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Research Article

Exploring the Antimicrobial and Antioxidant Properties of *Silybum marianum* Tissue-cultured Phenolic Extracts

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Abstract
This study contributes to understand the medicinal properties of *Silybum marianum* L., commonly known as milk thistle. The result demonstrates the plant's antioxidant and antibacterial activities have the potential to significantly impact the fields of botany, pharmacology, and medicine. The study was initiated by culturing different parts (root, stem, cotyledonary leaves, and leaves) on a Murashige and Skoog (MS) medium supplemented with benzyl adenine (BA) and naphthalene acetic acid (NAA) for callus induction. One of the key findings of this study is the effectiveness of the 0.5 mg L^{-1} BA and 2.0 mg L^{-1} NAA combination for callus initiation. As revealed by the results, this combination was the most effective among the various combinations tested. The study involved extracting the explants: the leaf, cotyledon, stem, root, callus of the leaf, and cotyledon of *S. marianum* L. The extraction process used a hexane solvent to remove fat. An ethanol solvent for phenolics separation after hexane was applied to the same sample. The high-performance liquid chromatography (HPLC) results showed that all the explants contain various phenolic compounds. The antibacterial activity has also been identified at 200 µg/mL concentrations against four pathogenic bacterial strains: *Salmonella typhi*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Staphylococcus aureus* by using the agar dilution method. The results showed significant cotyledonary leaf callus extract showed antibacterial activity, notably against *E. coli*, with a zone of inhibition measuring 31 mm. These extracts were also used as antioxidants. The root extract has antioxidant activity with a percentage of 79.0%. These extracts and their compounds could be used as a natural antioxidant and antibacterial source. As we unravel the full potential of plant tissue culture and the conventional antibiotics and antioxidants become less effective and the side effects of synthetic compounds rise, exploring plant-based solutions like those from the tissue culture of *S. marianum* could provide valuable and eco-friendly alternatives, and contribute to developing novel therapeutic agents from natural sources.

Keywords: *Silybum marianum* L., antioxidant, antibacterial, culturing explants, phenolic compounds, HPLC

1. INTRODUCTION

Silybum marianum L. (milk thistle) is a plant home in European, Asian, and American countries and it is spread globally. This medicinal plant, with a therapeutic history dating back 2000 years, is a testament to the importance of the plant in traditional medicinal use [1][2]. The *S. marianum* belongs to the family Asteraceae and is known by many names. From milk thistle to Mary thistle and Saint Mary's thistle to Cardus marianus, this plant's various names mirror its diverse uses and rich history, sparking curiosity about its potential. In Iran and Arabic countries, it is called Mary thiqhal [3][4]. *S. marianum* is a biennale, glabrous, pale-green, and spinescent plant with straight stems. The leaves of this plant have white spots around the veins. It has several pinnate parts, such as triangular -ovary parts [3]. *S. marianum* has simple, little-branched, or relatively thick branches that terminate to a green mass with longitudinal rakes. It grows up to 200 cm in height; the stem is usually hollow and conical. Its leaves are oval to lanceolate and either lobate or pinnate, with spiny edges and hairless, shiny green, and milk-white veins. The flower heads of this plant are up to 12 cm long and wide, with a red-purple colour [4][5]. *S. marianum* is a dioecious plant, meaning it has separate male and female flowers on different plants. Its seeds are spread by wind or animals, contributing to their wide distribution. Various studies have scientifically proven that *S. marianum* and its extracts have practical medical effects on many diseases, including renal protection, hypolipidemic hepatoprotection, anti-atherosclerosis activities, cardiovascular protection, prevention of insulin resistance, especially in cirrhotic patients, Alzheimer's prevention, cancer prevention, and liver disorders [6][7].

Despite the remarkable scientific progress in modern medicine, the traditional use of medicinal plants is still effective in maintaining human health, which is a testament to their enduring significance.

Table 1. Standards of phenolic compounds and their retention time.

Standards	Retention Time (min)	Concentration (ppm)	Area [1] (mAU.s)
Rutin	2.05	25	1203.65
Qurcetine	3.01	25	1598.80
Kaempferol	3.81	25	1652.65
Ferulic acid	4.23	25	1425.49
Caffeic acid	5.08	25	1741.05
Apigenin	5.85	25	1354.19
Gallic acid	6.21	25	1236.62
Luteolin	7.85	25	1320.25
p-Coumaric acid	8.50	25	1456.98
Chlorogenic acid	9.80	25	1230.65
Syringeic acid	11.05	25	1524.98
Sinapic acid	11.98	25	1321.02

Since ancient times, many wild plants have been utilized to combat various illnesses. Among this diverse array of medicinal plants, *S. marianum*, one of the most ancient plants systematically identified and used, has been widely employed as a natural remedy for various diseases. As a result of the importance of the plant *S. marianum* and the incredible history that this plant enjoys medicinally, and the significant demand for it by specialists in the field of pharmaceutical industries due to its diversity of secondary compounds [8]-[10]. The potential of *S. marianum* in producing natural compounds, especially phenolic compounds, in the laboratory is intriguing and promising. Also known as *in vitro* culture, it is a remarkable biotechnological technique that has revolutionized our ability to propagate and manipulate plant cells, tissues, and organs outside their natural environment. This method involves growing and maintaining plant material, such as cells or explants, in a sterile and controlled laboratory setting, free from external contaminants. Tissue culture is a tool for scientific inquiry and has a practical application with diverse uses. It plays a role in the large-scale production of bioactive compounds used in pharmaceuticals and other industries. The emergence of plant tissue culture has opened doors to a wide range of applications, including the large-scale production of bioactive compounds and the creation of genetically modified plants with desirable traits. This technology is poised to play an increasingly pivotal role in addressing global challenges, such as healthcare and bioprospecting [11]-[13]. *S. marianum* contains a mixture of the so-called flavonoids and flavonolignans, which are famous as silymarin. This active mixture includes silybin, silychristin, dihydrosilybin, and silydrianin in addition to a group of phenols. Phenolic compounds have a wide variety of chemical structures. They are characterised by unequal qualitative and quantitative distribution according to the species considered and the organs, tissues, and physiological stages [14]-[16].

S. marianum is a vital plant because of its antibacterial and antioxidant properties. Over the past few years, drug-resistant bacteria have risen because of the large-scale use of antibiotics. One of the main issues affecting the use of antibiotics is the resistance of many dangerous bacteria to them. Natural antimicrobial compounds that can replace synthetic antibiotics have been studied recently [17] -[19]. In addition to the above, the antioxidant activity of *S. marianum* is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers. In addition, they have a metal chelation potential, a process where the compounds bind to metal ions, preventing them from causing oxidative damage [16][20]. The study on natural antibacterials and antioxidants, particularly those found in *S. marianum*, is important as a successful

alternative in the pharmaceutical industry.

2. MATERIALS AND METHODS

2.1. Germination Experiment

The seeds of *S. marianum* were obtained from the local markets in Mosul. The seeds were washed with running tap water. Then, under the laminar air flow chamber, the seeds were surface sterilized according to slight modification [21]. The seeds were immersed for 15 min in a 2% sodium hypochlorite (NaOCl) solution; then, they were rinsed 3–5 times in sterile distilled water. Finally, the sterilized seeds were planted in a 250 mL glass jar containing 25 mL of MS (Phyto Tech LABS, USA) basal medium at a 3–4 seed/jar rate [22]. They were kept in the growth room under dark conditions at 25 ± 2 °C for the first five days (beginning germination Fig. 1(A)). Then, the seeds were transferred toa 16 h light/8 h dark photoperiod and 25±2 °C.

2.2. Establishment of Callus Cultures

Twenty-day-old aseptic seedlings (Fig. 1(B)) were used as a source of explants. Approximately 1.0–1.5 cm length of different explants (root, stem, cotyledonary leaves, and leaves) were inoculated on MS medium supplemented with growth regulator combination 0, 0.1, 0.2, 0.5, 1.0, and 2.0 mg/L of BA as well as 0, 0.1, 0.2, 0.8, 1.0, and 2.0 mg/L NAA. Solidification of the media was performed by adding 8 g/L agar, the pH was adjusted to 5.9, then all media were autoclaved for 15 min at 121°C. The specimens were incubated in the growth room under 16 h light/8h dark photoperiod at 25±2 °C. The callus was maintained initiated from all explants of *S. marianum* was maintained on the best medium of callus induction, and incubated in the growth room under 16 h light/8 h dark photoperiod at 25±2 °C.

2.3. *Extraction of Phenol*

The extraction of phenol involved a careful selection of organic solvents. The extraction process used a hexane solvent to remove fat and then, an ethanol solvent was used. After getting callus, the dried and powdered callus was treated with hexane for 5 h. The dried residue of the callus was washed with ethanol at 40 °C for 8 h. The ethanol was evaporated under a vacuum on a rotator evaporator instrument. And fractionated by column chromatography, Silica gel 60–120 mesh was used as the stationary phase. A colourless liquid (hexane) was used, and a slurry was prepared and poured into the column. The extract was added after mixing them homogeneously with a small amount of silica gel over the top of the column. The mobile phase/ eluent uses a precise combination of organic solvents, ranging from low to high polarity eluents, such as hexane, ethyl acetate, and ethanol. At the end of the process, we get several fractions of each extract.

The fractions were analyzed using thin-layer chromatography (TLC). From each extract, a specific fraction was chosen that has a known composition and does not contain any unknown substances. This selected fraction corresponds to the explant. The TLC test was performed on aluminium foil plates coated with 0.20 mm layers of silica gel 60F254 (Merck, Germany) to detect the presence of phytochemicals. Before use, the plates were activated at 80 °C for 25 min. Each extract

Figure 1. Seed germination of *Silybum marianum* (A) germinated seeds on the surface of a hormone-free MS medium (5 days old) and (B) 20-day-old seedlings on hormone-free MS medium.

Table 2. Callus initiation from different explants of *Silybum marianum* on MS medium supplemented with various concentration of BA and NAA.

BA (mg/ L)	NAA (mg/L)	Callus initiation percentage* (%)				Period of callus initiation (day)			
		Roots	Stems	Cotyl. Leaves	Leaves	Roots	Stems	Cotyl. Leaves	Leaves
0	0	0	0	0	0	0	0	0	0
0.1	0.2	66	50	100	88	10	11	10	8
0.2	0.1	63	25	37	44	3	6	7	7
0.5	0.8	88	83	87	70	6	11	10	8
0.5	2.0	100	100	100	80	8	13	9	12
1.0	2.0	62	14	100	50	11	10	10	9
2.0	1.0	40	50	100	100	10	10	10	10

Note: *Five replicates

was precisely measured at 2 µL and spotted onto the chromatographic plates using a micropipette, ensuring accurate loading. The TLC plate was developed using three mobile phases: chloroform, ethyl acetate, and formic acid (10:8:2 v/v/v). The chamber was pre-saturated with mobile phase vapors for 30 min at 20 ± 1 °C to ensure the TLC plate was evenly saturated with the mobile phase, which is crucial for a uniform separation. Components were identified using a UV-visible spectrophotometer. The selected fractions were further analysed using the high-performance liquid chromatography (HPLC) technique to confirm the identity of phenolic compounds.

2.4. *HPLC Conditions*

The HPLC technique was used to characterize the extracted phenolic components. Characterized by its high sensitivity and speed, HPLC is one of the best analytical systems for separating and characterizing isolated compounds from plants, such as phenolic compounds. The analytical HPLC system (reversed-phase HPLC with silica-based C18; an SYKAM HPLC system, Germany) was used to conduct the analysis. The samples (100 µL) were injected into the system. The mobile phase used was 95% acetonitrile + 0.01% trifluoroacetic acid (solvent A) and 5% acetonitrile + 0.01% trifluoroacetic acid (solvent B), which were combined to form the mobile phase, which had a flow rate of 1 mL/min. The gradient program was meticulously designed and executed, ensuring a thorough analysis that leaves no room for doubt: 10% A from 0 to 5 min, 25% A from 5 to 7 min, 40% A from 7 to 13 min, and then returning to initial conditions. A UV-visible detector at 278 nm identified phenolic chemicals. The identity of standard compounds is confirmed by matching the retention times of respective standards with those of the peaks in the extract, as shown in Table 1 [23].

2.5. *Origin and Selection of Microbial Strains*

The *S. marianum* extracts were meticulously examined against highly pathogenic microorganisms. This study carefully selected four human pathogenic bacteria to screen the *in vitro* antimicrobial activities. After a rigorous selection, all microbial strains were obtained from our laboratory stock culture. Gram-positive strains,

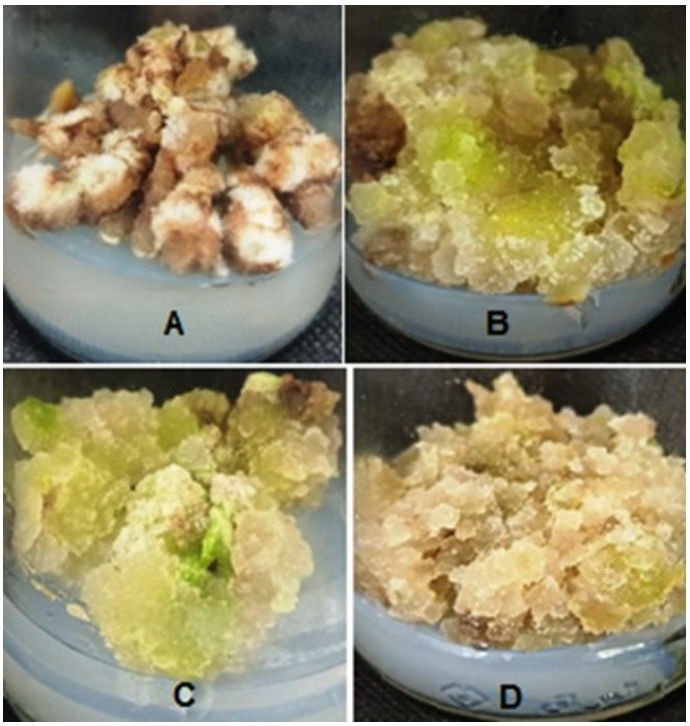


Figure 2. Callus induction from different explants of *Silybum marianum* on Ms medium + 0.5 mg L^{-1} BA +2.0 mg L^{-1} NAA. (A) Callus of root explant (30 day-old), (B) Callus of the stem(30-day-old), (C) Callus was initiated from cotyledonary leaves (30 days old), and (D) Callus of leaves explant (30-day-old).

including *Pseudomonas aeruginosa* (ATCC 9027) and *Staphylococcus aureus* (ATCC 6538), and Gram-negative strains, including *Salmonella typhi* (AATCC 6539) and *Escherichia coli* (ATCC 8739), were chosen for their relevance to the study.

2.6. Preparation of the Inoculums

The bacterial strains were grown and cultured onto the nutrient agar (NA), favorable for their growth (Mueller-Hinton broth) at 37 °C for 24 h with utmost precision. Following incubation, they were sub-cultured before any antimicrobial test. Bacteria were suspended in a sterile saline solution (0.85% NaCl) to prepare inoculums. The optical density (OD) of the suspensions was meticulously adjusted/maintained from 0.4 to 0.6 at 405 nm, which corresponds to a cell density close to that of 0.5 McFarland, matching an inoculum estimated at 10^6 to 10^8 colony forming units per mL (CFU/mL) [24].

2.7. Disk Diffusion Method on Agar

The conventional disc diffusion technique was carried out with meticulous attention to detail. Using a sterile swab, the Mueller-Hinton agar (MHA) plates were streaked by previously prepared inoculums. Then, 200 µg/mL of each extract were impregnated onto sterilized paper discs (6 mm, Whatman paper N5) in solvent (10% v/v dimethyl sulfoxide and 1% v/v tween 80 in deionized water). Under the same conditions, the antibiotics amikacin and gentamycin (5 g/mL) and the same solvent (10% v/v dimethyl sulfoxide and 1% v/v tween 80 in deionized water) employed in the dilution of extract were used as positive and negative control respectively. The plates were maintained at room temperature and then incubated for 24 h at 37 C. In the end, antibacterial activity was evaluated by measuring the nearest surrounding diameter of resulting inhibition zones (around and including disc diameter). Each experiment was done in triplicates, ensuring the thoroughness of the testing process [25].

2.8. DPPH Radical Scavenging Assay

The DPPH radical scavenging assay, a widely used method, is the primary tool for assessing antioxidant activity. A 0.04 g of DPPH was dissolved in 100 mL of methanol to get the concentration of 400 µg/mL. A meticulously prepared standard solution (vitamin C) was used, and a sample of 0.5 g was mixed with 100 mL of

methanol and distilled water. The other concentrations (330, 60, 120, 250, and 500 ppm) were made from vitamin C and samples (30, 60, 12, 250, and 500 ppm) using the scientifically rigorous dilution law. The concentration of the standard solution was 5000 ppm. After giving the mixture a good shake, it was left to stand at room temperature for 30 min. The absorbance was measured at 517 nm with a UV-VIS Shimadzu spectrophotometer. The IC_{50} value, representing the sample concentration needed to inhibit 50% of the DPPH free radical, is calculated using the log dose inhibition curve. A lower absorbance of the reaction mixture indicates higher free radical activity [26]. The formula used to calculate the % DPPH scavenging effect is as follows Eq. 1.

$$\text{DPPH scavenging effect (\%)} = \frac{(A0 - A1)}{A0} \times 100\% \quad (1)$$

Here, A_0 represents the absorbance of the blank and A_1 represents the absorbance in the presence of the test sample. This formula provides a clear and precise method for determining the DPPH scavenging effect, a vital aspect of the assay's results.

3. RESULTS AND DISCUSSIONS

3.1. Sterilisation Efficiency of Seeds and Production of Sterile Seedlings

The results indicated that various explants of *S.marianum* showed a good response for callus induction (Table 2). The best combination for root, stem and cotyledonary leaves was CI4 (MS medium containing 0.5 mg L^{-1} BA and 2.0 mg L^{-1} NAA (Fig. 2) with the percentage of callus induction of %100. The same medium was suitable for leaves, which gave a percentage of callus initiation of 80%. This medium was also used to maintain the callus. The color of the callus was graded between white to yellow and brown with a semi-reliable texture. Many studies indicated that callus colors and texture are closely related to the percentage of auxins and cytokinins added to the media [27].

Callus induction was affected by the type and concentrations of growth regulators in the culture media. Previous work mentioned that the optimum medium for callus induction from leaves explants of *S.marianum* was MS medium containing 0.25 mg L^{-1} of 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin (Kin) [28]. Furthermore, the B5 medium was enriched with 0.05 mg L^{-1} BA and 0.5 mg L^{-1} 2,4-D was the best medium for cotyledon callus production [29]. Interestingly, MS medium supplemented with 5.0 µM NAA and 2.5 µM BA and supported by adding 10% coconut water gave a friable callus from root explants of *S.marianum* [13].

3.2. Phenolic Content of S. marianum

Table 3 comprehensively analyses various phenolic compounds found in different plant extracts. The extracts analyzed, including leaf, cotyledon, stem, root, and callus from leaf, cotyledon, stem, and root, present a diverse range of plant materials for study. HPLC analysis results showed that the extracts contained the following phenolic compounds: rutin, quercetin, kaempferol, apigenin, gallic acid, syringic acid, ferulic acid, caffeic acid, p-coumaric acid, chlorogenic acid, luteolin, and sinapic acid, as shown in Fig. 3.

In the leaf extract, the identified compounds include rutin (14.7999 ppm, 2.05 min), quercetin (10.8070 ppm, 3.08 min), kaempferol (19.9373 ppm, 3.89 min), apigenin (11.1584 ppm, 5.80 min), gallic acid (13.0415 ppm, 6.25 min), and syringic acid (11.8737 ppm, 11.02 min) across six peaks. The cotyledon extract contains quercetin (17.8221 ppm, 3.06 min), kaempferol (7.7750 ppm, 3.88 min), apigenin (11.8686 ppm, 5.87 min), gallic acid (14.7666 ppm, 6.25 min), luteolin (11.4381 ppm, 7.89 min), *p*-coumaric acid (16.4242 ppm, 8.52 min), and sinapic acid (12.7288 ppm, 11.98 min) over seven peaks.

For the stem extract, the phenolic compounds include rutin (13.5379 ppm, 2.02 min), quercetin (16.9259 ppm, 3.05 min), kaempferol (16.7038 ppm, 3.88 min), ferulic acid (5.7944 ppm, 4.25 min), luteolin (15.7266 ppm, 7.82 min), *p*-coumaric acid (16.4242 ppm, 8.58 min), chlorogenic acid (23.8681 ppm, 9.90 min), and sinapic acid (19.0012 ppm, 11.95 min) across eight peaks. The root extract includes quercetin (11.5667 ppm, 3.05 min), ferulic acid (15.8578 ppm, 4.25 min), caffeic acid (8.6891 ppm, 5.08 min), apigenin (13.4959 ppm, 5.80 min), gallic acid (12.9431 ppm, 6.20 min), and luteolin (11.4037 ppm, 7.89 min) in six peaks.

The callus of leaf extract comprises rutin

Table 3. Phenolic profiling.

Extracts	Phenolic compounds					
	Rutin	**Quercetin**	**Kaempferol**	**Ferulic acid**	**Apigenin**	**Gallic acid**
Leaf	+	+	+	-	+	+
Cotyledon	+	+	-	+	+	-
Stem	-	+	+	-	+	+
Root	+	+	+	-	-	-
Callus of leaf	-	+	-	+	+	-
Callus of cotyledon	+	-	+	+	-	+
Callus of stem	-	-	+	+	-	+
Callus of root	+	+	-	+	+	+
Extracts	**Caffeic acid**	**Luteolin**	***p*-Coumaric acid**	**Chlorogenic acid**	**Syringeic acid**	**Sinapic acid**
Leaf	-	-	-	-	+	-
Cotyledon	+	-	+	+	-	-
Stem	-	+	+	-	-	+
Root	-	+	+	+	-	+
Callus of leaf	+	-	-	-	-	-
Callus of cotyledon	-	+	-	+	+	-
Callus of stem	+	-	-	+	+	+
Callus of root	-	-	+	-	+	+

Note: + Presence; - Absence

(18.9830 ppm, 2.05 min), kaempferol (11.0493 ppm, 3.80 min), ferulic acid (12.7072 ppm, 4.25 min), gallic acid (12.1896 ppm, 6.20 min), luteolin (18.9792 ppm, 7.88 min), chlorogenic acid (10.7097 ppm, 9.89 min), and syringic acid (19.0319 ppm, 11.08 min) with seven peaks. In the callus of cotyledon extract, the identified compounds are kaempferol (12.4819 ppm, 3.88 min), ferulic acid (11.2671 ppm, 4.28 min), caffeic acid (10.6216 ppm, 5.08 min), gallic acid (23.6045 ppm, 6.28 min), chlorogenic acid (22.2604 ppm, 9.89 min), syringic acid (11.0379 ppm, 11.08 min), and sinapic acid (18.1281 ppm, 11.90 min) over seven peaks. Finally, the callus of stem extract contains rutin (24.5004 ppm, 2.08 min), quercetin (14.1401 ppm, 3.05 min), apigenin (13.6560 ppm, 5.88 min), gallic acid (12.1883 ppm, 6.25 min), *p*-coumaric acid (13.2198 ppm, 8.58 min), syringic acid (10.5324 ppm, 11.89 min), and sinapic acid (21.7479 ppm, 11.94 min) across seven peaks.

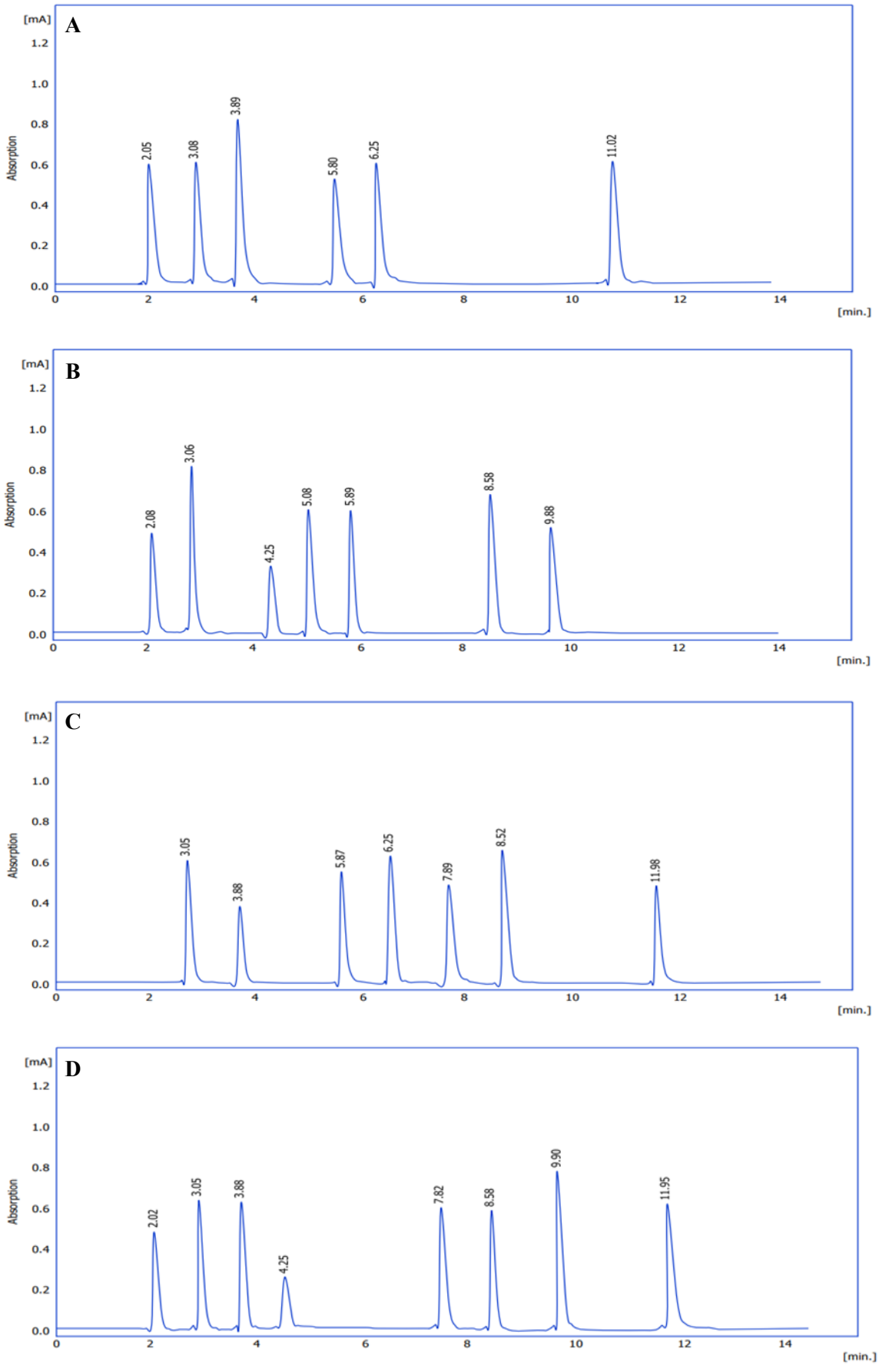
Comparing the extracts, quercetin and gallic acid are consistently present across most extracts, highlighting their commonality. In contrast, unique compounds such as chlorogenic acid in the callus of cotyledon and the callus of leaf, and sinapic acid in cotyledon, the callus of cotyledon, the callus of leaf, and the callus of stem provide distinct profiles. These compounds' retention times and concentrations vary significantly, reflecting the diversity in phenolic composition across different plant parts and their callus forms.

Phenols are widely distributed in the plant world, present in all parts of higher plants, and the great distinction between the parties appears in the level of wealth of some and the poverty of others. Previous studies have shown that the extraction yield increases significantly with ethanol or methanol compared to other organic solvent extractions. Whatever the extraction mode, ethanol is the best phenol extractor [30]. Some studies indicate that the highest percentage of phenols was found in the leaves and stems of the *S. marianum* plant, where it reached 21.79 and 17.29 mg GAE/g, respectively. On the other hand, flavonoids appeared in the leaves and stem at a rate of 129.66 and 114.29 QE/g, followed by seed extracts of 24.72 mg QE/g, respectively [16]. Another study showed that phenolic compound content in the seeds reached 2,582 and 3,911 mg/100 g, while the content of phenolic compounds in the extracts of the green group reached 23,337 mg GAE/100 g. A previous work conducted a study on the ethanol extract of milk thistle seeds revealed a total phenolic content of 620.0±4.93 µg GAE/g and a flavonoid content of 39.32±0.11 µg QE/g [31]. These values point to the ethanol extract of milk thistle seed as a source of potent antioxidant activity [32]. Serçe et al. described the content of phenolics and flavonoids in milk thistle [33]. Their study used milk thistle plants as dry material for infusion and pill preparation. The phenolic and flavonoid contents (expressed as GAE and catechin equivalent, respectively) were found to be 23.26±0.22 and 6.95±0.23 mg/g in infusions; and 20.92±0.45 and 3.88±0.13 mg/g in dietary supplements, respectively.

On the other hand, Tupe et al. reported a phenolic content of a methanolic extract of milk thistle was 18.33±0.16 mg GAE/g [34]. The high contents of phenolics and flavonoids obtained in infusions, dietary supplements, and methanolic milk thistle plant extract may depend on the part of the plant used. Phenolics and flavonoids are critical plant components. Giuliani et al. reported the relative content of total phenolics, flavonoids and condensed tannins in whole fruits and the different fruit fractions for all the investigated accessions of *S. marianum* [35]. These findings have significant implications for the field of antioxidants and antibacterial. For instance, chemotypes A and B display comparable relative amounts of total phenolics; the same trend is observed for flavonoids. The mutant C shows the highest contents for both total phenolics and flavonoids compared to the wild types (19.97%±0.23 and 8.05%±0.10, respectively), suggesting potential areas for further research. Regarding condensed tannins, chemotype A is characterized by the highest level (0.41%±0.01); chemotype B and mutant C presented the exact amounts (0.29% ±0.01).

3.3.Antibacterial Assay

Fig. 4 presents the antimicrobial activity of *S. marianum* explants extracts at a concentration of 200 µg/mL, measured by the zone of inhibition against four bacterial strains: *P. aeruginosa*, *S. typhi*, *S. aureus*, and *E. coli*. Extracts leaf,



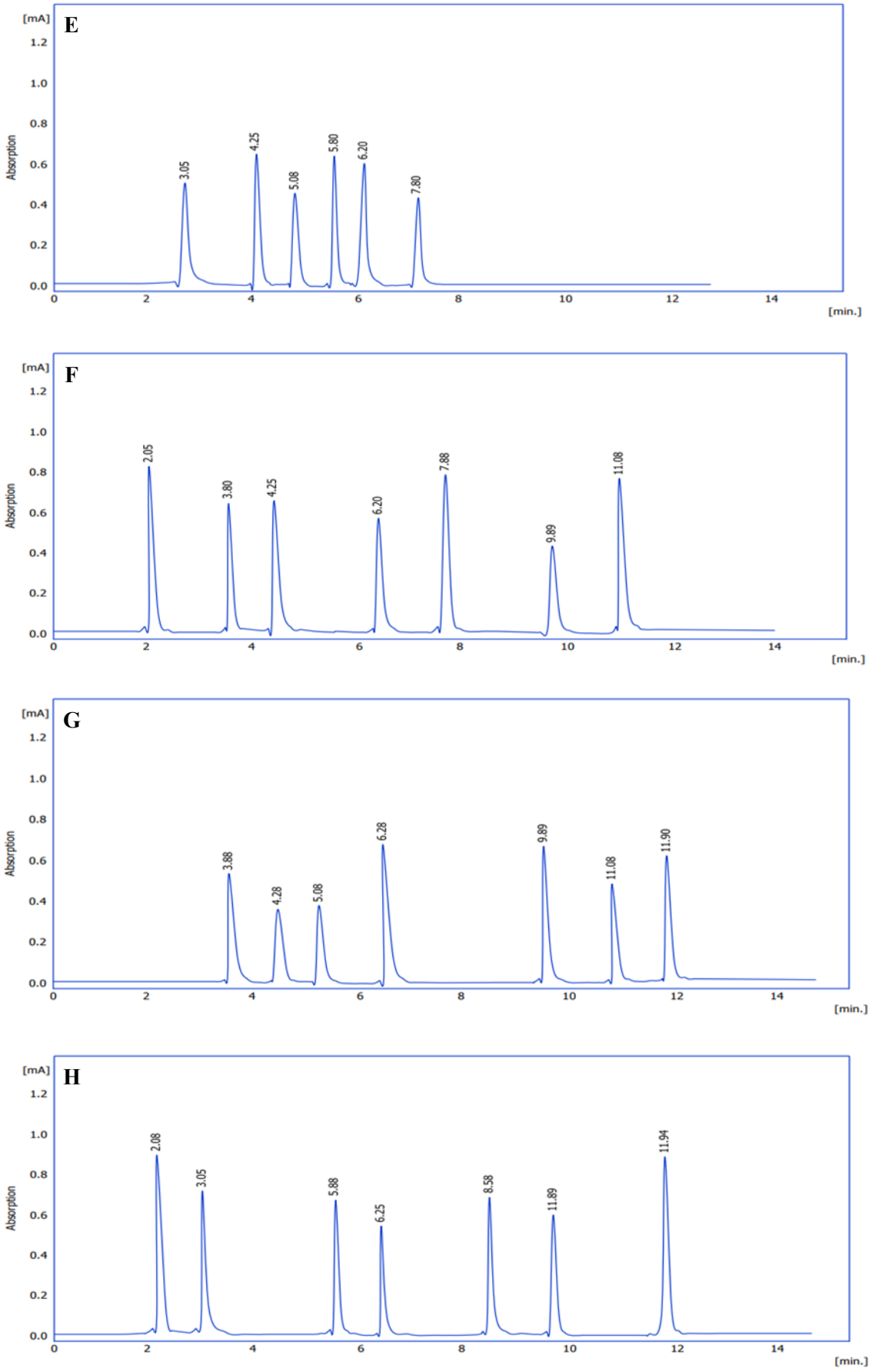


Figure 3. HPLC chromatogram of extracts (A) leaf, (B) cotyledon, (C) stem, (D) root, (E) callus of leaf, (F) callus of cotyledon, (G) callus of stem, and (H) callus of root.

cotyledonary leaf, stem and root showed no activity, with all zones of inhibition at 0 mm except for extract leaf against *S. typhi*, which had a zone of 12 mm. However, extract leaf callus stood out with its significant inhibition, measuring 23 mm against *P. aeruginosa*, 16 mm against *S. typhi*, 26 mm against S. aureus, and 19 mm against *E. coli*, impressing its potential. Extract cotyledonary leaf callus was the most effective overall, with inhibition zones reaching 31 mm against *E. coli*, 27 mm against *S. aureus* and 25 mm against *S. typhi*. Stem and root callus extracts showed moderate effectiveness, with inhibition zones ranging from 15 to 27 mm. The standard compounds (amikacin and gentamycin) consistently demonstrated good antimicrobial activity across all strains, with zones between 20 and 22 mm, highlighting the overall potential of these extracts against bacterial infections.

The irresponsible use of antibiotics by people suffering from bacterial infections has led to the emergence of resistant types of bacteria. Furthermore, the excessive use of these chemicals and their significant side effects highlights the potential of plant species as crucial antimicrobial alternatives. Numerous studies have emphasized the promising nature of these natural alternatives [36]. The survey of Yildiz showed that using an hexane extract from *S. marianum* seeds obtained from Türkiye is effective against many types of pathogenic bacteria [37]. Such as *S. typhi*, *E. coli*, *P. aeruginosa*, and *S. aureus* at different concentrations. Ahmad et al. showed that hexane, chloroform, and methanol extracts separated from *S. marianum* were obtained from other parts of Pakistan [38]. These results showed that the three extracts inhibited the growth of *Salmonella* spp., E at various concentrations. Another study used extracts of fruit parts of *S. marianum* in the form of EtOH, MeOH, and DCM extracts. Plant extracts were generally more effective against Gram-negative bacteria *A. baumannii*, *P. aeruginosa*, and *E. coli* at 25–400 µg/mL concentrations. As a result, it was determined that the fruit parts of *S. marianum* could be a natural antibiotic against the tested microorganisms [39]. Gök et al. study showed that oil extracts obtained from the seeds of *S. marianum* L. inhibited the growth of *P. mirabilis* (11 mm), *P. aeruginosa* (12 mm), and *L. pentoses* (13 mm) [40].

The ethanol extract containing the compound was more effective in inhibiting the growth of *L. plantarum* (14 mm) and *L. pentoses* (13 mm) species. Besides, it was detected that there was an antimicrobial effect against *B. subtilis* (12 mm). In another study by Abed et al., the results showed the sensitivity of the bacteria Gram-positive and Gram-negative towards *S. marianum* extracts [41]. The toxicity of these extracts can be attributed to the presence of phenolic compounds. These compounds function by establishing bonds, such as hydrogen

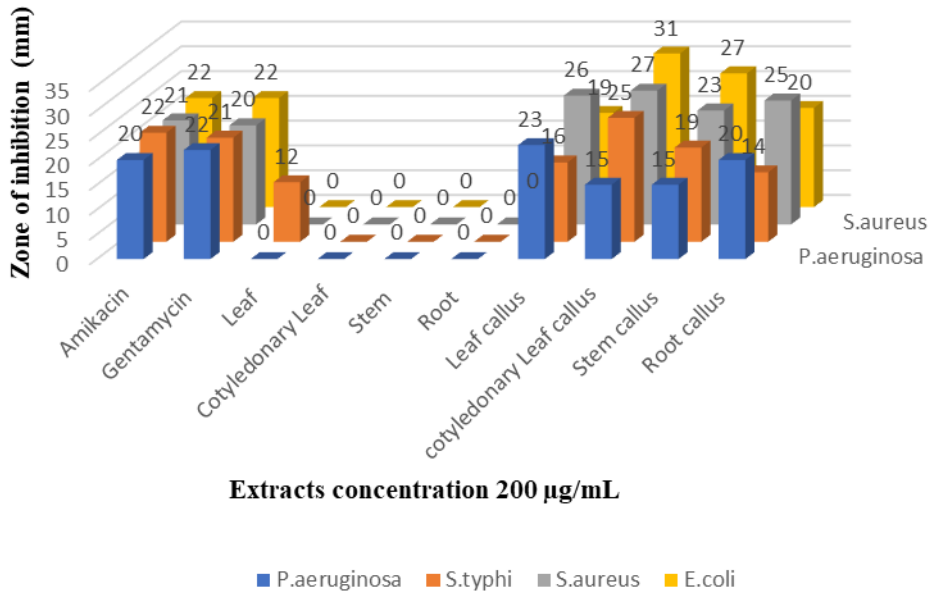


Figure 4. Antimicrobial activity of *S. marianum* explant extracts.

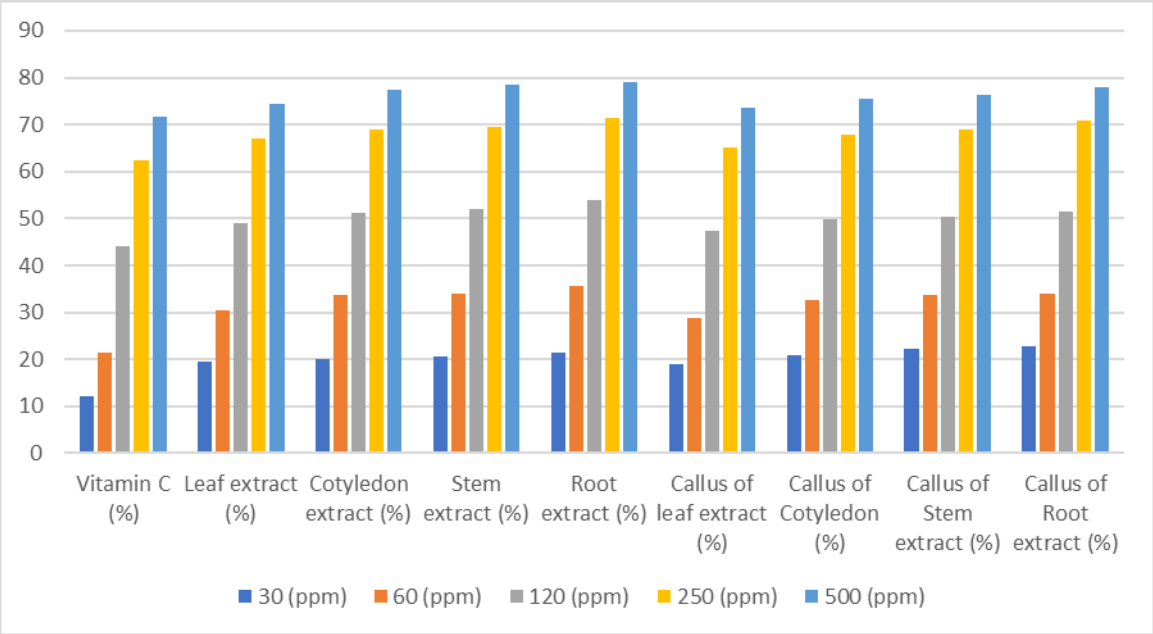


Figure 5. Antioxidant and free radical activity of *Silybum marianum* explants extracts.

bonds with the cell wall proteins or enzymes, chelation of metal ions, inhibition of bacterial metabolism, and sequestration of substances necessary for bacterial growth. A key mechanism of action is the role of the β-ring of flavonoids in intercalating with nucleic acids, thereby inhibiting DNA and RNA synthesis. This action, in particular, can inhibit the DNA gyrase of *E. coli*. Thus, it inhibits the growth of bacteria.

3.4. Antioxidants Assay

S. marianum, commonly known as milk thistle, is a plant rich in antioxidants, offering a promising avenue for various health benefits. Its high phenolic compounds contribute to potent antioxidant properties, making the extract a valuable natural antioxidant. Our study has demonstrated these phenolic compounds' antioxidant and radical scavenging mechanisms, which could reduce chronic diseases. These findings open up new possibilities for combating these health issues, instilling a sense of hope and optimism in medicine.

Fig. 5 presents data on the antioxidant activity (AA%) at varying concentrations of extracts. For vitamin C, the AA% at 30, 60, 120, 250, and 500 ppm are 12.15, 21.55, 44.18, 62.47, and 71.55, respectively. The Leaf extract shows AA% of 19.5, 30.5, 48.9, 66.9, and 74.5 at the same concentrations. For the cotyledon extract, the values are 20.1, 33.6, 51.2, 68.9, and 77.5. The stem extract exhibits AA% of 20.5, 33.9, 52.0, 69.4, and 78.4. The root extract has AA% values of 21.5, 35.6, 53.9, 71.5, and 79.0. The callus of leaf extract shows 19.0, 28.9, 47.5, 65.0, and 73.6. For the callus of cotyledon extract, the AA% are 21.0, 32.5, 49.8, 67.9, and 75.4. The callus of stem extract presents AA% values of 22.3, 33.6, 50.4, 68.9, and 76.4. Lastly, the callus of root extract exhibits AA% of 22.8, 33.9, 51.5, 70.9, and 77.9 at 30, 60, 120, 250, and 500 ppm, respectively.

Medicinal plants produce various secondary metabolites, of which phenolic compounds are essential antioxidants. These compounds' antioxidant activity is mainly due to their redox properties, which allow them to act as reducing agents or hydrogen-atom donors. Thus, natural antioxidants function as free-radical scavengers. Radical scavengers may directly react with and quench peroxide radicals to terminate peroxidation chain reactions and improve the quality and stability of products [42]. Assays based on DPPH radicals are among the most popular spectrophotometric methods for determining extracts' natural antioxidant capacity. Additionally, DPPH scavenging methods have been used to evaluate the antioxidant activity of natural compounds due to their simple, rapid, sensitive, and reproducible procedures. Chemical assays are based on the ability to scavenge synthetic free radicals using various radical-generating methods to detect

and reveal the ability of natural compounds, especially phenolics, to scavenge free radicals [43]. *S. marianum* is used for medicinal purposes to treat various diseases due to the presence of antioxidants, total phenolics, and flavonoids. Studies have reported the biological activities of silymarin seed oil, such as its ability to scavenge free radicals and the fact that it contains linoleic (omega-6) and oleic (omega-9) acids involved in preventing atherosclerosis, diabetes, and cancer, lung, and hepatocellular cancers.

Some other active components of *S. marianum* seeds are silybonol, apigenin, betaine, proteins, and free fatty acids. Moreover, the oil obtained from milk thistle seed has also been suggested as a rich source of vitamin E and a probable source of natural antioxidants. This antioxidant activity is significantly higher than artificially prepared flavonoids/flavonolignans [16]. A study by Serçe et al. [33] showed that the *S. marianum* extract demonstrated a strong DPPH radical scavenging ability in a concentration-dependent manner. The survey showed that the radical scavenging activities of *S. marianum*, with values of 92.0±5.2, 88.0±5.7, and 87.8±2.1 at 150 µg/mL, are significant as they indicate the potential of *S. marianum* as a powerful antioxidant. Javeed et al. [16] explained that the inhibition percentage (IC_{50}) results showed that all the extracts used in the study, such as the seeds, stem, and leaves, inhibited free radicals, conferring significant antioxidant activity in percentages of 75.98, 72.39, and 63.21%, respectively. The IC_{50} value represents the concentration of a substance required to inhibit a specific biological or biochemical function by 50%. A stem extract of 51.40 mmol/g afforded the test-reducing activity, followed by seed and leaf extracts of 46.60 and 41.30 mmol/g, respectively. In another study, the antioxidant activity of silymarin extracts and DPPH radical scavenging activity ranged from 2,866 to 4,239 µmol ascorbic acid/100 g dw (CV = 9.4%) [44]. A study shows that the significant variations in the DPPH-scavenging activity of the silymarin extracts across different milk thistle populations are particularly intriguing in the pharmaceutical industry. The DPPH radical scavenging activity, a vital aspect of the study, was found to be 3,489 µmol ascorbic acid/100 g dw across all populations. The substantial difference in TPC content among milk thistle populations indicates genetic diversity among different milk thistle populations, which may influence their antioxidant properties. The average TPC content across all populations was 2,477 mg GAE/100 g dw, with a CV of 13.3%. TPC content in several milk thistle genotypes ranged from 206 to 360 mg GAE/100 g. The total phenolic content in silymarin extracts is a crucial determinant of their antioxidant activity. Researchers have reported that silymarin extracts' antioxidant capacity is due to the synergic activity of phenolic constituents and flavonoids [45][46].

4. CONCLUSIONS

This study determined the antibacterial and antioxidant activities of *S. marianum* explants by culturing different explants (root, stem, cotyledonary leaves, and leaves) on a MS medium supplemented with BA and NAA for callus induction. The HPLC technique was used to separate and characterize phenolic compounds. The study determined that different plant explants could be a natural antioxidant and antibacterial source.

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Conflicts of Interest

The authors declare no conflict of interest.

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