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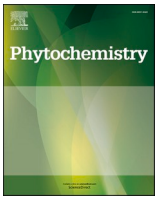

The chemical composition of the aerial parts of *Stachys spreitzenhoferi* (Lamiaceae) growing in Kythira Island (Greece), and their antioxidant, antimicrobial, and antiproliferative properties

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ABSTRACT

The *Stachys* L. genus has been used in traditional medicine to treat skin inflammations, stomach disorders, and stress. The aim of this study was to investigate the chemical profile and biological activity of the methanolic extract of *Stachys spreitzenhoferi* Heldr. (Lamiaceae) aerial parts, collected on the island of Kythira, South Greece. The analysis by liquid chromatography coupled with electrospray ionization and high-resolution mass spectrometry [LC-(−)ESI/HRMS^n] of the methanol extract revealed the occurrence of thirty-six compounds - flavonoids, phenylethanoid glycosides, iridoids, quinic acid derivatives, aliphatic alcohol glycosides, and oligosaccharides – highlighting the substantial presence, as main peaks, of the iridoid melittoside (**2**) along with flavonoid compounds such as 4′-*O*-methylisoscutellarein mono-acetyl-diglycoside/chrysoeriol mono-acetyl-diglycoside (**24**), trimethoxy- (**35**) and tetramethoxyflavones (**36**). This extract was tested for its antimicrobial properties against Gram-positive and negative pathogenic strains. The extract was not active against Gram-negative bacteria tested, but it possessed a good dose-dependent antimicrobial activity towards *S. aureus* (MIC: 1.0 mg/mL) and *L. monocytogenes* (MIC: 1.0 mg/mL) Gram-(+) strains. Furthermore, this extract has been tested for its possible antioxidant activity *in vitro*. In particular, it has been shown that these molecules cause a decrease in DPPH, ABTS, and H_2O_2 radicals. The extract of *S. spreitzenhoferi* exhibited anti-DPPH activity (IC_{50}: 0.17 mg/mL), anti-H_2O_2 activity (IC_{50}: 0.125 mg/mL), and promising antiradical effect with an IC_{50} value of 0.18 mg/mL for anti-ABTS activity. *S. spreitzenhoferi* extract caused a decrease in ROS (at the concentration of 200 μg/mL) and an increase in the activity of the antioxidant enzymes SOD, CAT, and GPX in OZ-stimulated PMNs. Furthermore, it exhibited antiproliferative activity against acute myeloid leukemia (U937 cell), causing 50% of cell death at the 0.75 mg/mL.

1. Introduction

Stachys L. is a genus distributed in temperate and tropical regions of the Mediterranean, Asia, America, and southern Africa, belongs to the Lamiaceae family and comprehends more than 300 species. It is characterized by a particular inflorescence called "spike of corn", so peculiar as to give the name to the entire genus, deriving from the Greek word (στάχυς) «stachys». One of the two subgenera, *Stachys*, includes 19 sections, while the other one, Betonica, is composed of 2 sections (Bhattacharjee, 1980). Folk medicine reports about its use to treat skin

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inflammations, stomach disorders, stress, and genital tumors. The traditional uses, phytochemistry, and bioactivity of this genus have been reviewed (Tomou et al., 2020; Tundis et al., 2014) as well as the occurrence of diterpene metabolites both in essential oils and non-volatile extracts (Piozzi and Bruno, 2009, 2011).

Among the various numerous species, there is a quite rare one, *S. spreitzenhoferi* Heldr., growing wild in Kythera and SE Laconia, in Elafonisos and the Maleas peninsula (Greece) (http://www.worldfloraonline.org/taxon/wfo-0000314628), on rock crevices, walls, limestone, and other sedimentary rocks, at altitudes of 5–50 m (Greek flora, 2021). It is a perennial semi-shrubby species, with tufts like "cushions" and airy shoots from 5 to 20 cm high. Its leaves are hairy and have serrated margins, colored gray-green on the upper surface and whitish on the lower, flowers are found in groups of 4–6, one on top of the other, at the edge of the shoots, showing white petals that form a tube with 2 lips, the upper dark purple and the lower with dark purple-red shapes.

On the mainland, it is possible to find another variety, the *Stachys spreitzenhoferi* subsp. *spreitzenhoferi*, accepted name of an infraspecific taxon of the species *Stachys spreitzenhoferi* Heldr. (http://www.worldfloraonline.org/taxon/wfo-0000314629), with different leaves, sparsely pubescent and green, that belongs, together with the other taxa, to section *Candidae* R. Bhattacharjee of the *Stachys* genus, also including *S. candida* Bory & Chaub., *S. chrysantha* Boiss. & Heldr., *S. saxicola* Coss. & Balansa and *S. iva* Griseb., the last one being assigned to subsection Stenophyllae Krestovsk (Table S1) (Krestovskaya, 2017).

S. candida (Skaltsa et al., 2000; Michailidou et al., 2021), *S. chrysantha* (Skaltsa et al., 2000; Michailidou, 2018), and *S. iva* (Lazarević et al., 2010; Pritsas et al., 2021) have been studied as far as it concerns the occurrence of flavonoids, phenylethanoid glycosides, phenolic derivatives, and iridoids, etc., while there are no data available about *S. spreitzenhoferi.* There are different studies that have investigated the biological activities of different *Stachys* plants. *S. anisochila* Vis. & Pancic, *S. beckeana* Dorfler & Hayek, *S. plumosa* Griseb. and *S. alpina* L. ssp. *dinarica* exhibited high anti-DPPH activity (IC_{50} < 50 μg/mL). Furthermore, *S. plumosa* extract achieved maximal activity of 60.67% at 100 μg/mL (Kukić et al., 2006). The ethanolic extract of *S. riederi* var. *japonica,* at 100 μg/mL, significantly inhibited the production of reactive oxygen species in ultraviolet A-irradiated human dermal fibroblasts (Hwang et al., 2019). Finally, two flavone glycosides and fatty acids isolated from the aerial parts of *S. byzanthina* C. Koch., exhibited moderate activity against Vero (African green monkey kidney), HeLa (human uterus carcinoma) and C6 (rat brain tumor) cells *in vitro*, compared with 5-fluorouracil (5-FU) (Demirtas et al., 2013).

Chemotaxonomic studies should be encouraged to help to distinguish and divide genera and species with certainty, as well as to clarify the relation of speciation processes with their geographical and climatic context. With a view to providing further information and data to our research on Mediterranean plants (Bruno et al., 2019; Gagliano Candela et al., 2021; Badalamenti et al., 2021a; D'Agostino et al., 2021; Catinella et al., 2021; Loizzo et al., 2021; Ilardi et al., 2022) and on biological properties (Rosselli et al., 2020; Sut et al., 2020; Badalamenti et al., 2022), and considering the promising activities shown by the different *Stachys* investigated at the biological level, MeOH extract of the aerial parts of *S. spreitzenhoferi* Heldr., collected on the cliffs of Avlemonas in the island of Kythira, South Greece, was studied to determinate its chemical composition as well as its antioxidant, antimicrobial, and antiproliferative properties.

2. Results and discussion

2.1. Analysis by LC-(−)ESI/HRMS^n of the MeOH extract of the aerial parts of S. spreitzenhoferi

In order to investigate the main chemical constituents of *S. spreitzenhoferi*, the LC-(−) ESI/HRMS^n analysis of the extract of the aerial parts was carried out (Fig. S1). The careful study of accurate masses, molecular formulae, and fragmentation patterns in comparison with literature data allowed to tentatively identify thirty-six compounds, mainly belonging to the classes of flavonoids (19, 22, 24, 25, 27–36), phenylethanoid glycosides (6, 14–18, 20, 26), and iridoids (2–5, 7, 8, 13). Furthermore, a little number of quinic acid derivatives (9–12), aliphatic alcohol glycosides (21, 23), and oligosaccharides (1) (Table 1) were evident. The chemical structures of some identified compounds are shown in Fig. 1.

Most of the detected chromatographic peaks could be assigned as flavonoids, distinguishable in flavones and flavanones, according to their mass spectrometric behavior and in agreement with the literature reports for the genus (Frezza et al., 2021). For example, the analysis of the HRMS/MS spectrum of compound 19 was characterized by a fragmentation pattern showing a main product ion at *m/z* 299.0554 ($C_{16}H_{11}O_6$), corresponding to the aglycon anion, along with a product ion having $C_{15}H_8O_6$ formula generated by neutral loss of a methyl radical from the latter ion (Table 1). On the basis of the above evidences compound 19 was assigned as a 4′-*O*-methylisoscutellarein diglycoside, likely the 4′-*O*-methylisoscutellarein-7-*O*-allosyl-(1 → 2)-glucoside, a flavone already described in *S. subnuda* Montbret & Aucher ex Benth. (Şen et al., 2019).

Noteworthy, also compounds 22 and 24 showed in their tandem mass spectrum the aglycon ion having $C_{16}H_{11}O_6$ formula but, in this case, the major product ion was that formed by neutral loss of C_2H_2O from the relative $[M-H]^-$ ion (Table 1). This observation suggested the occurrence of an acetyl group in both structures and, along with all mass spectrometric data, concurred to assign 22 and 24 as 4′-*O*-methylisoscutellarein mono-acetyl-diglycoside or chrysoeriol mono-acetyl-diglycoside isomers, by considering that metabolites similar to these, such as 4′-*O*-methylisoscutellarein 7-*O*-[6‴-*O*-acetyl]allosyl-(1 → 2)-glucoside, stachyspinoside, and isostachyspinoside, have been described respectively in *S. iva* Griseb and in *S. candida*, the first, and in *S. candida* the second two (Michailidou et al., 2021; Pritsas et al., 2021).

Furthermore, compound 33 showed both molecular formula and HRMS/MS spectrum supporting its assignment as chrysoeriol or 4′-*O*-methylisoscutellarein (Table 1), both methoxylated flavones occurring in *Stachys* species, being the former reported in *S. candida* and *S. chrysantha*, and the latter in *S. recta*, among the others (Skaltsa et al., 2000; Michailidou et al., 2021; Karioti et al., 2010). Similarly to 33, compounds 35 and 36 were characterized by HRMS/MS spectra showing product ions formed *via* consecutive neutral losses of methyl radicals from the $[M-H]^-$ ion, indicative of the occurrence of three and four methoxy groups, respectively, composing the aglycon structure (Table 1). Once again, the comparison with literature allowed to hypothesize for the trimethoxyflavone 35 the structures of penduletin, eupatorin, or xanthomicrol, all metabolites found in *S. candida*, and for the tetramethoxyflavone 36 those of calycopterin, casticine or polycladin, previously described in *S. candida* and *S. chrysantha* (Michailidou et al., 2021).

By proceeding in this way, compounds 25 and 27 were identified as di- and trimethoxyflavone-glycoside, respectively, both being characterized by a tandem mass spectrum showing the product ion formed by neutral loss of a hexose unit (Table 1). On this basis, the comparison with literature allowed to suppose for 25 the structure of tricin 7-*O*-glucoside, already found in *S. officinalis* and in other *Stachys* species (Marin et al., 2004), and for compound 27 the structure of sudachitin 7-glucoside, a metabolite reported in *Sideritis* specie, but whose aglycon (sideritoflavone) has been also described in *S. glutinosa* (Ruiu et al., 2015).

The assignment of the molecular formula to the product ions occurring in the HRMS/MS spectrum of 29 and 30 allowed to ascertain in both compounds the occurrence of a coumaroyl unit acylating the hexose sugar in turn glycosylated to the aglycon apigenin (Table 1). So, compounds 29 and 30 could be likely defined as apigenin 7-(6″-*p*-coumaroylglucoside) and apigenin 7-(3″-*p*-coumaroylglucoside), both reported in various *Stachys* species, and the former also in *S. candida*

Table 1
Metabolites identified in the extract of aerial parts of *S. spreitzenhoferi*.

n.	R_t	Compound	$[M-H]^-$ (*m/z*)	$[(M+FA)-H]^-$	Molecular Formula	Error (ppm)	HRMS/MS	Reference
1	1.75	Tetrasaccharide	665.2150	711.2198	$C_{24}H_{42}O_{21}$	2.32	545.1715 ($C_{20}H_{33}O_{17}$), 485.1499 ($C_{18}H_{29}O_{15}$), 443.1393 ($C_{16}H_{27}O_{14}$), 383.1184 ($C_{14}H_{23}O_{12}$), 341.1082 ($C_{12}H_{21}O_{11}$), 221.0661 ($C_8H_{13}O_7$), 179.0556 ($C_6H_{11}O_6$)	Yao et al. (2022)
2	4.27	Melittoside	523.1667	569.1715	$C_{21}H_{32}O_{15}$	1.80	463.1442 ($C_{19}H_{27}O_{13}$), 361.1129 ($C_{15}H_{21}O_{10}$), 343.1026 ($C_{15}H_{19}O_9$), 183.0658 ($C_9H_{11}O_4$), 181.0501 ($C_9H_9O_4$), 179.0558 ($C_6H_{11}O_6$)	Yao et al. (2022)
3	4.96	Gardoside	373.1134		$C_{16}H_{22}O_{10}$	1.36	193.0504 ($C_{10}H_9O_4$), 179.05567 ($C_6H_{11}O_6$), 149.0606 ($C_9H_9O_2$)	Zhou et al. (2018)
4	5.30	Harpagide	363.1293	409.1345	$C_{15}H_{24}O_{10}$	1.92	201.0766 ($C_9H_{13}O_5$), 183.0659 ($C_9H_{11}O_4$), 165.0555 ($C_9H_9O_3$)	Yang et al. (2021)
5	5.89	Geniposidic acid	373.1136	419.1190	$C_{16}H_{22}O_{10}$	1.87	211.0611 ($C_{10}H_{11}O_5$), 193.0503 ($C_{10}H_9O_4$), 167.0712 ($C_9H_{11}O_3$), 149.0609 ($C_9H_9O_2$), 123.0454 ($C_7H_7O_2$)	Zhou et al. (2018); Yao et al. (2022)
6	6.51	Dihydroxyphenyl-ethanol hexosyl-deoxyhexoside	461.1656		$C_{20}H_{30}O_{12}$	0.51	315.1079 ($C_{14}H_{19}O_8$), 297.0976 ($C_{14}H_{17}O_7$), 135.0456 ($C_8H_7O_2$)	Zhang et al. (2021)
7	6.64	8-Epiloganic acid	375.1292	421.1344	$C_{16}H_{24}O_{10}$	1.70	213.0766 ($C_{10}H_{13}O_5$), 169.0867 ($C_9H_{13}O_3$), 151.0766 ($C_9H_{11}O_2$)	Yao et al. (2022)
8	6.73	10-Deacetyl-asperulosidic acid	389.1084		$C_{16}H_{22}O_{11}$	1.45	345.1184 ($C_{15}H_{21}O_9$), 209.0453 ($C_{10}H_9O_5$)	Zhou et al. (2020)
9	7.24	3-Caffeoylquinic acid	353.0873		$C_{16}H_{18}O_9$	1.62	191.0557 ($C_7H_{11}O_6$), 179.0345 ($C_9H_7O_4$)	Karioti et al. (2010)
10	7.29	Caffeoyl-hexose-deoxyhexoside	487.1451		$C_{21}H_{28}O_{13}$	0.95	179.0346 ($C_9H_7O_4$)	Tian et al. (2017)
11	8.62	5-Caffeoylquinic acid	353.0875		$C_{16}H_{18}O_9$	2.35	191.0560 ($C_7H_{11}O_6$), 179.0345 ($C_9H_7O_4$)	Karioti et al. (2010)
12	8.80	4-Caffeoylquinic acid	353.0873		$C_{16}H_{18}O_9$	1.7	191.0557 ($C_7H_{11}O_6$), 179.0346 ($C_9H_7O_4$), 173.0453 ($C_7H_9O_5$), 135.0451 ($C_8H_7O_2$)	Karioti et al. (2010)
13	9.62	7-*O*-Acetyl-8-epiloganic acid	417.1395	463.1449	$C_{18}H_{26}O_{11}$	0.87	357.1188 ($C_{16}H_{21}O_9$), 255.0870 ($C_{12}H_{15}O_6$), 211.0975 ($C_{11}H_{15}O_4$), 195.0660 ($C_{10}H_{11}O_4$), 193.0868 ($C_{11}H_{13}O_3$), 151.0765 ($C_9H_{11}O_2$)	Zhang et al. (2003); Hanoglu et al., 2020
14	9.79	Dihydroxyphenyl-hydroxyethanol (caffeoyl) hexosyl-deoxyhexoside	639.1927		$C_{29}H_{36}O_{16}$	1.14	621.1812 ($C_{29}H_{33}O_{15}$), 529.1550 ($C_{23}H_{29}O_{14}$), 487.1443 ($C_{21}H_{27}O_{13}$), 459.1494 ($C_{20}H_{27}O_{12}$)	Zhang et al. (2021)
15	11.00	Dihydroxyphenyl-ethanol (caffeoyl)hexosyl-deoxyhexosyl-pentoside	755.2396		$C_{34}H_{44}O_{19}$	0.34	623.1966 ($C_{29}H_{35}O_{15}$), 593.2071 ($C_{25}H_{37}O_{16}$), 575.1957 ($C_{25}H_{35}O_{15}$), 461.1652 ($C_{20}H_{29}O_{12}$)	Sermukhamedova et al. (2017); Petreska et al. (2011)
16	11.21	Dihydroxyphenyl-ethanol (caffeoyl)hexosyl-deoxyhexosyl-pentoside	755.2397		$C_{34}H_{44}O_{19}$	0.51	623.1965 ($C_{29}H_{35}O_{15}$), 593.2070 ($C_{25}H_{37}O_{16}$), 575.1970 ($C_{25}H_{35}O_{15}$), 461.1650 ($C_{20}H_{29}O_{12}$)	Sermukhamedova et al. (2017); Petreska et al. (2011)
17	11.34	Dihydroxyphenyl-ethanol (caffeoyl)hexosyl-deoxyhexoside	623.1981		$C_{29}H_{36}O_{15}$	1.72	477.1388 ($C_{23}H_{25}O_{11}$), 461.1650 ($C_{20}H_{29}O_{12}$), 443.1546 ($C_{20}H_{27}O_{11}$), 315.1076 ($C_{14}H_{19}O_8$)	Zhang et al. (2021)
18	11.64	Dihydroxyphenyl-ethanol (caffeoyl)hexosyl-deoxyhexosyl-pentoside	755.2397		$C_{34}H_{44}O_{19}$	0.51	623.1968 ($C_{29}H_{35}O_{15}$), 593.2072 ($C_{25}H_{38}O_{16}$), 461.1651 ($C_{20}H_{29}O_{12}$)	Sermukhamedova et al. (2017); Petreska et al. (2011)
19	11.71	4′-*O*-methylisoscutellarein diglycoside	623.1619	669.1677	$C_{28}H_{32}O_{16}$	1.94	503.1182 ($C_{24}H_{23}O_{12}$), 461.1077 ($C_{22}H_{21}O_{11}$), 443.0972 ($C_{22}H_{19}O_{10}$), 341.0659 ($C_{18}H_{13}O_7$), 299.0554 ($C_{16}H_{11}O_6$), 284.0319 ($C_{15}H_8O_6$)	Pereira et al. (2012); Petreska et al. (2011); Karioti et al. (2010)
20	11.90	Dihydroxyphenyl-ethanol (caffeoyl)hexosyl-deoxyhexoside	623.1977		$C_{29}H_{36}O_{15}$	1.03	477.1387 ($C_{23}H_{25}O_{11}$), 461.1648 ($C_{20}H_{29}O_{12}$), 443.1552 ($C_{20}H_{27}O_{11}$), 315.1075 ($C_{14}H_{19}O_8$)	Zhang et al. (2021)
21	12.08	Octenol-(dihexosyl)hexoside	583.2607	629.2662	$C_{25}H_{44}O_{15}$	1.77	451.2169 ($C_{20}H_{35}O_{11}$), 421.2065 ($C_{19}H_{33}O_{10}$), 289.1647 ($C_{14}H_{25}O_6$)	Stojković et al. (2021)
22	12.46	4′-*O*-methylisoscutellarein mono-acetyl-diglycoside/ chrysoeriol mono-acetyl-diglycoside	665.1721	711.1774	$C_{30}H_{34}O_{17}$	1.25	623.1596 ($C_{28}H_{31}O_{16}$), 605.1492 ($C_{28}H_{29}O_{15}$), 503.1171 ($C_{24}H_{23}O_{12}$), 461.1066 ($C_{22}H_{21}O_{11}$), 299.0550 ($C_{16}H_{11}O_6$)	Petreska et al. (2011); Pereira et al. (2012); Karioti et al. (2010)
23	12.59	Octenol-(hexosyl)hexoside	451.2179	497.2236	$C_{20}H_{36}O_{11}$	1.20	289.1649 ($C_{14}H_{25}O_6$), 161.0454 ($C_6H_9O_5$)	Stojković et al. (2021)
24	13.07	4′-*O*-methylisoscutellarein mono-acetyl-diglycoside/ chrysoeriol mono-acetyl-diglycoside	665.1727		$C_{30}H_{34}O_{17}$	2.17	623.1611 ($C_{28}H_{31}O_{16}$), 605.1511 ($C_{28}H_{29}O_{15}$), 545.1295 ($C_{26}H_{25}O_{13}$), 503.1188 ($C_{24}H_{23}O_{12}$), 461.1081 ($C_{22}H_{21}O_{11}$), 443.0975 ($C_{22}H_{19}O_{10}$), 341.0661 ($C_{18}H_{13}O_7$), 299.0556 ($C_{16}H_{11}O_6$), 284.0322 ($C_{15}H_8O_6$)	Petreska et al. (2011); Pereira et al. (2012); Karioti et al. (2010)
25	13.84	Dimethoxyflavone-glycoside	491.1189		$C_{23}H_{24}O_{12}$	0.95	476.0951 ($C_{22}H_{20}O_{12}$), 329.0660 ($C_{17}H_{13}O_7$)	Shao et al. (2020)

(*continued on next page*)

Table 1 (*continued*)

n.	R_t	Compound	[M-H]$^-$(*m/z*)	[(M + FA)-H]$^-$	Molecular Formula	Error (ppm)	HRMS/MS	Reference
26	13.88	Methoxy-hydroxyphenyl-ethanol (feruloyl)hexosyl-deoxyhexoside	651.2290		$C_{31}H_{40}O_{15}$	1.00	505.1699 ($C_{25}H_{29}O_{11}$), 475.1808 ($C_{21}H_{31}O_{12}$)	Zhang et al. (2021)
27	14.62	Trimethoxyflavone-glycoside	521.1295		$C_{24}H_{26}O_{13}$	1.06	359.0765 ($C_{18}H_{15}O_8$)	Wang et al., (2013); Xie et al. (2014)
28	15.52	Stachysetin	577.1348 [(M-H)]$^{2-}$		$C_{60}H_{52}O_{24}$	1.35	885.2230 ($C_{45}H_{41}O_{19}$), 783.1922 ($C_{41}H_{35}O_{16}$), 673.1403 ($C_{31}H_{29}O_{17}$), 613.1186 ($C_{29}H_{25}O_{15}$), 471.0923 ($C_{23}H_{19}O_{11}$), 269.0449 ($C_{15}H_9O_5$)	El-Ansari et al. (1995)
29	16.39	Apigenin-coumaroylglycoside	577.1346		$C_{30}H_{26}O_{12}$	1.02	431.0972 ($C_{21}H_{19}O_{10}$), 269.0448 ($C_{15}H_9O_5$)	Petreska et al. (2011); Karioti et al. (2010)
30	16.64	Apigenin-coumaroylglycoside	577.1351		$C_{30}H_{26}O_{12}$	1.87	431.0982 ($C_{21}H_{19}O_{10}$), 269.0448 ($C_{15}H_9O_5$)	Petreska et al. (2011); Karioti et al. (2010)
31	17.33	Naringenin -(coumaroyl-glycoside)	579.1503		$C_{30}H_{28}O_{12}$	1.08	307.0815 ($C_{15}H_{15}O_7$), 271.0603 ($C_{15}H_{11}O_5$)	Morales-Soto et al. (2013)
32	17.68	Naringenin -(coumaroyl-glycoside)	579.1500		$C_{30}H_{28}O_{12}$	0.44	307.0817 ($C_{15}H_{15}O_7$), 271.0605 ($C_{15}H_{11}O_5$)	Morales-Soto et al. (2013)
33	18.80	4′-*O*-Methylisoscutellarei n/ Chrysoeriol	299.0557		$C_{16}H_{12}O_6$	2.39	284.0321 ($C_{15}H_8O_6$)	Mohammadi and Kharazian (2022); Noreen et al. (2021); Karioti et al. (2010)
34	21.82	Apigenin di-coumaroylglycoside	723.1714		$C_{39}H_{32}O_{14}$	0.85	577.1337 ($C_{30}H_{25}O_{12}$), 559.1232 ($C_{30}H_{23}O_{11}$), 453.1175 ($C_{24}H_{21}O_9$), 269.0446 ($C_{15}H_9O_5$)	Peron et al. (2020)
35	22.60	Trimethoxyflavone	343.0821		$C_{18}H_{16}O_7$	2.68	328.0584 ($C_{17}H_{12}O_7$), 313.0350 ($C_{16}H_9O_7$), 298.0117($C_{15}H_6O_7$)	Mari et al. (2015)
36	23.68	Tetramethoxyflavone	373.0926		$C_{19}H_{18}O_8$	2.21	358.0689 ($C_{18}H_{14}O_8$), 343.0456 ($C_{17}H_{11}O_8$), 328.0224 ($C_{16}H_8O_8$), 312.9988 ($C_{15}H_5O_8$)	Kumar et al. (2018)

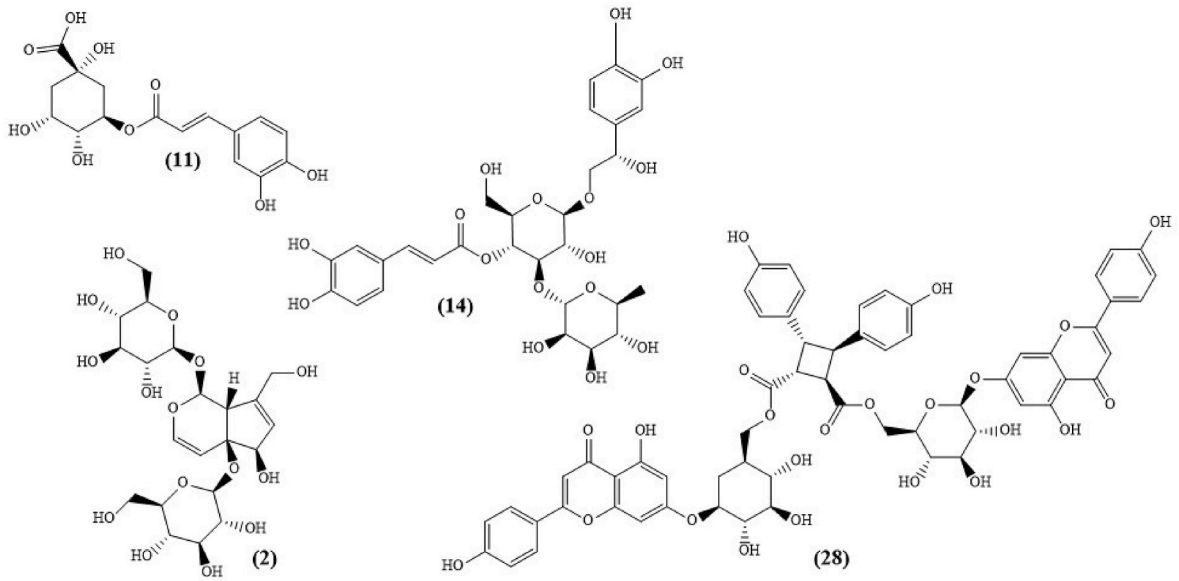

Fig. 1. Some compounds identified by LC-(−)ESI/HRMS^n analysis.

(Michailidou et al., 2021; Karioti et al., 2010).

Interestingly, compound 28 could be identified as the rare acylated flavonoid stachysetin, a dimer based on the skeleton of apigenin [i.e. diapigenin-7-*O*-(6″-*trans*,6″-*cis*-*p*,*p*'-dihydroxy-*μ*-truxinyl)-glucoside] previously described in *S. iva* Griseb. (Pritsas et al., 2021). Its mass spectrometric behavior, to the best of our knowledge, has been so far described only by FAB-mass spectrometry (El-Ansari et al., 1995). In particular, the analysis of the ESI-HRMS spectrum allowed to assign the molecular formula of $C_{60}H_{52}O_{24}$ to the [(M-H)]$^{2-}$ ion at *m/z* 577.1348, whose ESI-HRMS/MS spectrum was characterized by a main product ion at *m/z* 885.2230 formed by neutral loss of an apigenin unit ($C_{15}H_{10}O_5$). The inner fragmentation of the set free hexose determined the formation of the product ion at *m/z* 783.1922 from the [(M-$C_{15}H_{10}O_5$)-H)]$^-$ ion *via* neutral loss of $C_4H_6O_3$, while the breakdown of the cyclobutane allowed the neutral loss of a 4,4'-(1,2-ethenediyl)bis-phenol ($C_{14}H_{12}O_2$) unit from the [(M-$C_{15}H_{10}O_5$)-H)]$^-$ ion generating the product ion at *m/z* 673.1403 (Table 1). The two neutral progressive losses of 202 Da ($C_8H_{10}O_6$) from this latter product ion, at first, by cleavage of the double bond formed *via* removal of the 4,4'-(1,2-ethenediyl)bis-phenol and, subsequently, *via* removal of the second modified mono-dehydrated hexose, yielded the two product ions at *m/z* 471.0923 and 269.0449, this latter corresponding to the apigenin aglycon ion (Table 1).

The same aglycon ion could be detected in the tandem mass spectrum of compound 34 along with product ions formed by neutral loss of coumaroyl units (Table 1) allowing to define 34 as apigenin di-coumaroylglycoside, a type of metabolite occurring in various *Stachys*

species, such as the anisofolin A reported in *S. lanata* (Murata et al., 2008). Instead, compounds 31 and 32 showed a tandem mass spectrum characterized by a main product ion at *m/z* 271.0603, formed by neutral loss of a coumaroyl-hexose group ($C_{15}H_{16}O_7$) and corresponding to the aglycon naringenin (Table 1). Thereby compounds 31 and 32 could be tentatively identified as naringenin coumaroylglycoside isomers, likely naringenin 7-*O*-(6″-coumaroyl-glucopyranoside) and narigenin 7-*O*-(3″-coumaroyl)-glucopyranoside, metabolites occurring in various Lamiaceae plants such as *Eriophyton wallichii* and *Anisomeles indica*, whose aglycon has been reported also in *S. aegyptiaca* and *lavandulifolia* (Rahimi Khoigani et al., 2017; Fan et al., 2011; Chen et al., 2008; El-Ansari et al., 1995).

Furthermore, the analysis of HRMS/MS spectra of compounds 2–5, 7, 8, and 13 highlighted the occurrence of product ions generated by the neutral loss of one or two hexose units, along with, in the case of the tandem mass spectra of 3, 5, 7, 8, and 13, product ions generated from the aglycon moieties by neutral loss of 44 Da (CO_2) and/or 60 Da ($C_2H_4O_2$), allowing to ascertain the occurrence, in these latter compounds, of a carboxyl and/or an acetyl group, respectively (Table 1). The comparison of mass spectrometric data with literature reports concurred to identify 2, 3, 4, and 7 as melittoside, gardoside, harpagide and 8-epi-loganic acid, iridoids already described in *S. iva* Griseb (Pritsas et al., 2021), along with geniposidic acid (5), 10-deacetyl-asperulosidic acid (8), and 7-*O*-acetyl-8-epi-loganic acid (13), previously reported in species of this genus other than *S. iva* Griseb (Frezza et al., 2019; Kotsos et al., 2001).

The analysis of LC-(−)ESI/HRMS^n data allowed to ascertain the occurrence in the extract of *S. spreitzenhoferi* of a good number of phenylethanoid glycosides (6, 14–18, 20, 26), characterized by one or two monosaccharide units in addition to the glucose composing the core of the molecule with a hydroxytyrosol group, that in some case was further hydroxylated or methoxylated, and by the occurrence of a hydroxycinnamic acid, such as caffeic and ferulic acid, to esterificate the glucose core (Table 1) (Kite, 2020). In particular, the comparison of mass spectrometric data with literature reports suggested to define 6 as the known decaffeoylacteoside already reported in *S. lanata* and *S. sieboldii* (Murata et al., 2008; Nishimura et al., 1991), compound 14 as campneoside II, a phenylethanoid glycoside described, e.g., in *S. lanata* and *S. riederi* (Murata et al., 2008; Ikeda et al., 1994), the three isomers 15, 16 and 18 as lavandulifolioside, stachysoside B, and betonyoside F reported, among the others, in *S. iva* Griseb, *S. lanata*, and *S. schtschegleevii*, respectively (Pritsas et al., 2021; Murata et al., 2008; Nazemiyeh et al., 2006), as well as the two isomers 17 and 20 as acteoside and isoacteoside, already described in *S. iva* Griseb and in *S. recta*, respectively (Pritsas et al., 2021; Karioti et al., 2010), and finally compound 26 as martynoside, previously reported in *S. lanata* (Murata et al., 2008).

HRMS/MS spectra of 9, 11, and 12 showed the typical fragmentation pattern of quinic acid derivatives, allowing to identify these compounds as 3-, 5-, and 4-caffeoylquinic acids, respectively, in agreement with the chromatographic elution order and literature reports (Table 1) (Karioti et al., 2010; Chamorro et al., 2021). In particular, as well as in other species, compound 9 was already described in *S. iva* Griseb (Pritsas et al., 2021). Furthermore, compound 10 could be defined as a caffeoyl-hexose-deoxyhexoside (Table 1), likely the hydroxycinnamic acid derivative known as cistanoside F, already reported in *S. lanata* (Murata et al., 2008).

Instead, compounds, 21 and 23 could be assigned to the class of aliphatic alcohol glycosides (Table 1), having the octenol-hexoside ($C_{14}H_{26}O_6$) as base unit (Table 1). The comparison with literature reports for the genus allowed to likely define 21 and 23 as 1-ethenylhexyl *O*-*α*-L-arabinopyranosyl-(1 → 6)-*O*-[*β*-D-glucopyranosyl-(1 → 2)]-*β*-D-glucopyranoside and (1*R*)-1-ethenylhexyl-2-*O*-*β*-D-glucopyranosyl-*β*-D-glucopyranoside, two aliphatic alcohol glycosides of which the first was previously reported in *S. riederi* and the second in a plant species belonging to a different genus of the Lamiaceae family, the *Caryopteris incana* (Ikeda et al., 1994; Zhao et al., 2009).

Finally, the analysis of the HRMS/MS spectrum acquired for compound 1 (Table 1) suggested the occurrence of a tetrasaccharide consisting of four hexose units, like stachyose, an oligosaccharide composed of one glucose, one fructose, and two galactoses found in *Stachys* species such as *S. floridana* Schuttl. (Zhong et al., 2013).

2.2. Effect of S. spreitzenhoferi on bacterial survival

Pharmacological studies have demonstrated that extracts or components of plants belonging to the *Stachys* genus exert significant antibacterial, antifungal, antioxidant, and anti-inflammatory activities (Tundis et al., 2014). In this study, we evaluate the antimicrobial activity of MeOH extract of *S. spreitzenhoferi* aerial parts that were collected on the Avlemonas cliffs on the Kythira Island, in South Greece.

The *S. spreitzenhoferi* extract was tested using disc diffusion method, cell count, and minimum inhibitory concentration (MIC) determination, against the Gram-positive bacteria *Staphylococcus aureus* ATCC6538P, *Bacillus subtilis* PY79, *Bacillus cereus* ATCC10987, *Bacillus licheniformis* ATCC9789, *Listeria monocytogenes* ATCC7644, and Gram-negative bacteria *Escherichia coli* DH5*α*, *Pseudomonas aeruginosa* PAOI, *Shighella sonnei* ATCC25931, and *Salmonella tiphymurium* ATCC14028.

Chemical analyses of *S. spreitzenhoferi* extract showed the presence of compounds belonging to the class of flavonoids (19, 22, 24–25, 27–36), phenylethanoid glycosides (6, 14–18, 20, 26), and iridoids, with known antimicrobial activity.

The extract is not active against Gram-negative bacteria but, as shown in Fig. 2, was able to inhibit the Gram-positive growth, forming an inhibition halo almost comparable to the antibiotic ampicillin, thus acting on the microorganism cell wall used as positive control in the experiment.

The DMSO 80% used to resuspend the *S. spreitzenhoferi* extract represents the negative control of the experiment and does not affect microbial growth. Fig. 2 reports a quantitative analysis of inhibition halos. These first experiments allowed us to deepen the study on *S. spreitzenhoferi* extract antimicrobial activity.

Using the same indicator strains, we performed a more accurate assay in determining the substance antimicrobial efficiency by bacterial counts. Fig. 3 shows that the extract possesses a good dose-dependent antimicrobial activity, more directly towards *S. aureus* and *L. monocytogenes*.

S. spreitzenhoferi extract subsequent antimicrobial activity was analyzed according to broth microdilution method. By performing this assay, minimal inhibitory concentration (MIC) values were found to be comprised between 1.0 and 1.4 mg/mL against the tested strains as shown in Table 2.

Different studies mention similar results, relative to another species belonging to the *Stachys* genus. As assessed in a previous work, *S. officinalis* showed against *S. aureus* a MIC value of 1 mg/mL (Grujic-Jovanovic et al., 2004). However, since the extracts are complex mixtures of different compounds, it is difficult to attribute their antimicrobial activity to a single or a particular constituent. Usually, major compounds are the ones responsible for the biological activity of the extracts. However, since the extracts are complex mixtures of different compounds, it is difficult to attribute their antimicrobial activity to a single or a particular constituent. Usually, major compounds are the ones responsible for the biological activity of the extracts. However, there are studies showing that the whole essential oil has a higher activity than the combination of the major isolated compounds, and such studies indicate that minor components are critical to the biological activity of the oils. It is therefore likely that the oil bioactivity is presumably due to a synergistic effect between the various compounds present in the *Stachys* MeOH (Serbetçi et al., 2010). In our case, the activity may be related to the presence of flavonoids as main constituents. In fact, many studies have identified flavonoids to possess antifungal, antiviral, and antibacterial activity, and have demonstrated synergy between active flavonoids as well as between flavonoids and

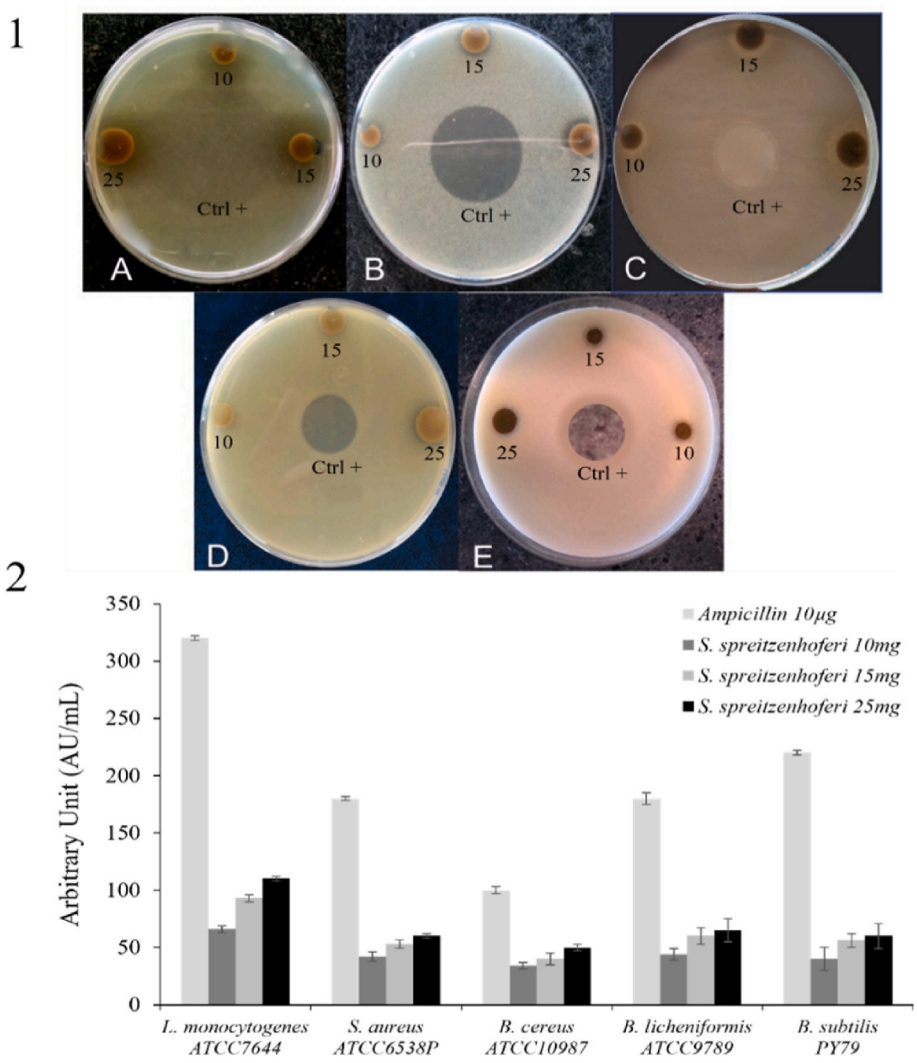

Fig. 2. 1. Inhibition halo *S. spreitzenhoferi* against (A) *L. Monocytogenes*, (B) *B. Subtilis*, (C) *B. licheniformis*, (D) *S. aureus*, and (E) *B. cereus*. Positive control is represented by Ampicillin, negative control is dimethyl sulphoxide (DMSO 80%). 2. The inhibition halo shown in panel 1 is expressed in AU/mL (see methods). Values are expressed as average of three different experiments; standard deviations were always less than 10%.

existing chemotherapeutics (Cushnie and Lamb, 2005).

2.3. Antioxidant activity of S. spreitzenhoferi in vitro

Previous studies demonstrated that plants of the *Stachys* genus may be a good source of natural antioxidants for medicinal uses, such as against aging and other diseases related to radical mechanisms (Tomou et al., 2020; Leporini et al., 2015). Previously, methanol extracts of aerial flowering parts of four *Stachys* taxa, such as *S. alpina* subsp. *dinarica, S. anisochila, S. beckeana* and *S. plumosa*, were investigated for their antioxidant activity (Kukić et al., 2006). This study demonstrated the presence of phenylethanoid glycosides as main constituents of *S. plumosa* (Bankova et al., 1999). These compounds were found to be strong antioxidants (Aligiannis et al., 2003). For the presence of phenylethanoid glycosides in methanol extracts of aerial parts in the species object of our study, radical scavenging capacity was evaluated.

In Fig. 4 panel A DPPH solution is purple in the absence of *S. spreitzenhoferi* extract. Increasing concentration (0.025–1 mg/mL) of extract increases the % of scavenging activities. The radical DPPH is reduced with the formation of a colorless product for the release of a hydrogen atom to the radical by the antioxidant molecule. In the same figure, panel B, the ABTS solution is green in the absence of *S. spreitzenhoferi* extract. In the presence of antioxidants from the green solution a colorless product is formed. The results shown in Fig. 4 are expressed in Table 3 as IC_{50}, i.e. the concentration of extract that causes a 50% decrease in DPPH, ABTS, and H_2O_2 radicals.

The extract *S. spreitzenhoferi* exhibited anti-DPPH (1,1-diphenyl 2-picryl hydrazyl) activity with and IC_{50} value of 0.17 mg/mL. In concentration range from 0.1 to 0.17 mg/mL extract scavenged OH radical about 40%; anti-H_2O_2 activity with IC_{50} values 0.125 mg/mL and the highest antiradical effect with an IC_{50} value of 0.18 mg/mL for anti-ABTS [2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)] activity.

In recent years, several studies on different taxa of genus *Stachys* have demonstrated that woundworts exert various biological effects, such as antioxidant, radical scavenging, and antiproliferative (Tundis et al., 2014; Háznagy-Radnai et al., 2012). Phytochemical studies in *Stachys* species revealed the presence of iridoids, polyphenols, including flavonoids, tannins, phenolic acids, phenylethanoid glycosides, diterpenes and triterpene saponins in addition to essential oil as minor constituents.

Recent studies summarized by Tomou et al. (2020) reported the high antioxidant potential of *Stachys* ssp., and confirmed the possibility of their use as natural antioxidants. The other *Stachys* species like *S. affinis* showed extremely high DPPH radical scavenging activity, several folds higher than the standard *α*-tocopherol, which was attributed to the abundance of phenolics and flavonoids. Another species, *S. mucronate*

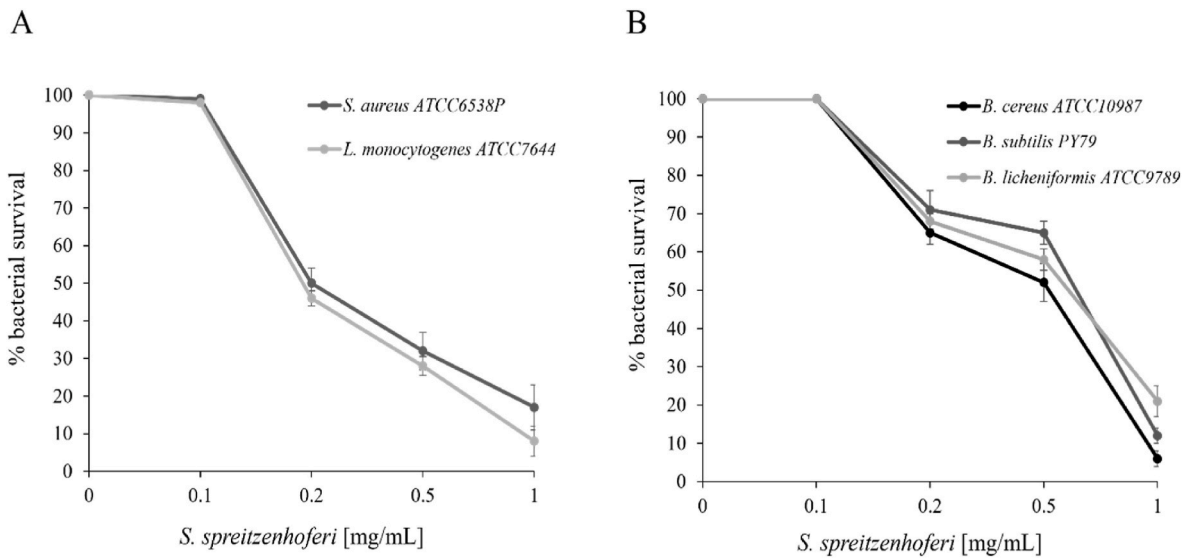

Fig. 3. Antimicrobial activity of *S. spreitzenhoferi* at different concentrations (0, 0.1, 0.2, 0.5, and 1 mg/mL) valuated by colony count assay, after 4 h of incubation, against *S. aureus* and *L. monocytogenes* strains (panel A), *B. subtilis, B. cereus* and *B. licheniformis* strains (panel B). The % of bacterial survival is represented on the y axis. The assays were performed in three independent experiments.

Table 2
Minimum inhibitory concentration (MIC100, mg/mL) values of S. spreitzenhoferi extract against a panel of Gram-positive bacteria. Values were obtained from a minimum of three independent experiments.

Strains	Concentration	Positive control
S. aureus ATCC6538P	1 mg/mL	0.0002 mg/mL
L. monocytogenes ATCC7644	1 mg/mL	0.00025 mg/mL
B. cereus ATCC10987	1 mg/mL	0.032 mg/mL
B. subtilis PY79	1.2 mg/mL	0.008 mg/mL
B. licheniformis ATCC9789	1.4 mg/mL	0.032 mg/mL

also demonstrated strong anti-radical activity due to the high content of polyphenols. Two acylated flavonoid glycosides from *Stachys bombycina* possessed a strong free radical scavenging activity as reported. Consequently, plants of genus *Stachys* are considered a great source of phytochemicals with therapeutic and economic applications. Given the increasing demand for natural products, many *Stachys* species have been cultivated for uses in traditional medicine, in food market, in cosmetic industry and for ornamental reasons.

2.4. ROS and antioxidant enzymes in PMN

As can be seen from Fig. 5, ROS production increases in OZ-stimulated PMNs compared to non-stimulated PMNs. After treatment with increasing concentrations of *S. spreitzenhoferi* extract in OZ-stimulated PMN, ROS levels tend to decrease until reaching a plateau of 0.2 mg/mL with values comparable to the control. The activity of the antioxidant enzymes SOD, CAT and GPX in OZ-stimulated PMNs increases compared to untreated PMNs. By treating the OZ-stimulated PMNs with increasing concentrations of *S. spreitzenhoferi* extract, there is a further increase in the activity of the antioxidant enzymes until reaching a plateau at the concentration of 0.2 mg/mL (Fig. 5).

Our results are in line with those obtained by Marinovic et al. (2015) who studied the antioxidant capacities of tea green tea extract on human neutrophils. Antioxidants are compounds that protect cells against the damaging effects of ROS/RNS. An imbalance between antioxidants and free radicals results in oxidative stress, leading to cellular damage.

Plant extracts rich in flavonoids present antioxidant activity through scavenging ROS, chelating redox active transition metal ions, inhibiting redox sensitive transcription factors, inhibiting pro-oxidant enzymes, and inducing antioxidant enzymes (Frei and Higdon, 2003; Stangl et al., 2007). It could therefore be assumed that the *S. spreitzenhoferi* extract contrasts the increase in ROS by acting at the level of the antioxidant defense system, increasing the activity of the antioxidant enzymes SOD, CAT, and GPX catalase.

2.5. Effect of S. spreitzenhoferi extract in acute myeloid leukemia, U937 cell

S. spreitzenhoferi extract induce proliferative block and cell death in cancer cells U937. In fact, as can be seen from Fig. 6, treating the cells with increasing concentrations of extract has a dose-dependent increase in cell death. Approximately 50% death is observed when cells are treated with 0.75 mg compared to the control. In Fig. 7, consistently with the data obtained by FACS analysis showing a dose-dependent increase in cell death, it is possible to observe, from the cell cycle phases by FACS analysis, that *S. spreitzenhoferi* extract, in a dose-dependent manner, causes a block of cells in the Pre-G1 phase associated to a decrease in the other phases of the cell cycle. These data suggest that *S. spreitzenhoferi* extract has good antiproliferative effects for acute myeloid leukemia, U937 cells and could be a possible candidate for the therapy of cancer patients. However, to better understand the effects of *S. spreitzenhoferi* extract, more studies are needed. Currently for many natural compounds, it is not completely clear whether for some observed beneficial effects, such as antineoplastic activity, a transcriptional action is necessary or whether they are mainly related to epigenetic action. For this reason, further studies should be carried out in order to evaluate whether some of these biological activities described could be attributable to a possible epigenetic action exerted by the second metabolites as demonstrated for other compounds of natural origin (Scafuri et al., 2020; Miceli et al., 2014).

3. Conclusion

In this work a not previously studied species of *Stachys* was investigated. The analysis of the methanol extract revealed the presence of thirty-six compounds - flavonoids, phenylethanoid glycosides, iridoids, quinic acid derivatives, aliphatic alcohol glycosides, and oligosaccharides. Several appealing biological properties of this extract has been also reported. First of all, the extract showed an interesting selective antimicrobial activity towards Gram positive bacteria. This makes it a

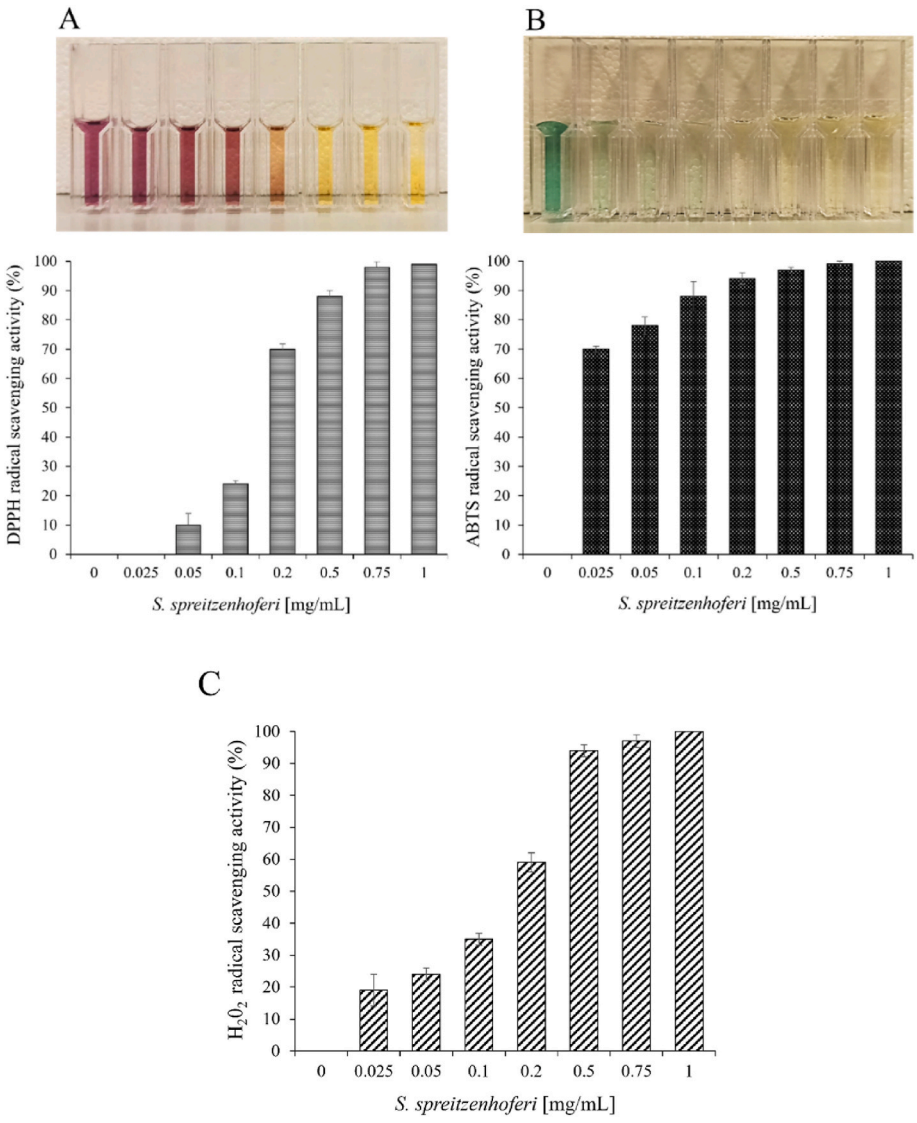

Fig. 4. Antioxidant activity of *S. spreitzenhoferi*. (A) DPPH radical scavenging activity was measured after 30 min of incubation and reported as % of DPPH removed respect to the control. (B) Radical scavenging activity was measured after 10 min of incubation and reported as % of ABTS removed respect to the control. (C) Hydrogen peroxide scavenging activity was measured after 30 min of incubation and reported as % of H_2O_2 removed respect to the control. Data are mean of three independent experiments ± SE (n = 5).

Table 3
Antioxidant activities of *S. spreitzenhoferi* extract.

Sample	IC_{50} of DPPH method (mg/mL)	IC_{50} of ABTS method (mg/mL)	IC_{50} of H_2O_2 method (mg/mL)
Extract	0.17 ± 1.70	0.18 ± 1.85	0.125 ± 1.30
Positive control	0.03 ± 0.50	0.05 ± 0.63	0.03 ± 0.60

IC_{50}: concentration which inhibited 50% free radicals; DPPH: 1,1-diphenyl-2-picrylhydrazyl; ABTS: 2,20-azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid); H_2O_2 hydrogen peroxide. Positive control was represented by ascorbic acid for DPPH and ABTS; and resveratrol for H_2O_2.

useable compound for treating skin infections. It is known in the literature that the typical microorganisms that colonize the skin are generally Gram-positive species, such as *Staphylococcus epidermidis, Corynebacterium species, Staphylococcus aureus* and *Streptococcus pyogenes* known to be pathogenic for the skin. (Todar, 2008; Ki and Rotstein, 2008). From our study we have also highlighted the intrinsic antioxidant properties of the *Stachys* extract. Additionally, *S. aureus* and *S. pyogenes* produce toxins that can elicit a superantigen response, causing massive release of cytokines. Staphylococcal burn skin syndrome, toxic shock syndrome, and scarlet fever are all superantigen mediated (Chiller et al., 2001). These two activities make this compound even more interesting for its probable topical use. Furthermore, *S. spreitzenhoferi* extract caused a decrease in ROS and an increase in the activity of the antioxidant enzymes SOD, CAT, and GPX in OZ-stimulated PMNs and exhibited antiproliferative activity against U937 cells. Thus, results of this work give new insight into the potential application of this extract as antimicrobial, antioxidant and antitumoral agent.

The biological potential that has emerged from this work suggests the promising use of this plant in pharmaceutical and nutraceutical preparations, and in particular, the next step requires the need to isolate the main metabolites in order to evaluate their antioxidant, antiproliferative, and antibacterial capacities.

4. Experimental

4.1. Plant material

Aerial parts from *S. spreitzenhoferi* Heldr. (Ss) were collected on the cliffs of Avlemonas on the island of Kythira, South Greece, at about 4 m s/l, 36°13′32.75″ longitude N and 13°04′51.83″ latitude E, in August 2021. One of the samples, identified by Prof. Vincenzo Ilardi, has been stored in the University of Palermo Herbarium (Voucher No. 109719).

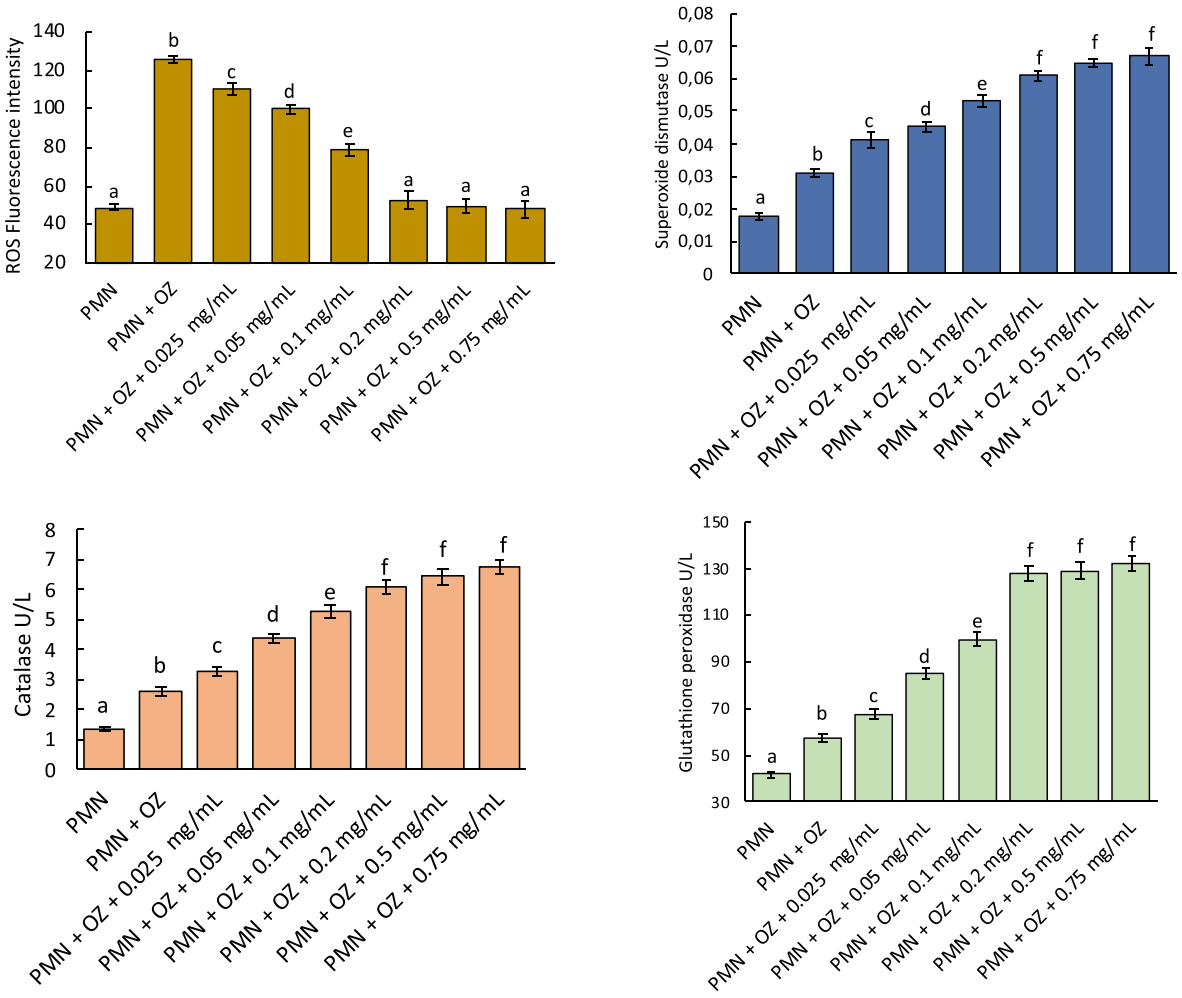

Fig. 5. ROS production and on activities of antioxidant enzymes (superoxide dismutase; catalase; glutathione peroxidase) in polymorphonuclear cells treated with extract of *Stachys spreitzenhoferi* on at the concentration of 0, 0.025, 0.05, 0.1, 0.2, 0.5, 0.75 mg/mL without or with of OZ (0.50 mg/mL). Data were presented as mean and standard error and they were analyzed with a paired *t*-test. Bars not accompanied by the same letter were significantly different at $p < 0.05$.

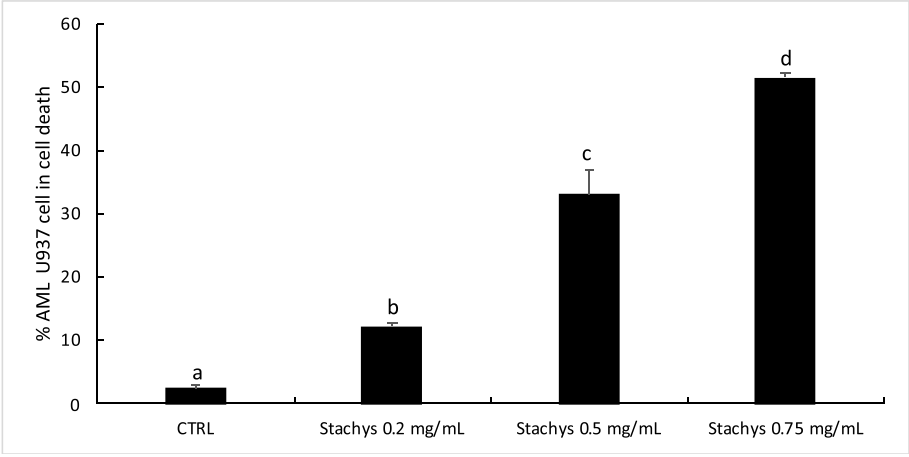

Fig. 6. AML U937 cells treated with *S. spreitzenhoferi* extract at several concentration for 24 h Cell death by FACS analysis. Data were presented as mean and standard error and they were analyzed with a paired *t*-test. Bars not accompanied by the same letter were significantly different at $p < 0.05$.

4.2. Extraction of plant material

S. spreitzenhoferi aerial parts were dried at room temperature for 7 days and then the dry material (100 g), finely ground, was exhaustively extracted by maceration (3 × 72 h) by using methanol as solvent. The extracts were filtered through Whatman No. 4 filter paper and the solvent was completely evaporated by using a rotary evaporator (Buchi model R-210, Cornaredo, Italy) under reduced pressure to obtain crude extract (17.9 g) (yields with respect to dry plant 17.9%).

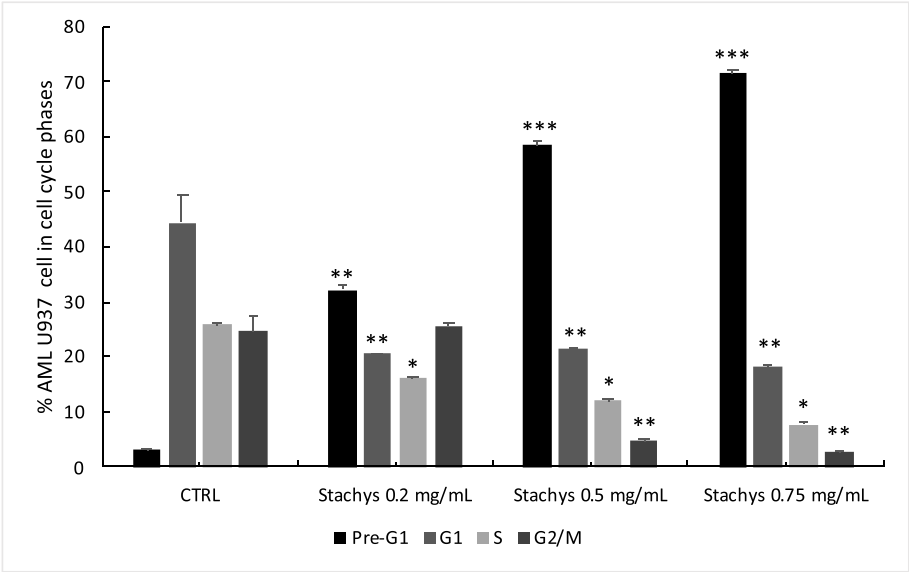

Fig. 7. AML U937 cells treated with *S. spreitzenhoferi* extract at several concentration for 24 h Cell cycle by FACS analysis Data were presented as mean and standard error and statistical significance was calculated with one-way ANOVA followed by Tukey's test. * ($p < 0.0003$), **($p < 0.002$) and ** ($p < 0.0001$) indicate significant differences between control and treatments.

4.3. LC-(−)ESI/HRMS^n analysis

For LC-(−)/HRMS analysis a system of liquid chromatography coupled to electrospray ionization and high-resolution mass spectrometry (LC-ESI/HRMS) consisting of a quaternary Accela 600 pump and an Accela autosampler coupled to a linear ion-trap-Orbitrap hybrid mass spectrometer (LTQOrbitrap XL) (ThermoScientific, San Jose, CA) operating in negative ionization mode was used. The chromatographic separation was carried out on a Luna C-18 column (RP-18, 2.0 × 150 mm, 5 μm; Waters; Milford, MA), at a flow rate of 0.2 mL/min. The mobile phase consisted of a combination of A (0.1% formic acid in water, v/v) and B (0.1% formic acid in acetonitrile, *v/v*). A linear gradient from 5 to 65% B (v/v) in 26 min was used. The autosampler was set to inject 2 μL of extract (0.5 mg/mL). The following experimental conditions for the ESI source were adopted: sheath gas at 20 (arbitrary units); auxiliary gas at 5 (arbitrary units); source voltage at 3.5 kV; the capillary temperature at 280 °C; the capillary voltage at −48 V and tube lens at −176.47 V. A mass range from *m/z* 150–2000 Da was explored. To perform data-dependent scan experiments yielding tandem mass (HRMS/MS) product ions, the first and the second most intense ions from the HRMS scan event were selected and submitted to collision-induced dissociation (CID) by applying the following conditions: a minimum signal threshold at 250, an isolation width at 2.0, and a normalized collision energy at 30%. Both in full and in MS/MS scan mode, resolving power of 30000 was used. Data were collected and analyzed using the Xcalibur 2.2 software provided by the manufacturer. LC-HRMS experiment was performed in triplicate. The variance (ANOVA) and *t*-test were applied to estimate differences (considered to be significant at $p \leq 0.05$). Microsoft Excel 2016 was used for statistical analyses.

4.4. Bacterial strains

The antimicrobial activity was evaluated using Gram-positive and Gram-negative strains: *Staphylococcus aureus* ATCC6538P, *Bacillus subtilis* PY79, Bacillus cereus ATCC10987, *Bacillus licheniformis* ATCC9789, *Listeria monocytogenes* ATCC7644, *Escherichia coli* DH5*α*, *Pseudomonas aeruginosa* PAOI, *Shigella sonnei* ATCC25931, and *Salmonella tiphymurium* ATCC14028.

4.5. Antimicrobial activity assay

The presence of antimicrobial molecules in the extract of *S. spreitzenhoferi* was detected using the agar diffusion assay following the method of Kirby-Bauer with slight modifications (Bauer et al., 1966). Briefly, three different extract amounts (10, 15, and 25 mg) were placed on Luria bertani agar plates which were overlaid with approximately 10 mL of soft agar (0.7%) pre-mixed with 10 μL of Gram-positive and negative strains grown for 24 h at 37 °C. The negative control was dimethylsulfoxide (DMSO) 80% used to resuspend the *S. spreitzenhoferi* extract; the positive control was represented by the antibiotic colistin for *Pseudomonas aeruginosa* and ampicillin for the other strains. Plates were incubated overnight at 37 °C and the antimicrobial activity was calculated according to the relation cited below (Di Napoli et al., 2019). A single colony of Gram-positive strains was resuspended in 5 mL of LB medium (Difco, Detroit, MI) and incubated overnight at 37 °C. The culture in the stationary phase was diluted at 1:100 in 20 mM, pH 7.0 NaP buffer. Samples with a final volume of 500 μL were prepared; they contained bacterial cells for 1/25 of the final volume, extract of *Stachys spreitzenhoferi* (resuspended in DMSO 80%) at different concentrations (0.1, 0.2, 0.5, and 1 mg/mL), and 20 mM pH 7.0 of NaP buffer up to final volume. Samples without extract were used as a positive control. The negative control contained bacterial cells with DMSO 80% used to resuspend the *S. spreitzenhoferi* extract. After 4 h of incubation at 37 °C with stirring at 150 rpm, serial dilutions (1: 100, 1: 1000) of all samples were prepared and then plated on LB-agar in Petri dishes that were finally incubated at 37 °C overnight. The following day, the surviving percent of bacterial cells was estimated by counting the number of colonies (Zanfardino et al., 2010). Each experiment was performed in triplicate and the reported result was an average of three independent experiments. (P value was <0.05).

4.6. Determination of minimal inhibitory concentration

Minimal inhibitory concentrations (MICs) of *S. spreitzenhoferi* extract against the Gram-positive strains were determined according to the microdilution method established by the Clinical and Laboratory Standards Institute (CLSI). ~5×10^5 CFU/mL were added to 95 μL of cation-adjusted Mueller-Hinton broth (CAM-HB; Difco) supplemented or not with various concentrations (0.2–2 mg/mL) of *S. spreitzenhoferi* extract

(Prencipe et al., 2021). Following overnight incubation at 37 °C, MIC_{100} values were determined as the lowest extract concentration responsible for no visible bacterial growth.

4.7. Antioxidant activity

4.7.1. DPPH scavenging capacity assay

The measurement of the DPPH (2,2-diphenylpicrylhydrazyl hydrate) radical scavenging activity was carried out according to Kedare and Singh (2011). Different concentrations of extract (0.025–1 mg/mL) were added in a final volume of 1 mL of 100% methanol containing 0.1 mM of freshly prepared DPPH (giving absorbance ≤1.0). The reaction was allowed to proceed for a maximum time of 30 min at 25 °C and the absorbance was measured at 517 nm. The DPPH free radical scavenging activity was calculated according to the following equation (Mazzoli et al., 2019) DPPH radical scavenging activity (%) = (1− AS/AC) × 100, where AS is the absorbance of the reacted mixture of DPPH with the extract sample, and AC is the absorbance of the DPPH solution.

4.7.2. ABTS scavenging capacity assay

This assay was performed according to the reported method (Re et al., 1999), with some modifications, which are based on ABTS radical cation scavenging. A stock solution was prepared by stirring 7 mM ABTS and 2.45 mM (final concentration) potassium persulfate in water and incubating at room temperature in the dark, for 16 h before use. The concentrated ABTS was diluted with phosphate-buffered saline (PBS) to a final absorbance of 0.72 (±0.02) at 734 nm. Then 1 mL ABTS solution was added to 100 μL of extracts (0.025–1 mg/mL concentrations). The absorbance of ABTS was measured on a 1700 PharmaSpec UV/Vis spectrophotometer (Shimadzu) after 6 min incubation in dark, at 734 nm. Finally, the absorbance was measured at 734 nm against a blank, and the percentage inhibition of ABTS radical was determined from the following equation: $ABTS^{+}$ radical scavenging activity (%) = (1 − AS/AC) × 100, where AC is the absorbance of the ABTS solution and AS is the absorbance of the sample at 734 nm. The concentration required for 50% inhibition was determined and represented as IC_{50}.

4.7.3. Hydrogen peroxide scavenging assay

The hydrogen peroxide stability was measured by following absorbance at 240 nm of 1 mL of fresh hydrogen peroxide solution (50 mM Potassium Phosphate Buffer, pH 7.0; 0.036% (*w/w*) H_2O_2). Quantitative determination of H_2O_2 scavenging activity was measured by the loss of absorbance at 240 nm as previously described by Beers and Sizer (Petruk et al., 2018; Beers and Sizer, 1952). Briefly, different concentrations of *S. stachys* extract (0.025–1 mg/mL) were incubated at 20 °C in 1 mL of hydrogen peroxide solution (50 mM Potassium Phosphate Buffer, pH 7.0; 0.036% (*w/w*) H_2O_2). After 30 min, aliquots were centrifuged for 1 min at 13000 g and the hydrogen peroxide concentration in the supernatant was determined by measuring the absorbance at 240 nm. The percentage of peroxide removed was calculated as follows: peroxide removed (%) = (1 − AS/AC) × 100, where AC is the absorbance of 1 mL of hydrogen peroxide solution and AS is the absorbance of the sample at 240 nm.

4.8. Antioxidant activity on polymorphonuclear leukocytes (PMN)

4.8.1. Blood collection and polymorphonuclear leukocytes (PMN) isolation

Whole blood was obtained with informed consent from healthy volunteers at University "Federico II" in Naples, Italy. Between 08.00 and 09.00 a.m., three healthy fasting donors were subjected to peripheral blood sampling with K3EDTA vacutainers (Becton Dickinson, Plymouth, UK). PMN were isolated following the protocol described by Badalamenti et al. (2021b). The isolated PMNs was measured in the presence or absence of various concentrations extract of *S. spreitzenhoferi*, without or with Opsonised zymosan (OZ).

4.9. Reactive oxygen species ROS generation

Dichlorofluorescein (DCF) assay was performed to quantify ROS generation according to (Manna et al., 2012). The PMN were treated with extract of *S. spreitzenhoferi* at the concentration of 0, 0.025, 0.05, 0.1, 0.2, 0.5, 0.75 mg/mL without or with OZ (0.5 mg/mL) for 6 h and then incubated with the non-polar and non-fluorescent 2′,7′-dichlorodihydrofluorescin diacetate (DCFH-DA), at 10 μM final concentration, for 15 min at 37 °C. ROS quantity was monitored by fluorescence (excitation wavelength of 350 nm and an emission wavelength of 600 nm) on a microplate reader. Results were expressed as fluorescence intensity.

4.10. Antioxidant enzymes measured in PMN cells

A commercial kit (BioAssay System, San Diego, CA, USA) was used to determine superoxide dismutase (SOD), catalase (CAT), and glutathione *S*-transferase (GST) enzymatic activity in PMN cells according to the manufacturer's recommendations. The activity of enzymes was expressed as U/L (Barbosa et al., 2016). Extract of *S. spreitzenhoferi* were tested at the concentration of 0, 0.025, 0.05, 0.1, 0.2, 0.5, 0.75 mg/mL. The experiments were performed in the presence and absence of OZ (0.5 mg/mL).

4.11. Cell lines and culture conditions

The cell line U937 was obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were grown at 37 °C in 5% CO_2 atmosphere in Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco, NY, USA), then supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% l-glutamine, 1% ampicillin or streptomycin, and 0.1% gentamicin. All cell lines, initially plated at 1000 cells/mL, were treated using different dosages of *S. spreitzenhoferi* extract, corresponding to 0.2, 0.5, 0.75 mg/mL to 24 h.

4.12. FACS analysis

FACS analysis using BD FACS Celesta Flow Cytometer from BD Biosciences was used to estimate cell population percentages at different cell cycle stages and to estimate dead cell population percentages. The cells examined were collected after exposure to the compounds. After washing with phosphate buffered saline (1x PBS), to examine the percentage of cells at different stages of the cell cycle, the cells were treated with Cycle Buffer (1x PBS, 10% NP-40, 10% sodium citrate and propidium iodide 2 mg/mL) for 15 min at room temperature, instead to examine the percentage of dead cell population, the cells were treated with PI Buffer (1x PBS and 2 mg/mL propidium iodide) (Bontempo et al., 2021; De Masi et al., 2020).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Appendix B. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.phytochem.2022.113373.

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