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## Metabolomics and molecular networking studies to uncover the chemodiversity of three *Pulicaria* species in Qatar and their correlation with antioxidant and anticancer potential

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Qatari *Pulicaria* species—*Pulicaria undulata* (L.) C. A. Mey., *Pulicaria sicula* (L.) Moris and *Pulicaria gnaphalodes* (Vent.) Boiss have been a source of food and medicine among locals. However, these species have taxonomic issues and alternative uses. Hence, this study aimed to comparatively investigate the chemical diversity of three Qatari *Pulicaria* species, including their antioxidant and anticancer potential. HPLC-QTOF-MS-based untargeted metabolomics and molecular networking approaches were used to study the chemical diversity. The total phenolic and flavonoid contents, including antioxidant activity, were determined using standard colorimetric microplate-based assays. The anticancer activity was evaluated in human breast cancer MDA-MB-231 cells using the MTT assay. The methanolic extracts of three *Pulicaria* species had distinct chemical signatures, with a notable portion of similar compounds. Phytochemically, *P. undulata* and *P. sicula* were more similar to each other than *P. gnaphalodes*. Among the 36 compounds identified across all extracts, sesquiterpene lactones represented the most abundant chemical class. Interestingly, the methanolic extract of *P. undulata* was particularly qualitatively rich in sesquiterpene lactones, whereas *P. sicula* was comparatively richer in flavonoids, quantitatively. The observed antioxidant activity in *P. sicula* and *P. undulata* was nearly identical, despite *P. sicula* having significantly higher contents of total phenolics and flavonoids. The anticancer activity of extracts from all three species was dose-dependent, with *P. undulata* exhibiting the highest potency among them. This study provides valuable insights into the comparative chemodiversity and bioactivity profiles of three *Pulicaria* species, crucial for their quality control, and precise and sustainable use as anticancer or nutraceutical agents.

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## 1 Introduction

The genus *Pulicaria* (family: Asteraceae) consists of around 82 accepted species that are distributed from Europe to North Africa and Asia.<sup>1,2</sup> This genus mainly consists of essential oil-bearing plants. Reported phytochemicals in the *Pulicaria* genus include phenolics, terpenes, triterpenes, and steroids. However, sesquiterpenoids and flavonoids are the main classes of reported phytochemicals within the genus *Pulicaria*.<sup>3</sup> Several species of *Pulicaria* have been found to possess various medicinal properties, including anti-inflammatory, antipyretic, antioxidant, antimicrobial, and anticancer properties.<sup>4</sup> Of the reported *Pulicaria* species, three species have been reported in Qatar, namely *Pulicaria undulata* (L.) C. A. Mey., *Pulicaria*

*gnaphalodes* (Vent.) Boiss, and *Pulicaria sicula* (L.) Moris.<sup>5</sup> Among these, *P. undulata* is the most widely distributed species in Qatar, followed by *P. gnaphalodes* and *P. sicula*, respectively. Traditionally, these species are used as medicine to treat various ailments, such as infections, epilepsy, etc., and also as herbal tea by locals.<sup>1,5</sup>

Generally, *Pulicaria* is considered a taxonomically problematic genus.<sup>6</sup> Such taxonomic discrepancies have also been observed in Qatari *Pulicaria* species. In the flora of Qatar, along with *P. undulata*, it has been reported that there is a possibility of a subspecies of *P. undulata* (*Pulicaria undulata* subsp. *undulata*) in Qatar.<sup>5</sup> However, this fact has not yet been scientifically validated. Moreover, it has also been observed that *P. gnaphalodes* has two distinct seasonal forms, one of which has a resemblance to *P. undulata*.<sup>5</sup> The less morphological variability is the main reason for these taxonomic ambiguities in Qatari *Pulicaria* species. Hence, more detailed molecular and phytochemical studies are needed to resolve these taxonomic issues. Besides genomics, metabolomics has proven to be a valuable tool in plant taxonomy, especially for identifying and

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classifying plant species based on their unique metabolite profiles. These profiles not only indicate the genetic basis of plant species but also their biofunctional potential. Recently, besides metabolomics, feature-based molecular networking (FBMN) has been successfully employed to visually investigate differences in the recorded metabolome of the studied species of *Momordica*, *Onosma*, and *Centaurea*, including metabolite annotation.<sup>7–9</sup>

So far, several compounds have been isolated, identified, and quantified in a single Qatari species of *Pulicaria*. However, there has been no comprehensive comparative metabolome study on Qatari *Pulicaria* species with their bioactivities, such as antioxidant and anticancer potential. Thus, in this study, the comparative metabolome and molecular networking analysis of the methanolic extracts of three Qatari *Pulicaria* species were investigated using the high-pressure liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (HPLC-TOF-MS) technique. Additionally, the comparative anti-cancer and antioxidant potentials of these species were evaluated, along with their correlation to total phenolic and flavonoid contents. The findings of this study can be valuable in understanding the chemodiversity among Qatari *Pulicaria* species, which could be useful for their molecular-level classification, including the authentic and effective use of these species as herbal medicine or nutraceutical.

## 2 Materials and methods

### 2.1 Chemicals and reagents

Aluminum chloride ( $\text{AlCl}_3$ ), ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)), Folin-Ciocalteu reagent, gallic acid (GA), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), penicillin-streptomycin, quercetin, sodium hydroxide, sodium nitrite, trolox, and umbelliferone were obtained from Sigma Chemical Co., USA. Dimethylsulfoxide and Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS) were procured from HiMedia, India. Absolute ethanol and hexane were purchased from VWR, France. Hypergrade methanol and water (for LC-MS analysis) were obtained from Supelco (Merck), USA. All other chemicals used were of analytical grade.

### 2.2 Plant materials and preparation of extracts

The aerial parts of *P. undulata*, *P. gnaphalodes*, and *P. sicula* were collected from various locations in Qatar in February 2024. These species were authenticated by Ms. Muneera Al-Mesaifri, Biological Sciences, College of Arts and Sciences, Qatar University. All collected plant samples were then cleaned and dried in a hot air oven (50 °C for 72 h). After drying, the plant material was ground into a fine powder using a home grinder and sieved to achieve a uniform particle size. To prepare the extract, each powdered plant material was first defatted with hexane (1 : 10, w/v). Subsequently, the dried defatted samples were extracted with methanol (1 : 10, w/v). The methanol layer was separated by centrifugation at 6000 rpm for 15 min and then dried at ambient temperature. The yield of each extract

was recorded, and finally, these extracts were stored in the dark at 4 °C for further studies.

### 2.3 HPLC-Q-TOF-MS analysis

For HPLC-Q-TOF-MS analysis, 20 mg of dried methanolic extract from each sample was dissolved in 1 mL of methanol containing the internal standard (IS), umbelliferone. After vortexing, sonicating, and syringe filtering, chemical profiling of each of these samples was carried out using an Agilent HPLC-QTOF-MS system (1260 Infinity II LC System coupled with 6530 Q-TOF-MS). A sample of 5  $\mu\text{L}$  volume was injected into the HPLC system, and separation was performed using Zorbax SB C18 (250 mm × 4.6 mm (i.d.), 5  $\mu\text{m}$  particle size). Mobile phases A and B were 0.1% formic acid and methanol, respectively. The analysis time was 45 min and the elution gradient for the mobile B phase was as follows: initially equilibrated at 3% B; 0–10 min, 3–50% B; 10–25 min, 50–90% B; 25–35 min, 90–95% B; 35–42 min, 95–100% B; 42–45 min, 100–3% B. The flow rate was 0.5  $\text{mL min}^{-1}$ , and the column temperature was set at 40 °C. Nitrogen was utilized as the drying and collision gas in the dual AJS ESI source. The ion source parameters of the mass spectrometer were as follows: heated capillary temperature at 350 °C, drying gas flow rate at 10  $\text{L min}^{-1}$ , nebulizer pressure at 45 psi, and VCap at 3500V. The mass spectra were acquired in MS and auto MS/MS mode across the mass range of *m/z* 100–1500 in positive mode. Agilent Mass Hunter software was used for the data acquisition.

### 2.4 HPLC-QTOF-MS data processing, multivariate analysis, and compound annotation

The raw d.MS and d.MS/MS data files were converted to mzML files using the MSConvert software (ProteoWizard, Palo Alto, CA, USA). All mzML data files from the MS analysis were uploaded to the XCMS open-source software (<https://www.xcmsonline.scripps.edu>) to detect, align, and extract all features in Excel format. The signal intensities of all mass features were normalized using the signal intensity of the IS.<sup>10</sup> The normalized Excel data file was converted to a csv file for further multivariate analysis using MetaboAnalyst 6.0 (<https://www.metaboanalyst.ca>). Similarly, mzML files of MS/MS analysis were processed using SIRIUS 6.0.7 software for the identification of molecular formulas and a class of compounds.<sup>11</sup> Online databases such as PubChem, ChemSpider, MassBank, HMDB, and Phenol-Explorer were utilized to identify compounds by analyzing the exact mass and fragments, including considering the mass error of observed peaks. Moreover, a list of previously reported compounds in *Pulicaria* species was referenced during the tentative identification of compounds.

To construct FBMN, the mzML files of d.MS/MS data were uploaded to the publicly accessible Global Natural Product Social Molecular Networking (GNPS) platform (<https://gnps.ucsd.edu>, accession on 26 March 2025) by the WinSCP software (<https://wincp.net/eng/download.php>). A molecular network (MN) was created with a cosine score of 0.7 and 6



matched fragments. Cytoscape version 3.10.2 was subsequently used to visualize and analyze MN.<sup>8,12</sup>

## 2.5 Determination of total phenolics, total flavonoids, and antioxidant activity

All dried methanolic extracts were dissolved in methanol and then used to estimate the total phenolics and flavonoid content using the Folin-Ciocalteu and  $\text{AlCl}_3$  assays, respectively, with slight modifications.<sup>13</sup> Briefly, for the estimation of total phenolics, 20  $\mu\text{L}$  of the extract was mixed with 20  $\mu\text{L}$  of Folin-Ciocalteu reagent (2N). After 5 min incubation, 200  $\mu\text{L}$  of 7% sodium carbonate and 20  $\mu\text{L}$  of distilled water were added. Following an incubation in the dark (120 min), the absorbance was measured at 760 nm using the Varioskan Lux Multimode microplate reader (Thermo Fisher Scientific, USA). Results of total phenolics were reported as mg of GA equivalents (GAE) per g of extract.<sup>13</sup>

For the estimation of total flavonoids, 25  $\mu\text{L}$  of the sample extract or standard solution was added to a well containing 100  $\mu\text{L}$  of methanol and 10  $\mu\text{L}$  of sodium acetate (5%). Subsequently, after 5 min incubation, 15  $\mu\text{L}$  of  $\text{AlCl}_3$  was added to the well and incubated for 6 min. Later on, 50  $\mu\text{L}$  of 1 M NaOH was added, and the absorbance was finally measured at 434 nm using a microplate reader. The results of total flavonoids were reported as mg of quercetin equivalents (QE) per g of extract.<sup>13</sup>

The antioxidant potential of each sample was measured using the ABTS assay. The ABTS reagent was prepared as per our previously described method.<sup>14</sup> In a 96-well plate, 100  $\mu\text{L}$  of the fresh working solution of ABTS reagent was mixed with 10  $\mu\text{L}$  of the extract or standard solution. After an incubation in the dark (5 min), the absorbance was measured on a plate reader at 730 nm. Appropriate blanks (methanol) and standards (Trolox) were run simultaneously. The results of the ABTS assay were expressed in mg Trolox per g of extract.<sup>14</sup>

## 2.6 Anticancer activity

For the anticancer assay, MDA-MB-231 human triple-negative breast cancer (TNBC) cells were obtained from the National Centre for Cell Science (NCCS), Pune, India, and maintained in DMEM with 10% FBS and 1% penicillin-streptomycin. To ensure consistent growth and viability, the cells were grown in a humidified atmosphere at 37 °C with 5%  $\text{CO}_2$  and passaged regularly.

Cell viability in human breast cancer MDA-MB-231 cells was assessed to examine the anti-proliferative effects of methanolic extracts of three *Pulicaria* species studied.<sup>15</sup> The cells were seeded at a density of  $1 \times 10^4$  cells per well in 96-well culture plates and allowed to adhere overnight in a humidified incubator at 37 °C with 5%  $\text{CO}_2$  to ensure optimal growth. Following incubation, the cells were treated with varying concentrations (10 to 100  $\mu\text{g mL}^{-1}$ ) of the methanolic extracts dissolved in DMSO, and incubated for an additional 24 h. The treatment concentrations used were 10, 20, 25, 50, 75, and 100  $\mu\text{g mL}^{-1}$  to observe a dose-dependent response. Following the 24 h treatment, 20  $\mu\text{L}$  of MTT was added to each well, and the plates were

incubated for the next 4 h to allow live cells to convert MTT into formazan crystals at the bottom of the wells. After incubation, the culture medium and unreacted MTT reagent were carefully removed. To dissolve the formazan crystals from metabolically active cells, 100  $\mu\text{L}$  of DMSO was added to each well. The absorbance of each well was measured at 540 nm using a BioRad 680 microplate reader (BioRad, USA). The absorbance readings were proportional to the number of live cells following treatment. Cell viability percentage for each extract was calculated by comparing the treatment and control well absorbances by using the formula:

$$\text{Cell viability (\%)} = [(\text{OD of treated})/(\text{OD of control})] \times 100$$

## 2.7 Morphological analysis by phase contrast microscopy

The morphological alterations linked to different plant extract treatments in MDA-MB-231 cells were also evaluated as described previously.<sup>15</sup> Briefly, the cells were plated in a 96-well plate at a density of  $1 \times 10^4$  cells per well. Following an overnight incubation, the cultured cells were exposed to different concentrations of three plant extracts (10–100  $\mu\text{g mL}^{-1}$ ). After a 24 h exposure, the cellular morphology of the treated and control cells was examined under an inverted phase contrast microscope (Nikon ECLIPSE Ti-S, Japan).

## 2.8 Statistical analysis

The results were expressed as mean  $\pm$  standard error (SE). The experiments were conducted three times in triplicate. Microsoft Excel and the trial version of GraphPad Prism software were used for data analysis and visualization.

# 3 Results and discussion

## 3.1 Untargeted metabolomic profiling of *Pulicaria* species

The comparative representative total ion chromatogram (TIC) of HPLC-QTOF-MS analyses of methanolic extracts of *Pulicaria* species (Fig. S1) showed considerable phytochemical variability among the three species. However, these chemical fingerprint profiles were insufficient to understand the metabolic similarities and variations among *Pulicaria* species. Hence, the HPLC-QTOF-MS data were further processed and subjected to unsupervised multivariate analyses, such as Principal Component Analysis (PCA) and Hierarchical Clustering Analysis (HCA). Results of PCA (which is usually carried out for pattern recognition on the datasets) showed that samples of each species of *Pulicaria* formed distinct clusters, indicating the chemical uniqueness of each species from the others (Fig. 1a). In PCA plot, the observed total percentage of variance explained by PC1 and PC2 was 84%, further indicated the chemical profiles of the three species are well-differentiated in the PCA space. This high level of discrimination suggests that PCA can be effectively used for quality control and chemotaxonomic classification of these species. The replicates of *P. gnaphalodes* and *P. sicula* in the PCA score plot were tightly clustered, indicating low chemical variability

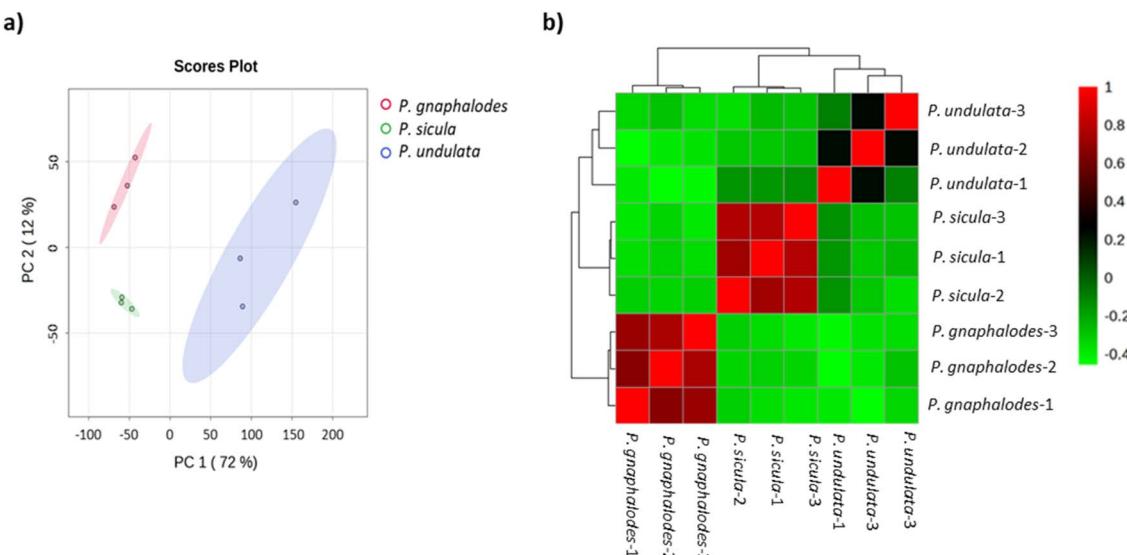


Fig. 1 (a) Principal Component Analysis (PCA) and (b) Hierarchical Clustering Analysis (HCA) plots showing the clustering patterns of samples from three *Pulicaria* species.

within species. In contrast, the scattered or loosely clustering pattern observed among *P. undulata* replicates could be due to the geographical locations of the samples collected or the presence of subspecies. However, this fact needs to be confirmed in future in-depth targeted and untargeted metabolomics studies, especially to prove the earlier observation about the presence of subspecies of *P. undulata* in Qatar.

To further investigate the grouping and chemotaxonomic relationship among *Pulicaria* species studied, HCA was performed. The results of the HCA clearly showed that the samples of the three species studied formed two main clusters. The observed single cluster for *P. undulata* and *P. sicula* indicated overall metabolome similarity among these two species. This result further indicated that the phytochemistry of *P. gnaphalodes* was distinct from the other two *Pulicaria* species. The considerable color variation in the heatmap cells among *P. undulata* replicates further indicates quantitative differences in feature intensities, which may contribute to the observed scattered or loosely clustered pattern in the PCA score plot.

### 3.2 MS/MS-based molecular networking

MS/MS-based molecular network analysis was performed to trace the chemical diversity and identify major chemical classes in three *Pulicaria* species (Fig. 2). In the MN, compounds with similar structures are joined together and grouped into clusters based on the similarity of their MS/MS fragments.<sup>16</sup> On the contrary, spectra not clustered into molecular families were represented as self-loop nodes positioned at the bottom of the network.<sup>7</sup> Different colors, such as red (*P. gnaphalodes*), green (*P. sicula*), and blue (*P. undulata*), were used to denote comparative metabolite distribution among three *Pulicaria* species (Fig. 2a). The computed MN of

three species showed 565 nodes (representing unique MS/MS spectra or features), 634 edges (indicating spectral similarity), and 386 connected components (representing distinct molecular families) based on GNPS spectral matching. These results collectively demonstrate substantial chemical diversity with a considerable portion of structurally similar compounds across these species (Fig. 2a).

The MolNetEnhancer platform is further used to depict major classes in a computed MN. Due to the chemical complexity and lack of comprehensive spectral libraries, identifying all molecular families was difficult. Eleven chemical classes such as flavonoids, phenylpropanoids amino acids, nucleosides, saccharides, fatty acids, phenolic acids, steroids, terpenoids, alkaloids and benzopyran were identified (Fig. 2b). There was no clear species-specific pattern observed, except for some classes such as fatty acids, which were predominantly observed in *P. gnaphalodes*. The distribution of the majority of the above-identified classes of compounds was wide across the species studied.

### 3.3 Identification of major compounds in three *Pulicaria* species

Major peaks obtained from the HPLC-ESI-QTOF-MS/MS analysis of the methanolic extracts of three *Pulicaria* species were also tentatively identified. Based on SIRIUS data processing, along with information from online databases (PubChem, GNPS, ChemSpider, MassBank, HMDB, and Phenol-Explorer) and relevant literature, a total of 36 compounds were tentatively identified across the three *Pulicaria* species. The four confidence levels (1–4) proposed by the Metabolomics Standards Initiative (MSI)<sup>17</sup> were used to denote the level of each compound identification. Compounds identified based on the database and literature, including the possibility of sole



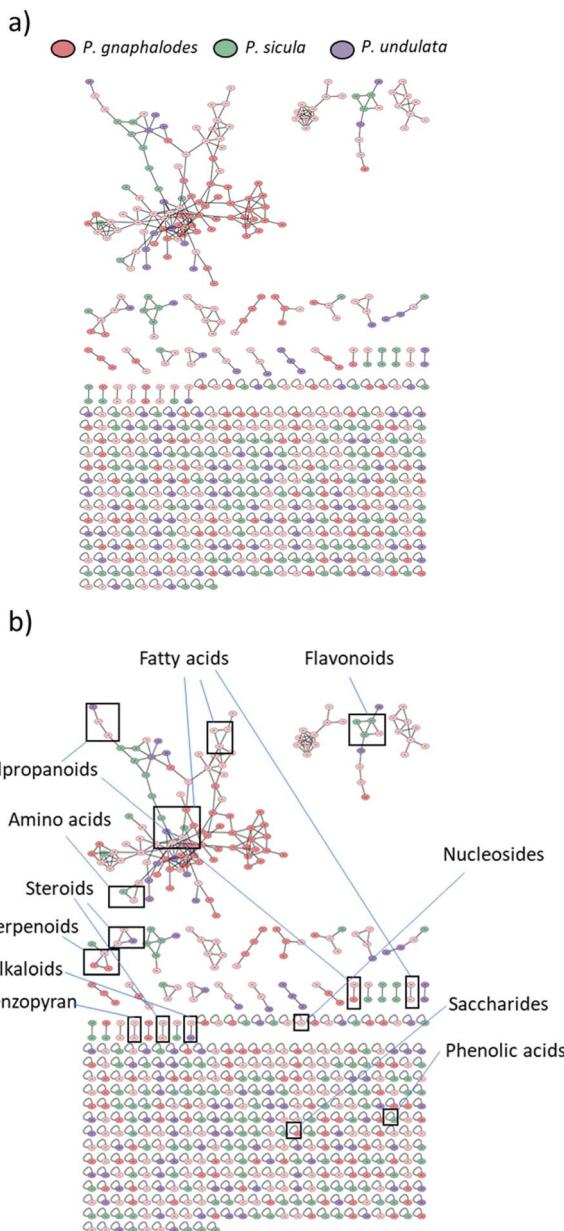


Fig. 2 The molecular network of the methanolic extracts of three *Pulicaria* species, based on MS/MS data acquired in positive mode. Only nodes (features) with a cosine score  $>0.7$  are connected by edges in the network. (a) Comparative metabolite distribution in *P. gnaphalodes* (red), *P. sicula* (green), and *P. undulata* (blue). (b) The annotated clusters of eleven chemical classes, such as flavonoids, phenylpropanoids, amino acids, nucleosides, saccharides, fatty acids, phenolic acids, steroids, terpenoids, alkaloids, and benzopyran in a computed MN.

existence, were categorized as MSI level 2, while compounds with possible structural isomers were categorized as MSI level 3, following the annotation confidence levels proposed by Schymanski *et al.*<sup>18</sup> However, further confirmation of the structure of each of these compounds using reference standards is warranted. However, database-dependent identification of compounds has limitations, as it may not distinguish between

structural isomers, stereoisomers, or compounds with similar exact masses and fragmentation patterns details of these compounds, including their respective retention time, precursor ion mass, observed mass, mass errors, main MS/MS fragments, and molecular formula, MSI ID, as well as their occurrence in the studied *Pulicaria* species, are listed in Table 1. Among the identified compounds, some have been reported in their respective *Pulicaria* species, while others have been found in other studied or non-studied *Pulicaria* species.<sup>1</sup> For example, tuberonic acid glucoside was not reported in the studied *Pulicaria* species but was found in the methanolic extract of *Pulicaria dysenterica* (L.) Bernh.<sup>19</sup> In addition, compounds such as fatty amides were identified for the first time in *Pulicaria* species studied. These fatty amides, along with silyamandin and 2-palmitoylglycerol, have not been previously identified in *Pulicaria* species studied but have been reported to be present in the Asteraceae family.<sup>20–22</sup>

Among the identified compounds in the methanol extract of the three species, sesquiterpene lactone was the major class of compounds (nine compounds). Subsequently, among other identified compounds were: five flavonoids, five amino acids and their derivatives, including four fatty amides. In addition to these, three glucosides, two fatty acids, two saccharides, one alkaloid, one lignan (phenylpropanoid), one monoterpene, one nucleoside, and one phenolic acid were also identified (Table 1).

Nearly twelve compounds, such as fructosyl leucine/ isoleucine, valine, phenylalanine, palmitamide, 2-palmitoylglycerol, palmitoleamide, silyamandin, deoxyguanosine, chlorogenic acid, glucose, sucrose, ivalin/confertin/pseudoivalin/8-epiconfertin were commonly found in all three studied species (Table 1). Among the nine sesquiterpene lactones identified in three species, eight compounds were found in *P. undulata* alone. Of these eight compounds, six were also present in both *P. undulata* and *P. gnaphalodes*. This collectively indicates that the methanolic extract of *P. undulata* was rich in sesquiterpene lactones, followed by *P. gnaphalodes*. Among the five flavonoids identified, four were found exclusively in *P. sicula*, highlighting the flavonoid richness of *P. sicula* compared to the other two species. Interestingly, two identified fatty acids, 3-hydroxydecanoic acid and 6-hydroxypentadecanedioic acid, were found solely in *P. gnaphalodes*.

### 3.4 Total phenolics and flavonoids, and antioxidant activity

The findings of the total phenolics and flavonoid content, including the antioxidant activity in terms of ABTS<sup>+</sup> radical scavenging activity, in methanolic extracts of *Pulicaria* species studied, are tabulated in Table 2. All three extracts had more phenolics than flavonoids. The total phenolics and flavonoid content observed in *P. sicula* were significantly higher than in *P. undulata* and *P. gnaphalodes*. The observed total phenolics content in *P. undulata* and *P. gnaphalodes* was not significant. However, the total flavonoid content found in *P. undulata* was significantly higher than in *P. gnaphalodes*. Despite significant differences in the total phenolics and flavonoid content, the



Table 1 HPLC-ESI-QTOF-MS/MS-based tentative identification of major compounds in *Pulicaria* species. PU: *P. undulata*, PS: *P. siccula*, and PG: *P. gnaphalodes*. MSI ID indicates confidence levels (1–4) proposed by the Metabolomics Standards Initiative (MSI)

Sr. no.	RT (min)	Precursor ion ( $m/z$ )	Observed ( $m/z$ )	Theoretical mass ( $m/z$ )	Mass error (ppm)	MS/MS fragments	Molecular formula	Compound (MSI ID)	Class of compounds	Pulicaria species	Reference
1	4.6	[M + Na] <sup>+</sup>	203.0530	203.0526	1.93	162	C <sub>6</sub> H <sub>12</sub> O <sub>6</sub>	Glucose (2)	Saccharides	PU, PS, PG	—
2	5.2	[M + H] <sup>+</sup>	118.0862	118.0862	0.18	—	C <sub>5</sub> H <sub>11</sub> NO <sub>2</sub>	Valine (2)	Amino acids	PU, PS, PG	—
3	5.2	[M + H] <sup>+</sup>	116.0707	116.0705	1.72	—	C <sub>5</sub> H <sub>11</sub> NO <sub>2</sub>	Proline (2)	Amino acids	PU, PS	—
4	5.3	[M + H] <sup>+</sup>	365.1060	365.1054	1.64	—	C <sub>12</sub> H <sub>22</sub> O <sub>11</sub>	Sucrose (2)	Saccharides	PU, PS, PG	—
5	6.9	[M + H] <sup>+</sup>	144.1018	144.1019	-0.72	118	C <sub>7</sub> H <sub>13</sub> NO <sub>2</sub>	Proline betaine (2)	Alkaloids	PU, PS	—
6	9.2	[M + H] <sup>+</sup>	130.0503	130.0498	3.31	—	C <sub>5</sub> H <sub>7</sub> NO <sub>3</sub>	Pyroglutamic acid (2)	Amino acid derivatives	PU, PG	—
7	10.1	[M + H] <sup>+</sup>	268.1041	268.1040	0.37	—	C <sub>10</sub> H <sub>13</sub> N <sub>5</sub> O <sub>4</sub>	Deoxyguanosine (2)	Nucleoside	PU, PS, PG	—
8	10.4	[M + H] <sup>+</sup>	294.1550	294.1547	0.92	—	C <sub>12</sub> H <sub>23</sub> NO <sub>7</sub>	Fructosyl leucine/isoleucine (3)	Amino acid derivatives	PU, PS, PG	—
9	12.5	[M + H] <sup>+</sup>	355.1003	355.1022	-0.53	—	C <sub>16</sub> H <sub>18</sub> O <sub>9</sub>	Chlorogenic acid (2)	Phenolic acids	PU, PS, PG	—
10	12.6	[M + H] <sup>+</sup>	166.0870	166.0862	4.48	120	C <sub>9</sub> H <sub>11</sub> NO <sub>2</sub>	Phenylalanine (2)	Amino acids	PU, PS, PG	—
11	13.4	[M + Na] <sup>+</sup>	367.1367	367.1363	1.36	—	C <sub>16</sub> H <sub>24</sub> O <sub>8</sub>	Dihydroconiferin/8- <i>epi</i> -iridotriol glucoside (3)	Glucosides	PS	—
12	16.3	[M + Na] <sup>+</sup>	411.1619	411.1626	-1.70	388	C <sub>18</sub> H <sub>28</sub> O <sub>9</sub>	Tuberonic acid glucoside (3)	Glucosides	PU, PS	19
13	17.5	[M + H] <sup>+</sup>	499.1234	499.12348	-0.17	364, 163	C <sub>25</sub> H <sub>22</sub> O <sub>11</sub>	Silyamandin (2)	Ligan (sub-class-phenyl propanoid)	PU, PS, PG	20
14	18.3	[M + Na] <sup>+</sup>	371.1680	371.1680	1.0	163	C <sub>16</sub> H <sub>28</sub> O <sub>8</sub>	Terpene glycoside (2,4,7-thujanetriol 4-glucoside) (3)	Glucosides	PS	23
15	18.0	[M + H] <sup>+</sup>	263.1271	263.1277	-2.28	245	C <sub>15</sub> H <sub>18</sub> O <sub>4</sub>	Xanthanin-epoxide/epi-xanthanin-epoxide (3)	Sesquiterpene lactones	PU	24
16	19.0	[M + H] <sup>+</sup>	495.1130	495.1132	-0.48	318, 333	C <sub>22</sub> H <sub>22</sub> O <sub>13</sub>	Quercetagetin-ethyl-ether-glucoside (3)	Flavonoids	PU	25
17	19.9	[M + Na] <sup>+</sup>	211.1305	211.1305	0.00	193	C <sub>10</sub> H <sub>20</sub> O <sub>3</sub>	3-Hydroxydodecanoic acid (2)	Fatty acids	PG	24
18	20.2	[M + Na] <sup>+</sup>	311.1822	311.1822	0.00	253	C <sub>15</sub> H <sub>28</sub> O <sub>5</sub>	6-Hydroxypentadecanoic acid (2)	Fatty acids	PG	24
19	23.6	[M + H] <sup>+</sup>	247.1328	247.1328	0.00	229, 187, 114	C <sub>15</sub> H <sub>18</sub> O <sub>3</sub>	Xanthanin/epi-xanthanin (3)	Sesquiterpene lactones	PU, PG	24
20	23.7	[M + Na] <sup>+</sup>	331.1510	331.1516	-1.81	—	C <sub>17</sub> H <sub>24</sub> O <sub>5</sub>	Isoxanthanol (3)	Sesquiterpene lactones	PU, PG	26
21	24.3	[M + H] <sup>+</sup>	249.1478	249.1484	-2.40	231	C <sub>15</sub> H <sub>20</sub> O <sub>3</sub>	Confertin/8-epiconfertin/ivalin/pseudovalin (3)	Sesquiterpene lactones	PU, PS, PG	1
22	24.8	[M + H] <sup>+</sup>	247.1331	247.1328	1.21	229, 187, 114	C <sub>15</sub> H <sub>18</sub> O <sub>3</sub>	Xanthanin/epi-xanthanin (3)	Sesquiterpene lactones	PU, PG	24
23	25.0	[M + H] <sup>+</sup>	251.1635	251.1635	-2.38	—	C <sub>15</sub> H <sub>22</sub> O <sub>3</sub>	Desacetyl xanthanol (3)	Sesquiterpene lactones	PU, PG	27 and 28
24	25.2	[M + Na] <sup>+</sup>	271.1281	271.1305	-8.85	—	C <sub>15</sub> H <sub>20</sub> O <sub>3</sub>	Parthenolide (3)	Sesquiterpene lactones	PU	29
25	25.3	[M + H] <sup>+</sup>	249.1480	249.1484	-1.60	231	C <sub>15</sub> H <sub>20</sub> O <sub>3</sub>	Confertin/8-epiconfertin/ivalin/pseudovalin (3)	Sesquiterpene lactones	PU, PG	1
26	26.5	[M + H] <sup>+</sup>	249.1479	249.1484	-2.0	231	C <sub>15</sub> H <sub>20</sub> O <sub>3</sub>	Confertin/8-epiconfertin/ivalin/pseudovalin (3)	Sesquiterpene lactones	PU, PG	1
27	26.9	[M + H] <sup>+</sup>	317.0650	317.0655	-1.82	—	C <sub>16</sub> H <sub>12</sub> O <sub>7</sub>	Isorhamnetin (2)	Flavonoids	PS	30



Table 1 (Cont'd.)

Sr. no.	RT	Precursor ion ( $m/z$ )	Observed ( $m/z$ )	Theoretical mass ( $m/z$ )	Mass error (ppm)	MS/MS fragments	Molecular formula	Compound (MSI ID)	Class of compounds	<i>Pulicaria</i> species	Reference
28	27.5	[ $M + H$ ] <sup>+</sup>	375.1080	375.1074	1.48	—	$C_{19}H_{18}O_8$	Quercetatin-tetramethyl ether (3)	Flavonoids	PS, PG	25
29	29.1	[ $M + H$ ] <sup>+</sup>	331.0810	331.0812	-0.69	—	$C_{17}H_{14}O_7$	Quercetin-dimethyl ether (3)	Flavonoids	PS	—
30	29.6	[ $M + Na$ ] <sup>+</sup>	219.1715	219.1715	-1.82	—	$C_{13}H_{24}O$	Citronellylacetone (3)	Monoterpenes	PG	1
31	29.6	[ $M + H$ ] <sup>+</sup>	345.0967	345.0968	-0.51	—	$C_{18}H_{16}O_7$	Dihydroxy-trimethoxyflavone (3)	Flavonoids	PS	27
32	37.5	[ $M + H$ ] <sup>+</sup>	254.2480	254.2477	1.17	—	$C_{16}H_{31}NO$	Palmitoleamide (2)	Fatty amides	PU, PS, PG	22
33	38.0	[ $M + H$ ] <sup>+</sup>	280.2626	280.2634	-3.19	—	$C_{18}H_{33}NO$	Linoleamide (2)	Fatty amides	PS, PG	22
34	40.4	[ $M + H$ ] <sup>+</sup>	256.2631	256.2634	1.11	—	$C_{16}H_{33}NO$	Palmitamide (2)	Fatty amides	PU, PS, PG	22
35	41.1	[ $M + Na$ ] <sup>+</sup>	353.2663	353.2662	0.23	331	$C_{19}H_{38}O_4$	2-Palmitoylglycerol (2)	Monoacylglycerols	PU, PS, PG	21
36	41.5	[ $M + H$ ] <sup>+</sup>	282.2799	282.2790	2.95	—	$C_{18}H_{35}NO$	Oleamide (2)	Fatty amides	PU, PS	22

resultant of ABTS<sup>+</sup> radical scavenging activity in *P. sicula* and *P. undulata* was non-significant. This finding suggests that the extracts of both these plants may contain comparable amounts of compounds that are capable of scavenging ABTS<sup>+</sup> radicals. To further understand whether these compounds are phenolics or flavonoids, correlation studies were conducted, and the results are tabulated in the SI Table S1. The results of ABTS<sup>+</sup> radical scavenging activity showed a highly significant ( $P < 0.001$ ), very strong correlation with total flavonoid content ( $r = 0.943$ ), and a non-significant moderate correlation with total phenolics content ( $r = 0.625$ ), indicating that flavonoids may contribute more significantly to the observed antioxidant activity in *Pulicaria* species than phenolics.

### 3.5 Anticancer activity

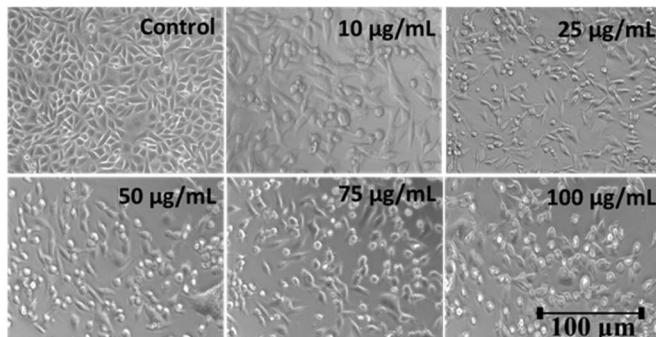
The results of the MTT assay showed that the extracts of all three *Pulicaria* species had dose-dependent cytotoxic effects against human breast cancer MDA-MB-231 cells (Fig. 3). The observed IC<sub>50</sub> values for methanolic extracts were in the order *P. gnaphalodes* (98.03  $\mu\text{g mL}^{-1}$ , 95%, CI: 90.04–109.1  $\mu\text{g mL}^{-1}$ ) > *P. sicula* (89.41  $\mu\text{g mL}^{-1}$ , 95% CI: 83.44–97.30  $\mu\text{g mL}^{-1}$ ) > *P. undulata* (85.66  $\mu\text{g mL}^{-1}$ , 95% CI: 80.21 to 92.36  $\mu\text{g mL}^{-1}$ ) indicating that *P. undulata* has more anticancer potential compared to the other two species. Previous studies have shown that essential oils, extracts, fractions, and isolated compounds from *Pulicaria* species possess promising anticancer properties, particularly against liver, breast, lung, and colon cancers.<sup>1</sup> Among the three *Pulicaria* species studied, *P. undulata* has been extensively investigated for its anticancer activity, but studies on the anticancer potential of *P. gnaphalodes* and *P. sicula* are almost negligible.<sup>1</sup> The present study is the first comparative study on the anticancer activity of these species against human breast cancer MDA-MB-231 cells. According to the available literature, the observed anticancer potential of these species has been generally attributed to their sesquiterpene lactones and flavonoids.<sup>1</sup> The results of the phytochemical profiling of the present study indicated that *P. undulata* exhibited a relatively higher diversity of sesquiterpene lactones and a lower abundance of flavonoids compared to *P. sicula*. Conversely, *P. sicula* had a comparatively higher concentration of flavonoids than *P. undulata* but a lower diversity of sesquiterpene lactones. In connection with this, the observed high anticancer potential of *P. undulata* compared to *P. sicula* highlights the potential role of sesquiterpene lactones as anticancer agents.

In addition, the results of inverted phase-contrast microscopy showed that the studied plant extracts exhibited cytotoxicity in MDA-MB-231 cells through apoptosis (Fig. 3). In all three species, the observed apoptosis effect was concentration-dependent. Initially, cells shrank and became more refractile, especially at doses  $\geq 50 \mu\text{g mL}^{-1}$ . In extract-treated cells, membrane blebbing, a sign of early apoptosis, was apparent. Detachment and rounding off were common, especially in high-dose treatments, suggesting cytoskeletal disturbance. Apoptotic structures, comprising shattered nuclei and membrane-bound vesicles, formed at 75–100  $\mu\text{g mL}^{-1}$ .

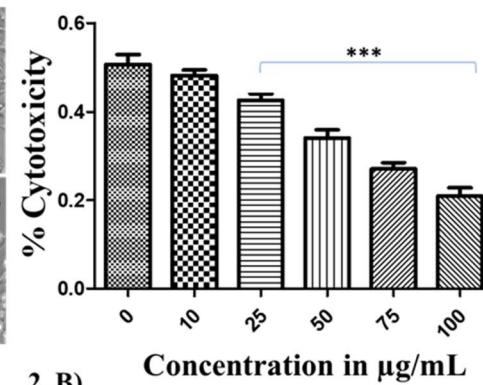
**Table 2** Comparative analysis of total phenolic content, total flavonoid content, and antioxidant activity in methanolic extracts of three *Pulicaria* species. Values are presented as mean  $\pm$  standard error (SE) from three independent replicates. Different superscript letters within the same column indicate statistically significant differences between means ( $P < 0.05$ ; one-way ANOVA followed by Tukey's HSD test). GAE is gallic acid equivalents and QE is quercetin equivalents

	Total phenolics (mg GAE per g extract)	Total flavonoids (mg QE per g extract)	ABTS (mg trolox per g extract)
<i>P. undulata</i>	$64.6 \pm 11.6^a$	$13.7 \pm 0.4^a$	$35.9 \pm 0.4^a$
<i>P. sicula</i>	$106.2 \pm 8.2^b$	$18.9 \pm 1.5^b$	$36.7 \pm 0.2^a$
<i>P. gnaphalodes</i>	$51.9 \pm 7.0^a$	$2.2 \pm 1.0^c$	$21.2 \pm 0.5^b$

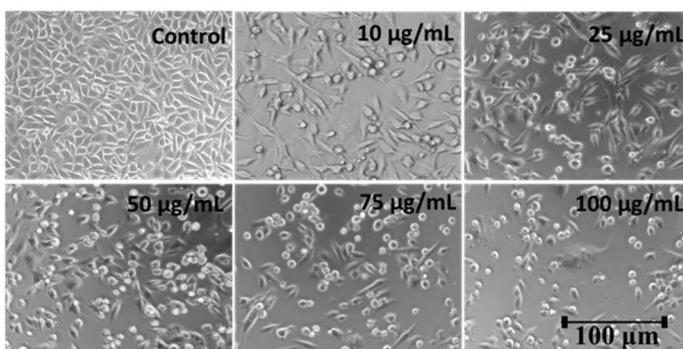
1.A)



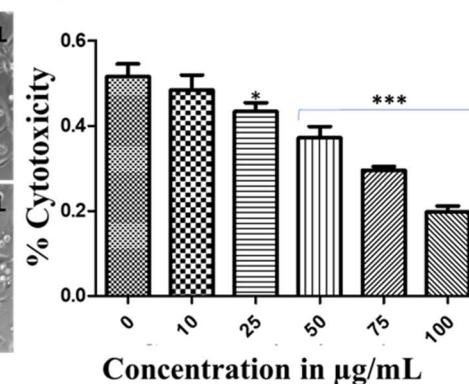
1. B)



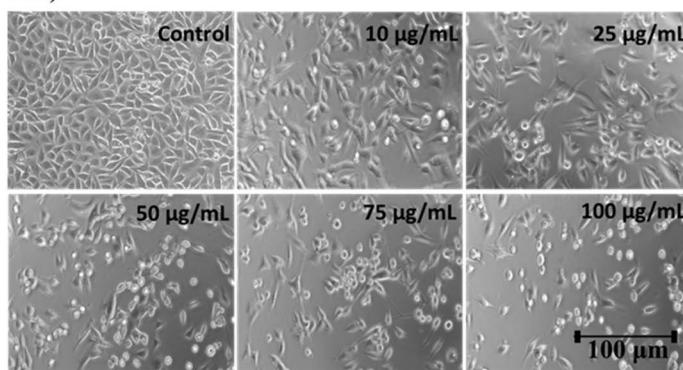
2. A)



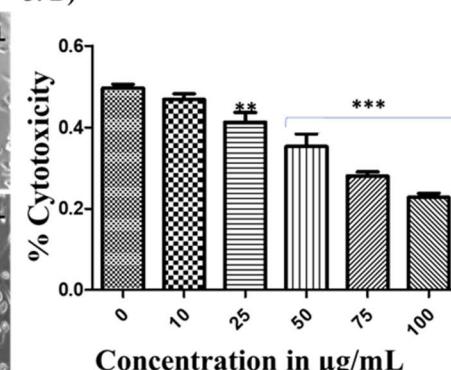
2. B)



3. A)



3. B)



**Fig. 3** *In vitro* cytotoxic efficacy of *P. undulata*, *P. sicula*, and *P. gnaphalodes* against MDA-MB-231 breast cancer cells. (1A), (2A), and (3A) Photomicrographs show morphological analysis of living and dead MDA-MB-231 cells subjected to different concentrations ( $10$ – $100 \mu\text{g mL}^{-1}$ ) of methanolic extracts of *P. undulata*, *P. sicula*, and *P. gnaphalodes*, respectively. (1B), (2B), and (3B) Denote the dose-dependent percentage of cell cytotoxicity of MDA-MB-231 cells subjected to different concentrations ( $10$ – $100 \mu\text{g mL}^{-1}$ ) of methanolic extracts of *P. undulata*, *P. sicula*, and *P. gnaphalodes*, respectively. Values are shown as mean  $\pm$  SM of three independent experiments,  $^{**}p < 0.01$  and  $^{***}p < 0.001$ , in comparison to the control.



## 4 Conclusions

In this study, the comparative chemical diversity among three commonly occurring *Pulicaria* species in Qatar was investigated for the first time using an HPLC-QTOF-MS-based untargeted and molecular networking approach. Moreover, the comparative antioxidant and anticancer potentials of these species were also explored. Results of untargeted metabolomics and molecular networking studies showed that the methanolic extracts of three *Pulicaria* species had distinct chemical signatures with a considerable portion of similar sorts of compounds across these species. Phytochemically, *P. undulata* and *P. sicula* were found to be more similar than *P. gnaphalodes*, which can also be reflected in the morphology of these species. Besides this, 36 different compounds were also tentatively identified in the methanol extract of these *Pulicaria* species using HPLC-ESI-QTOF-MS/MS analysis data. Among the identified compounds, sesquiterpene lactone was the major class of compounds, accounting for 25% of the total compounds. The methanolic extract of *P. undulata* exhibited a relatively higher diversity of sesquiterpene lactones, followed by *P. gnaphalodes*. In contrast, *P. sicula* was found to be rich in flavonoids compared to the others.

Similar results were also found in the analyses of total phenolics and flavonoids contents, where *P. sicula* had significantly higher amounts of total flavonoids, including phenolics, compared to the other two species. However, interestingly, the observed ABTS<sup>+</sup> radical scavenging activity in *P. sicula* and *P. undulata* was almost the same despite significant differences in their total phenolics and flavonoids. In anticancer assessment studies, the methanolic extracts of all three *Pulicaria* species showed dose-dependent cytotoxic effects against human breast cancer MDA-MB-231 cells. Among the three species, *P. undulata* had the strongest anticancer potential compared to the other two species studied, which can be attributed to its high and diverse content of sesquiterpene lactones. Altogether, the present study provides valuable insights into the comparative chemodiversity and bioactivity profiles of three *Pulicaria* species, offering a foundation for their quality control and supporting their potential development as anticancer or nutraceutical agents.

## Author contributions

MA performed analytical assays and contributed to revising the manuscript draft. JR and KA conducted cell-line assays, compiled the results of anticancer activity, and contributed to manuscript writing and editing. DK contributed to conceptualization, study design, and supervision, conducted HPLC-TOF-MS analysis and data analysis, and prepared the original draft. All authors reviewed and approved the manuscript.

## Conflicts of interest

The authors declare no conflicts of interest.

## Data availability

All data included in this study are available upon request by contacting the corresponding author.

The data supporting the findings of this study are available within the article. Fig. S1: total ion chromatogram (TIC) of *P. sicula*, *P. undulata* and *P. gnaphalodes*; Table S1: correlation between total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activity as measured by the ABTS assay. See DOI: <https://doi.org/10.1039/d5ra03980k>.

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