | Muco-Inositol | |
| 11. | α-D-Glucopyranoside, O-α-Dglucopyranosyl-(1.fwdarw.3)-ß-d-fru | |
| 12. | Myo-Inositol, 4-C-methyl- | |

**Table 3.** *Cont.*

| Sr. No. | Name of Compounds | Nature of Compounds |
|---|---|---|
| 13. | 2,20-Cycloaspidospermidine-3-carboxylic acidVindolinine | Alkaloid |
| 14. | Aspidospermidine-3-carboxylic acid, | |
| 15. | Aspidospermidine-3-carboxylic acidvindoline | |
| 16. | Ascaridole epoxide | Monoterpenoid |
| 17. | Phytol | Terpenoid (diterpenoid) |
| 18. | 3′,5′-Dimethoxyacetophenone | Ketone |
| 19. | 4H-Pyran-4-one/Maltol | Ketone |
| 20. | 1,2,3-Propanetriol, 1-acetate/acetin | Acetate |
| 21. | 5Hydroxymethylfurfural | Aldehyde |
| 22. | Deoxyspergualin | Polyamine |
| 23. | Desulphosinigrin | Glucosinolate (Flavanoid) |
| 24. | Catechol/Resorcinol | Phenol |
| 25. | Benzofuran, 2,3-dihydro | Heterocyclic aromatic |
| 26. | 1,2,3,5-Cyclohexanetetrol+ | Polyhdroxyl compound |
| 27. | Condyfolan, 14,19-didehydro-12-methoxy-, (14E)- | Nitrogen compound |
| 28. | (1R,3R,4R,5R)-(-)-Quinic acid | Quinic acid |
| 29. | Phthalic acid, di(oct-3-yl) ester | Plasticizer compound |

Sucrose, octadecadienoic acid (Z,Z)-,methyl ester, myo-inositol, phytol, 4H-pyran-4-one, and hexadecanoic acid were found in all studied accessions with a high percentage of peak area. The peak area percentage of sucrose ranged from 9.57 to 13.36 in different accessions. The peak area percentage of myo-inositol ranged from 5.46 to 44.16. Hexadecanoic acid ranged from 5.71 to 11.18. The peak area percentage of 9,12-octadecadienoic acid (Z,Z)-, methyl ester ranged from 5.39 to 33.66%. The relative percentages of some components such as quinic acid (43.88%), hexadecanoic acid, methyl ester (15.00%), 1,2,3,5-cyclohexanetetrol (9.24%), 3′-5′-dimethoxyacetophenone (7.37%), phytol (3.89%), and pentadecanoic acid (3.47%) were found to be at a maximum for the accession Cr00DP. Several compounds observed in the extracts of *C. roseus*, including 1,2,3-propanetriol, 1-acetate/acetin, 4Hpyran-4-one, benzofuran, 2,3-dihydro, vindolinine and vindoline, have been reported to have good antineoplastic, antioxidant, and antimicrobial activities.

### *3.2. Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA) of GC–MS*

To differentiate the accessions, hierarchical cluster analysis was applied to the chromatographic data obtained from all samples (Figure 5). The similarity between accessions was closely related to the relative proportions of the 29 different metabolites. In the compound dendrogram, phytol was found at the maximum amount (in all the studied accessions), followed by 9,12,15-octadecatrienoic acid, pentadecanoic acid, vindoline, and myoinositol. Catechol was present in the lowest amounts in the accessions, followed by 5-hydroxymethylfurfural and desulphosinigrin. Other components, including quinic acid, vindolinine, hexadecanoic acid, and phthalic acid, etc., were found to be present in moderate amounts in the accessions. In the accessions dendrogram, 25 accessions were divided into 2 clusters at a level of 10% similarity by heat map hierarchical clustering. Cluster I comprised six accessions (Cr00WFRE, Cr00WFYE, Cr00WFRE2, Cr00LPNF, Cr00CAF, and Cr00SAF), out of which Cr00WFRE was demarcated from the others at a level of 50% similarity. Cluster II comprised 19 accessions, out of which Cr00DPF was demarcated at a 60% similarity level from other accessions.

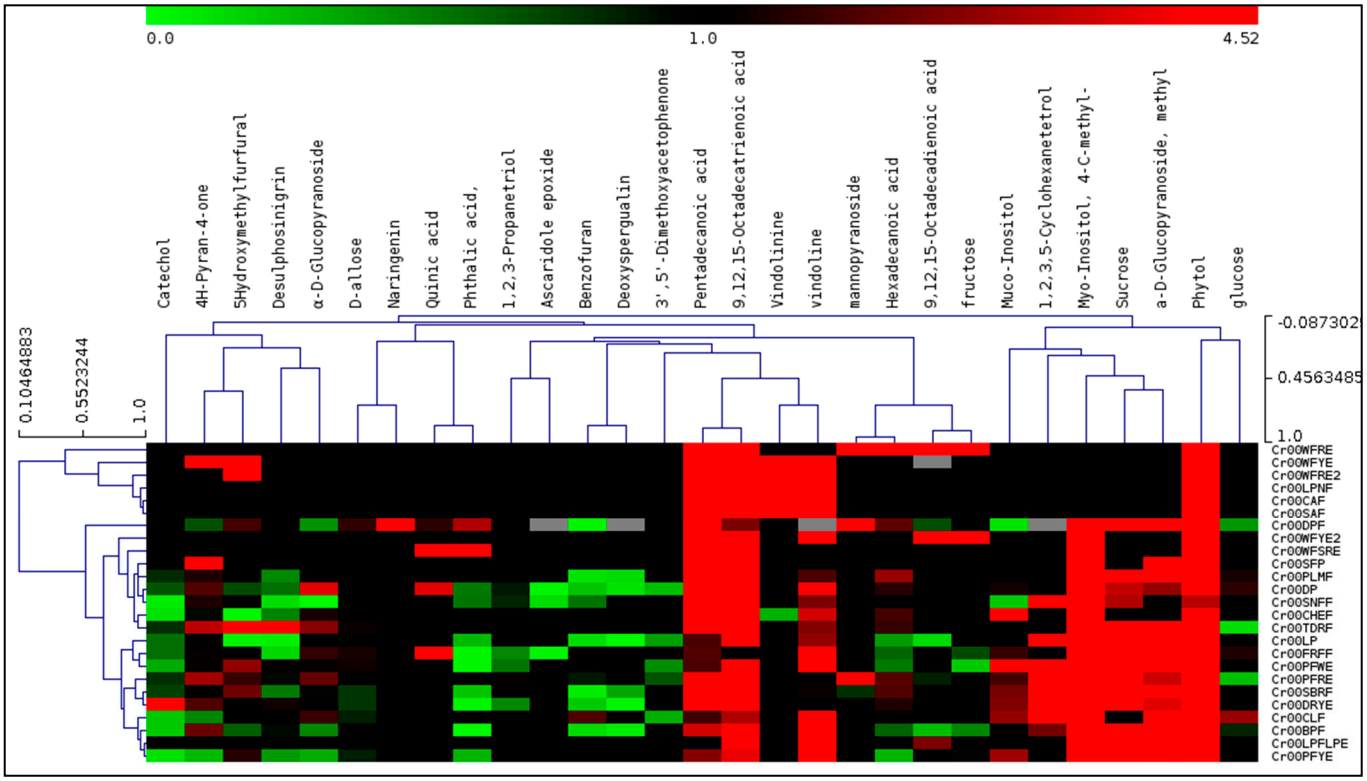


**Figure 5.** Heat map of secondary metabolites (columns) identified by GC–MS analyses in 25 different accessions of *Catharanthus roseus* (rows). The dendrograms were created using correlation-based distances and hierarchical clustering using the Ward linkage method ($p < 0.05$). Colours represent the relative abundance in the samples from minimum (green) to maximum (red).

To differentiate the collected accessions, PCA was also performed using the peak area percentage of 29 metabolites for the calculation of the eigenvalue, variability percentage and cumulative percentage (Table 4) for the grouping of the 25 accessions of *C. roseus*, based on which, a distance biplot was calculated. To display the points, two principal components, PC1 and PC2 were chosen to represent the information.

**Table 4.** The principal components of GC–MS showing the Eigenvalues, variability percentage and cumulative variance percentage.

| | F1 | F2 | F3 | F4 | F5 |
|---|---|---|---|---|---|
| Eigenvalue | 6.250 | 3.533 | 2.887 | 2.342 | 2.077 |
| Variability (%) | 21.550 | 12.181 | 9.955 | 8.076 | 7.162 |
| Cumulative (%) | 21.550 | 33.732 | 43.687 | 51.762 | 58.924 |

Figures 6 and 7 show the score plots of PC1 and PC2 and also depict the relationship between metabolites and accessions. PC1 explained 21.55% of the total variance and discriminated the accessions based on the concentration of 9,12,15-octadecatrienoic acid methyl ester, phthalic acid, quinic acid, and phytol. Accessions Cr00SAF, Cr00CAF, Cr00LPNF and Cr00WFSRE showed variability for these components. The PC2 explained a further 12.18% of the variance and discriminated the accessions based on fructose, D-mannopyranoside, hexadecanoic acid, and 9,12,15-octadecatrienoic acid. As per PC2, Cr00WFRE and Cr00WFYE2 showed variability. Based on the scatter plot, the twenty-five accessions could be classified into four groups, and similar clusters were also formed by HCA clustering.

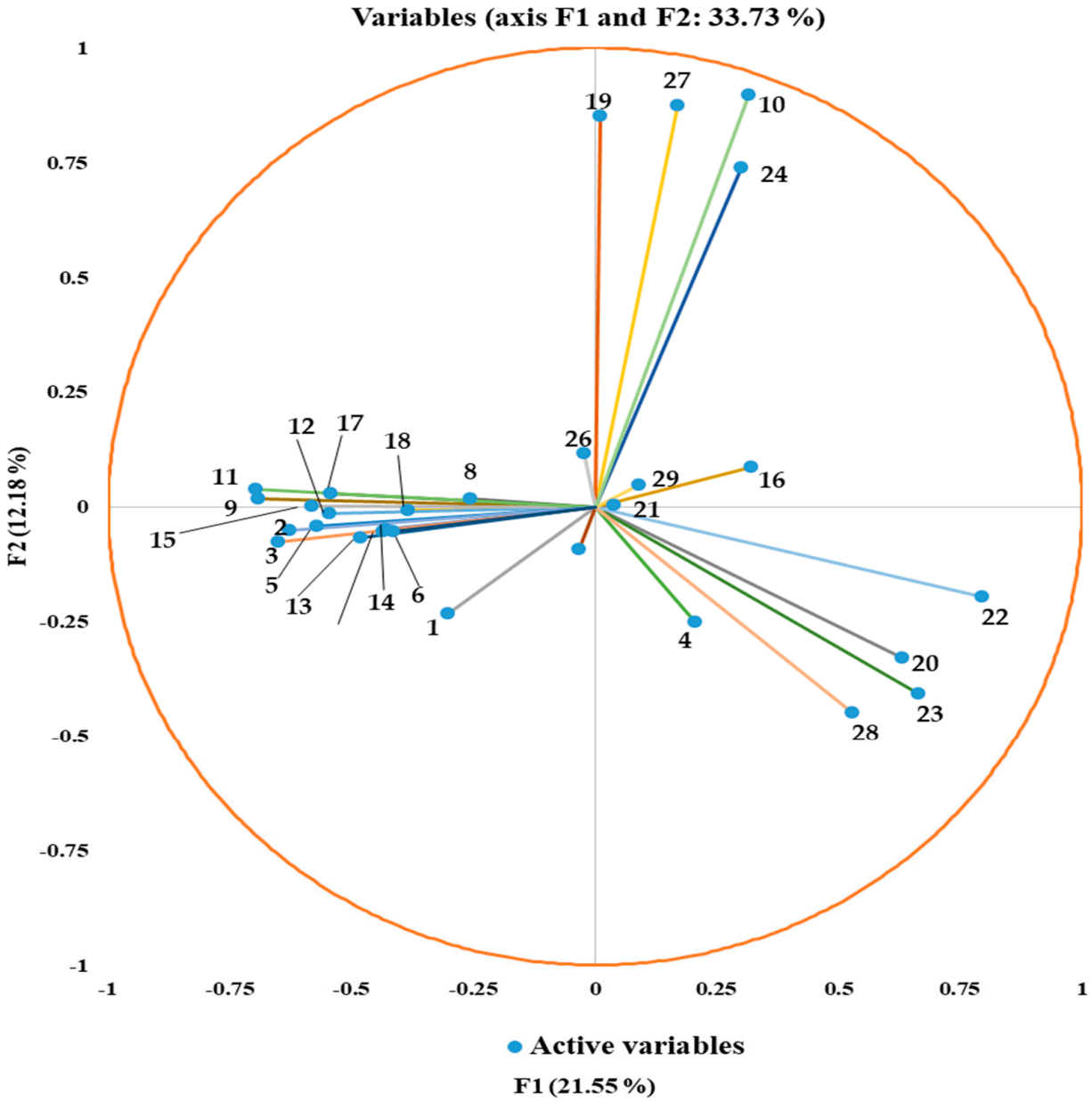


**Figure 6.** Loading plot of compounds detected in 25 different accessions of *Catharanthus roseus* by GC–MS: (**1**) 4H-Pyran-4-one; (**2**) Catechol; (**3**) Benzofuran, 2,3-dihydro; (**4**) 5-Hydroxymethylfurfural; (**5**) 1,2,3-Propanetriol, 1-acetate/acetin; (**6**) Ascaridole epoxide; (**7**) Ascaridole epoxide; (**8**) Deoxyspergualin; (**9**) Sucrose; (**10**) D-fructose; (**11**) D-allose; (**12**) Desulphosinigrin; (**13**) 3′,5′-Dimethoxyacetophenone; (**14**) α-D-Glucopyranoside, O-α-D-glucopyranosyl-(1.fwdarw.3)-ß-d-fru); (**15**) 1,2,3,5-Cyclohexanetetrol+; (**16**) Quinic acid; (**17**) Myo-Inositol, 4-C-methyl-; (**18**) Muco-inositol; (**19**) Hexadecanoic acid, methyl ester; (**20**) Pentadecanoic acid; (**21**) Phytol; (**22**) 9,12,15-Octadecatrienoic acid, methyl ester; (**23**) 2,20-Cycloaspidospermidine-3-carboxylic acid, Vindolinine; (**24**) 9,12,15-Octadecatrienoic acid; (**25**) Octadecane, 3-ethyl-5-(2-ethylbutyl)-; (**26**) Aspidospermidine-3-carboxylic acid vindoline; (**27**) Condyfolan, 14,19-didehydro-12-methoxy-, (14E)-; (**28**) Aspidospermidine-3-carboxylic acid; (**29**) Phthalic acid, di(oct-3-yl) ester.

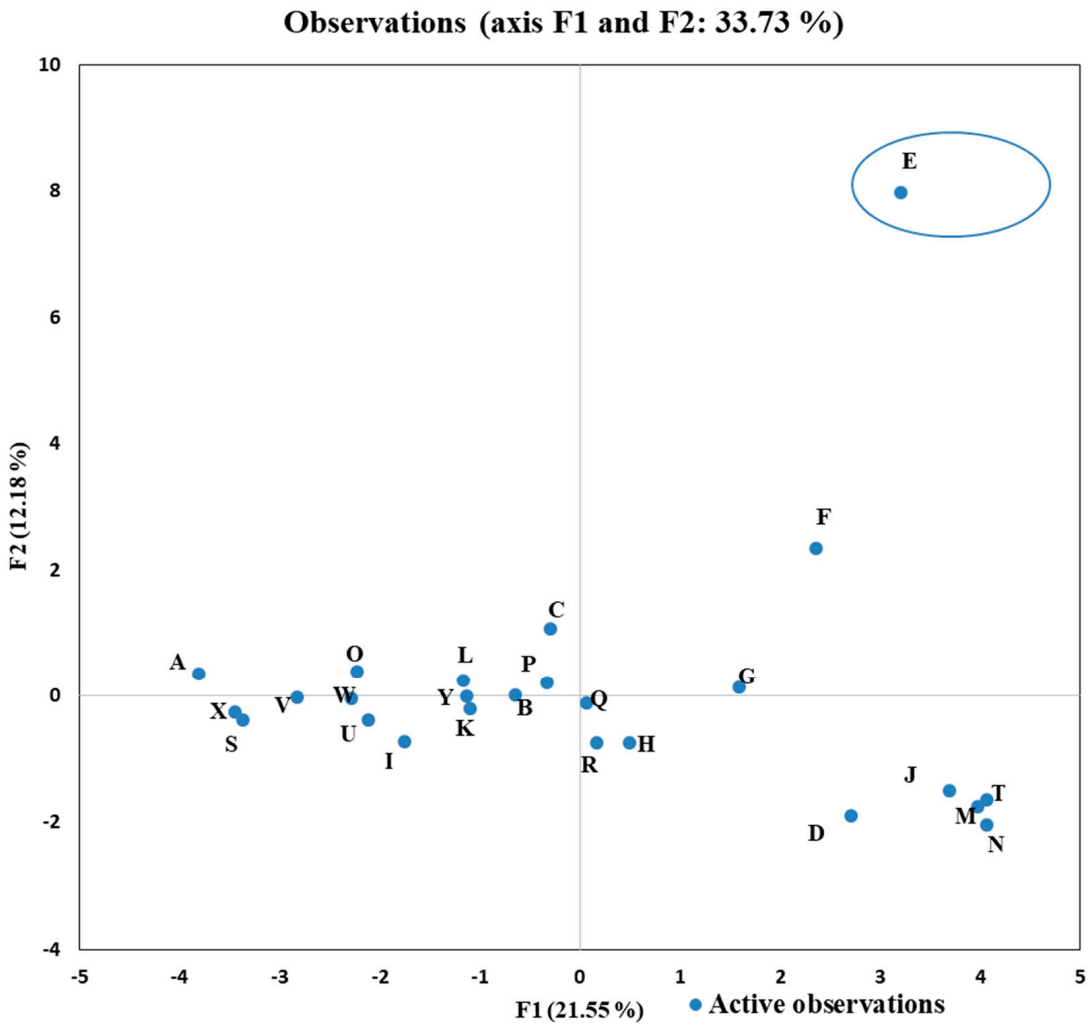


**Figure 7.** Loading plot of 25 different accessions of *Catharanthus roseus* based on GC–MS analysis: (**A**) Cr00PFRE, (**B**) Cr00LPFLPE, (**C**) Cr00DPF, (**D**) Cr00WFYE, (**E**) Cr00WFRE, (**F**) Cr00WFRE2, (**G**) Cr00WFSRE, (**H**) Cr00WFYE2, (**I**) Cr00DP, (**J**) Cr00LP, (**K**) Cr00BPF, (**L**) Cr00SFP, (**M**) Cr00CAF, (**N**) Cr00LPNF, (**O**) Cr00SBRF, (**P**) Cr00PLMF, (**Q**) Cr00CHEF, (**R**) Cr00SNFF, (**S**) Cr00CLF, (**T**) Cr00SAF, (**U**) Cr00TDRF, (**V**) Cr00 DRYE, (**W**) Cr00FRFF, (**X**) Cr00PFWE, and (**Y**) Cr00PFYE).

## 4. Discussion

### *4.1. Gas Chromatography–Mass Spectrometry (GC–MS) Analysis*

As indicated earlier, a total of 29 compounds were recognized from 25 accessions of *C. roseus*. Out of these, the relative amounts of quinic acid (43.88%) and hexadecanoic acid (15.00%) were most prominent. 1,2,3,5-Cyclohexanetetrol (9.24 %), phytol (3.89%), pentadecanoic acid (3.47%), 3′-5′–dimethoxyacetophenone (7.37%) and sucrose (4.52 %) were present in moderate amounts. *C. roseus* has a wide range of pharmacological activities and is used to treat various serious conditions such as cancer, hypertension, cardiovascular diseases, and chronic fatigue syndrome. Most notably, the plant possesses potent antineoplastic properties and is commonly used for this purpose. Its use has also been explored for a variety of other medicinal applications, such as treating chronic inflammation, fighting off bacterial and fungal infections, and minimizing oxidative stress in the body.

Large amounts of the aldehyde component 5-hydroxymethylfurfural were found in the examined accessions, and it demonstrated antioxidant and antiproliferative properties that were previously noted in *Derris trifoliate* and *Lepidium sativum* [19,45,46]. Linoleic acid, an important fatty acid, was also found in *C. roseus*. It is known to have a range of beneficial health effects, including anticancer, anti-arthritic, anti-inflammatory, anti-acne,

hypocholesterolemic, hepatoprotective, antihistaminic, and nematicide properties [47]. Specific forms of linoleic acid found in *C. roseus* included 9,12,15-octadecatrienoic acid (Z,Z,Z)- and 9,12,15-octadecatrienoic methyl ester (Z,Z,Z)- [48,49]. The presence of 9-octadecenoic acid was also noted in the extract, just as it was in the ethanolic root extract of *Plumbago zeylanica* [50]. Palmitic acid ester is a versatile and beneficial compound with many different uses. As an antioxidant, it helps protect against oxidative damage and can even be used in insecticides and anti-androgenic agents. Additionally, this ester can be used in nematicides for pest control, as a flavouring agent for food, and as a hypocholesterolemic agent to reduce high cholesterol levels [51,52].

Bioactive volatile compounds, such as phenolic compounds, aldehydes, alkanes, alkenes, esters, and ketones, are present in many plants. These compounds have an array of beneficial effects, including anti-arthritic, antidiabetic, anti-ulcer, anti-inflammatory, cytotoxic, and hypolipidemic activities. Additionally, these volatile compounds may possess antioxidant, analgesic, and anticancer properties. Researchers are searching for novel and safe solutions for various diseases and health conditions using these compounds [53]. Phytol has been found to possess multiple beneficial properties, including antidiuretic, antioxidant, neuroprotective, antimicrobial, antineoplastic, and anti-inflammatory activities. It also possesses central nervous system depressive properties and has been used in ancient medicine to treat a variety of illnesses. Its antioxidant activity helps protect the body against oxidative damage, and its anti-inflammatory properties help reduce inflammation and swelling. In addition, phytol has the potential to inhibit tumour growth, control bacterial and fungal infections, and reduce excessive urination [54,55]. The GC-MS analysis of *C. roseus* aqueous methanolic infusion revealed the presence of mannitol and sucrose carbohydrates, as well as oxygenated hydrocarbons and phenolic hydrocarbons. This suggests that *C. roseus* has a complex chemical profile, with important concentrations of carbohydrates and hydrocarbons. These phytochemicals are responsible for various pharmacological activities such as antimicrobial, antioxidant, and antiproliferative activities. The chemical composition of *C. roseus* morphotypes was found to be very complex, with multiple compounds present. These varied significantly across morphotypes, with some detected in trace amounts. It is important to monitor variations in the phytoconstituents of *C. roseus* morphotypes to better understand their effects.

### *4.2. GC-MS Data Used for Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA)*

The cluster analysis of metabolites in the leaf extracts of *C. roseus* divided the 25 accessions of *C. roseus* into two clusters. PCA multivariate statistical analysis methods were used for the analysis of secondary metabolites. Some important components were selected as potential biomarkers, including phytol, sucrose, glucose, vindoline, myo-inositol, and 9,12,15-Octadecatrienoic acid, for clustering of accessions. Similar to our study, Ghosh et al. [56] also studied the relative peak areas of phytol, desulphosinigrin, hexadecenoic acid, etc. and used them for multivariant statistical analysis. Their results also showed positive correlation patterns between the phytochemical profiles of *Clerodendrum infortunatum*. In the present study, quinic acid, hexadecanoic acid, 9,12,15, octadecatrienoic acid, vindoline, pentadecanoic acid, and 1,2,3,5-cyclohexanetetrol were the significant biomarkers of *C. roseus*, which suggests that the metabolomics technology platform used in this study was relatively effective for discriminating the morphotypes.

As per the results of this study, we identified the accession with the maximum number of phytoconstituents, i.e., the best-performing accession. The accession Cr00DP was found to be our best performing accession, with 22 phytochemicals. However, to the best of our knowledge, no previous studies have reported on it in the available literature. The accessions contained fatty acids, sugar moieties, aldehydes, ketones, alkaloids, flavonoids, and phenolic compounds. A variety of therapeutic properties have been attributed to these compounds including antidiabetic and anticancer properties. With further clinical research and trials, regular consumption of *C. roseus* leaves may be recommended for preventing

oxidative stress and improving human health. An innovative approach to treating disease caused by free radicals involves isolating active components from natural sources that can reduce or inhibit the production of these compounds in the body. The isolated ingredients can then be formulated into supplements and medicines that can help reduce the harmful effects of free radicals. The use of natural ingredients also results in the treatment being safer and more effective since the ingredients have already been tested and safely used for centuries. Further research is needed to understand how these compounds work together to give this plant its unique properties.

## 5. Conclusions

According to our review of the literature, this study seems to be the first work that focuses on identifying the numerous bioactive phytochemicals from the leaves of 25 *C. roseus* accessions using GC-MS analysis. The majority of these chemicals have various pharmacological and therapeutic effects. Various breeding techniques have been developed to produce new cultivars of *C. roseus* with varying chemical compositions. Therefore, it is important to classify the different accessions of *C. roseus* based on their phytochemistry. Additionally, given the use of *C. roseus* as a source of anticancer substances, a more comprehensive understanding of its chemical composition is important for new drug discovery. These 29 metabolites possessing antitumour, immunomodulatory, antihyperlipidemic, anti-ulcer, and anticholinesterase activities, were identified by GC-MS analysis. The data collected in this investigation revealed a distinctive qualitative chemical composition of the studied plants based on the high prevalence of quinic acid, 5-hydroxymethylfurfural, phytol and hexadecanoic acid, and the high 1,2,3,5-cyclohexanetetrol content. Finally, the findings of this investigation provide further information on *C. roseus* accessions that may be beneficial for future explorations of metabolite applications in scientific research. More research is necessary to develop novel drugs from the bioactive compounds found in *C. roseus*. Further research in this area could lead to the discovery of new medicines and treatments to improve the health and well-being of many people.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/separations10060340/s1, Figure S1: Map of India showing states from where various accessions were collected; Figure S2: Districts of Haryana from where 14 accessions were collected; Figure S3: GC-MS chromatogram of methanolic leaf extracts of 25 accessions of *Catharanthus roseus*; Table S1: Comparative analysis of phytochemicals in 25 *Catharanthus roseus* accessions.

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