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Codiaeum variegatum Zanzibar (Pictum spot): LC-MS/MS Phytochemical Profile and *In vitro* Antioxidant and Antitumor Activities

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Abstract

This research aimed to study the phytochemical and biological properties of eleven clone cultivars of *C. variegatum* (*Mrs. Iceton or Red Iceton, Gold Star, Petra, Oak leaf, Norma, Eleanor Roosevelt, Spirale, Majesticum, Zanzibar pictum spot, Andreanum, Red spot*) to evaluate their therapeutic potentials, values, and variability. Methanol-soluble portion (MSP) of the total 70% aqueous methanol aerial part extracts was prepared using conventional techniques (low temp., low pressure) to prevent the degradation of their active metabolites. Their phytochemical screening and chromatographic analysis revealed the presence of a high content of terpenes, flavonoids, alkaloids, phenolic acids, and saponins. Moreover, the LC-MS technique, using UPLC-qTOF ESI-MS/MS was optimized for the tentative identification of 114 phytoconstituents from the MSP of *C. variegatum Zanzibar (Pictum spot)* as a representative instance for all eleven cultivars investigated. The identification is mainly based on matching their R_t-values, monoisotopic molecular, specific fragment ion masses, and their relative abundances with the respectable open library database software. The antioxidant activity for MSP of the eleven *C. variegatum* cultivars revealed a promising antioxidant capacity due to their high polyphenolic content, especially flavonoids and phenolic acids detected by preliminary and LC-MS/MS phytochemical screening. Moreover, a significant effect was noticed for all examined cultivars on the viability of Ehrlich ascites carcinoma cells (EACC), which reflects their antitumor activity. The promising *in vitro* antioxidant and cytotoxicity findings of the target 11MSP revealed strong broad-spectrum activities that were encouraging to extend special attention to the isolation and biological evaluation of the major constitutive polyphenols as antioxidant and anticancer agents. Current findings draw attention to the fact that *C. variegatum Zanzibar* can be considered a potent, safe antioxidant and antitumor agent that could be used in many pharmaceutical, food, and folk medicine applications to treat certain diseases.

Keywords: Euphorbiaceae; Codiaeum; LC-MS/MS; Polyphenolics; Antioxidant; Antitumor

1. Introduction

The genus *Croton* belongs to the Euphorbiaceae family and contains approximately 1300 species of trees, shrubs, and herbs, which are widely distributed throughout tropical and subtropical regions of the world [1]. Many *Croton* species have been used as folk medicines in Africa, South Asia, and South America, for the treatment of many diseases such as stomachache, abscesses, inflammation, and malaria [2]. *Codiaeum variegatum* (L.) is a popular ornamental foliage plant that displays an anomalous range of variations in its leaf size, shape, and color pattern. By fixing such leaf variations, more than 300 cultivars have been produced around the world.

Reactive oxygen species (ROS) generation that is excessive is associated with oxidative stress. This is crucial in accelerating the development of liver disorders [3]. The antioxidant and free radical scavenging activities of phytochemicals, particularly polyphenols, are recognized to be responsible for several disorders, including diabetes, cancer, aging, and cardiovascular issues [4]. *C. variegatum*, commonly referred to as Croton, was formerly known as "garden croton"; its leaves are thought to possess sedative, purgative, antioxidant, anti-amoebic, anti-inflammatory, and anti-cancer qualities [5]. Gastritis is treated using a decoction of the roots. *C. variegatum* fresh latex and leaf extract have antiviral properties

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against the herpes simplex and influenza A viruses. There are reports of immunostimulant, antifungal, antibacterial, insecticidal, antimalarial, and kidney stone therapy characteristics [5].

The international cancer burden doubled between 1975 and 2000 and then doubled again by 2020 and is set to nearly triple by 2030. There were around 12 million new cancer cases and 7 million cancer deaths worldwide in 2008, with 20-26 million new cases and 13-17 million deaths projected for 2030 [6]. Therefore, the need for effective management, treatment, and cure of cancer is undoubtedly crucial. The control of cancer, one of the leading causes of death worldwide, may benefit from the potential that resides in alternative therapies [7]. Conventional therapies cause serious side effects and, at best, merely extend the patient's lifespan by a few years. There is thus a need to utilize alternative concepts or approaches to the prevention of cancer [8]. An integrative approach for managing a patient with cancer should target the multiple biochemical and physiological pathways that support tumor development and minimize normal-tissue toxicity. Interestingly, both laboratory experiments and clinical trials have demonstrated that when combined with chemotherapy, herbal medicines could raise the efficacy level and lower toxic reactions. These facts raised the feasibility of the combination of herbal medicine and chemotherapy [9]. The anticancer and antioxidant effects have been reported among the common activities on *Croton*, one of the largest genera of Euphorbiaceae plants [10]. Meanwhile, many *Croton* species are widely used in ethnomedicine for the treatment of several diseases including cancer [11]. As such there has been a growing interest in this genus for phytochemical screening and isolation of anticancer compound/s if any. The search for improved cytotoxic agents continues to be an important line in the discovery of modern anticancer drugs. Synergistic interactions of such substances with chemotherapeutic agents may be studied. Also, the molecular mechanism of the anticancer activity of the isolated compound/s may be a subject of research in the near future.

The genus *Croton* is abundant in diverse diterpenoids, including clerodane, tigliane, kaurane, labdane, cembrane, and pimarane, with a wide range of biological activities, such as cytotoxicity [12], anti-inflammatory [13], and anti-microbial [14]. Due to the great structural diversity and broad relevant bioactivities of their metabolites, *Croton* species have attracted increasing research attention. Several authors have provided many reviews about their chemical constituents and biological activities [13], [15], [16]. The current study is a phytochemical screening approach of the target eleven clone cultivars of *C. variegatum* together with an evaluation of their therapeutic potentials, values, and variability as antioxidant and cytotoxic agents. Furthermore, an extensive and fast precise LC/MS/MS determination was carried out for the constitutive secondary metabolites in the MeOH-soluble portion (MSP) of *C. variegatum Zanzibar (Pictum spot)* aerial parts as an instance for other cultivars.

2. Materials and methods

2.1. Chemicals and reagents

All chemical reagents and solvents were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA), Thermo-Fisher Scientific (Waltham, MA, USA), or Merck (Darmstadt, Germany) for the *in vitro* studies as HPLC- or analytical grades that were used before for similar biological investigations [17].

2.2. Plant materials

In October 2019, aerial parts samples from the eleven clone cultivars of *C. variegatum* L. were collected from the Suez Canal University Garden in Ismailia, Egypt. Dr. Thérèse Labib, the head of Taxonomists at Al-Orman Botanical Garden Herbarium in Giza, Egypt, kindly identified all samples. Voucher specimens (coded as R-Ci-I, R-Cg-II, R-Cp-III, R-Co-IV, R-Cn-V, R-Ce-VI, R-Cs-VII, R-Cm-VIII, R-Cz-IX, R-Ca-X, R-Cr-XI) were then submitted to the herbarium section of the Botany Department, Faculty of Science, Cairo University, Cairo, Egypt for future reference.

2.3. Extraction

A quantity of 50 g, from each plant, was subjected to extraction with 70% aqueous MeOH (10 x 150 mL) at a temperature of 80℃ under reflux conditions [17]. The extract cuts were individually collected and dried under reduced pressure and temperature on a rotatory evaporator, and then successively taken with MeOH under reflux at 65℃ to obtain the MeOH-soluble portion/s (MSP). Subsequently, each MSP was screened for the presence of all-natural product types [18].

2.4. Preliminary phytochemical screening

The powder of the naturally air-dried samples for eleven *C. variegatum* plants, cultivated in Egypt (1-Mrs. Iceton or Red Iceton, 2-Gold Dust, 3-Petra, 4-Oak leaf, 5-Norma, 6-Eleanor Roosevelt, 7-Spirale, 8-Majesticum, 9-Zanzibar (Pictum spot), 10-Andreanum, 11-Red spot) were screened separately for volatile substances [19], carbohydrates and/or glycosides [20], flavonoids [20], saponins [20], alkaloids [20], Tannins [21], anthraquinones [20], coumarins [20], unsaturated sterols and/or triterpenes [20], and iridoids [20]. To confirm the presence of the different polyphenolic types, a two-dimensional paper chromatography (2D-PC) technique was employed, with BAW (n-BuOH-AcOH-H_2O, 4:1:5, upper layer)

for the first run and 15% aqueous AcOH for the second run [22]. The spots were visualized using UV light and spray reagents, including ammonia vapors, $AlCl_3$, $FeCl_3$, and NA/PE. This allowed for a thorough analysis of the samples' chemical composition.

2.5. UPLC/ ESI-qTOF-HRMS/MSanalysis

The LC/MS analysis was conducted at the proteomics and metabolomics unit located in a children's cancer hospital (CCHE 57357) in Cairo, Egypt. The analysis utilized an HPLC standard interface (Exion LC, Sciex) coupled with a quadruple time-of-flight (QTOF) mass spectrometer (Triple TOF 5600+, Sciex) equipped with HR-TOF scan capabilities. This instrument allowed for MS/MS selective fragmentation analysis and the collection of structural information [22], [23]. The analysis was performed in negative mode. The estimation and assignment of MS data were done by Analyst TF (1.7.1) and MS-DIAL 4.8 open-source software together with Respect negative (1573 records) reference databases. Enhanced product ion (EPI) scan in a linear QTOF with information-dependent data acquisition (IDA) enabled the generation of MS fragment data even from minor metabolites.

Chromatographic separation was achieved using an X-select HSS T3 column (2.1x150mm, 3.5µm) from Waters, which was maintained at a temperature of 40°C. An In-line filter disks pre-column (3.0mm x 0.5µm) from Phenomenex was utilized. The mobile phases employed were as follows: Mobile phase A consisted of a 5 mM $HCOONH_4$ buffer (*p*H=8) containing 1% MeOH, and mobile phase B consisted of 100% MeCN. The flow rate was set to 0.3 ml/min, and the injection volume was 10 µl of 2.5 µg/µl solution in H_2O-MeOH-CH_3CN (50: 25: 25 v/v) after vortex for 2 min followed by ultra-sonication for 10 min to prepare stock solution (50 mg of sample/1 ml) and then dilution 50 to 1000 µl. The separation process involved a series of linear gradients (Table 1). Solvents, HPLC grade: MeOH, HCOOH, and NaOH for *p*H adjustment (Fisher Scientific, UK); $HCOONH_4$, and CH_3CN (Sigma-Aldrich, Germany); H_2O Milli-Q (Millipore, USA).

Table 1. Time program of UPLC/HRESI-MS analysis for MSP of *C. variegatum Zanzibar (Pictum spot)*

Time/min	0	1	21	25	25.01	28
%A	90.0	90.0	10.0	10.0	90.0	90.0
%B	10.0	10.0	90.0	90.0	10.0	10.0

MSP: MeOH-soluble portion of 70% aq. methanol extract of aerial parts

2.6. Biological evaluation

2.6.1. Assays for antioxidant capacity

To detect antioxidant properties, several chemical assays with different mechanisms namely, DPPH radical scavenging, reducing power ABTS radical scavenging, ferric reducing power ability and metal chelating were employed. The obtained results were expressed as equivalents of the standard compounds, BHA or Trolox. All assays were applied according to previous studies [24], [25].

2.6.2. Cell viability assay

In vitro, cytotoxic activity was determined according to previous literature [24] with a slight modification. Using Trypan Blue cytotoxicity assay briefly, methanol extracts of the 11 cultivars of *C. variegatum* were dissolved in saline to a final concentration of 500 µg/ml and made up to 800 µl with Phosphate buffered saline (PBS). EAC (100 µl) with a concentration of about 10^6 cells/ml was added to the tubes. This was then incubated at 37^0C for 3 hours followed by the addition of 100 µl (0.4% in PBS) of trypan blue dye (Sigma-Aldrich, St. Louis, USA) to all the tested cultivars extracts. The control consisted of PBS in place of the extracts. The cells that did not take up the dye and those that took up the dye were viable and non-viable, respectively. Cells were counted using a haemocytometer slide.

2.7. Statistical analysis

An excel spread sheet program was used to calculate the mean and standard deviation.

3. Results

3.1. Phytochemical screening

Samples of the 11 *C. variegatum* cultivars (*1- Mrs. Iceton or Red Iceton, 2-Gold Dust, 3-Petra, 4-Oakleaf, 5- Norma, 6-Eleanor Roosevelt, 7-Spirale, 8-Majesticum, 9-Zanzibar (Pictum spot), 10-Andreanum, 11-Red spot*) were preliminary screened for their constitutive natural products classes by the application of the different chemical and chromatographic examinations published in the literature [19–22] see section 2.4. The results recorded in Table 2 showed the presence of carbohydrates and/or glycosides, flavonoids, terpenoids, alkaloids, and tannins in all 11 *C. variegatum* cultivars with traces of saponins and iridoids and the absence of volatile oils, coumarins, and anthraquinones**.**

Table 2. Preliminary phytochemical screening findings of the MSP for different eleven *C. variegatum* cultivars

Family of compounds	Type of test	Inference											Refs
		1	2	3	4	5	6	7	8	9	10	11	
volatile substances	Micro sublimation	-	-	-	-	-	-	-	-	-	-	-	[19]
Carbohydrates	Molish's test	++	+	++	++	+	++	++	++	++	++	++	[20]
	Fehling's test	++	++	++	++	++	++	++	++	++	++	++	
Flavonoids	Shinoda's test	±	±	±	±	±	±	±	±	±	±	±	[20]
	Free flavonoids	+	+	+	+	+	+	+	+	+	+	+	[26]
	Free flavonoids	+	+	++	+	+	±	+	++	+	+	+	
	Combined flavonoids	++	+	+	+	±	±	+	++	+	+	++	[27]
Saponins	Forth test	±	±	±	±	±	±	±	±	±	±	±	[20]
	Blood haemolysis	+	+	+	+	+	+	+	+	+	+	+	
Alkaloid	Dragendorff's test	+	+	+	+	+	+	+	+	+	+	+	[20]
	Meyer's test	+	+	+	+	+	+	+	+	+	+	+	
Tannins	$FeCl_3$	++	++	++	++	++	++	++	++	++	++	++	
	Matchstick	±	±	±	±	±	±	±	±	±	±	±	
	Vanillin hydrochloric acid	±	±	±	±	±	±	±	±	±	±	±	[21]
	Gelatin	±	±	±	±	±	±	±	±	±	±	±	
Anthraquinones	Borntrager's	-	-	-	-	-	-	-	-	-	-	-	
Coumarins	-	-	-	-	-	-	-	-	-	-	-	-	
Unsaturated sterols and/or Triterpenes	a-Liebermann Burchard test	+	+	+	+	+	+	+	+	+	+	+	[20]
	b-salkowiski's test	+	+	+	+	+	+	+	+	+	+	+	
Iridoids	-	+	+	+	+	+	+	+	+	+	+	+	

(-) Absent, (±) Present in traces, (+) Present, (++) Present in abundance

3.2. LC-ESI-qTOF-HRMS profile of *C.variegatum Zanzibar (Pictum spot)*

The current study demonstrates the effectiveness of UPLC/ESI-qTOF-HRMS/MS in rapidly identifying 114 compounds in the MSP of *C. variegatum Zanzibar(Pictum spot)* extract using negative ionization mode (Table 3). It was chosen for the analysis of aerial parts extract due to the great stability of major phenolic compounds in their phenolate form (Figures 1S-114S). The large number of the MS peaks recorded in both TIC and BPC chromatograms represented how much MSP is crowded with different types of secondary metabolites (Fig. $1_{A, B}$).

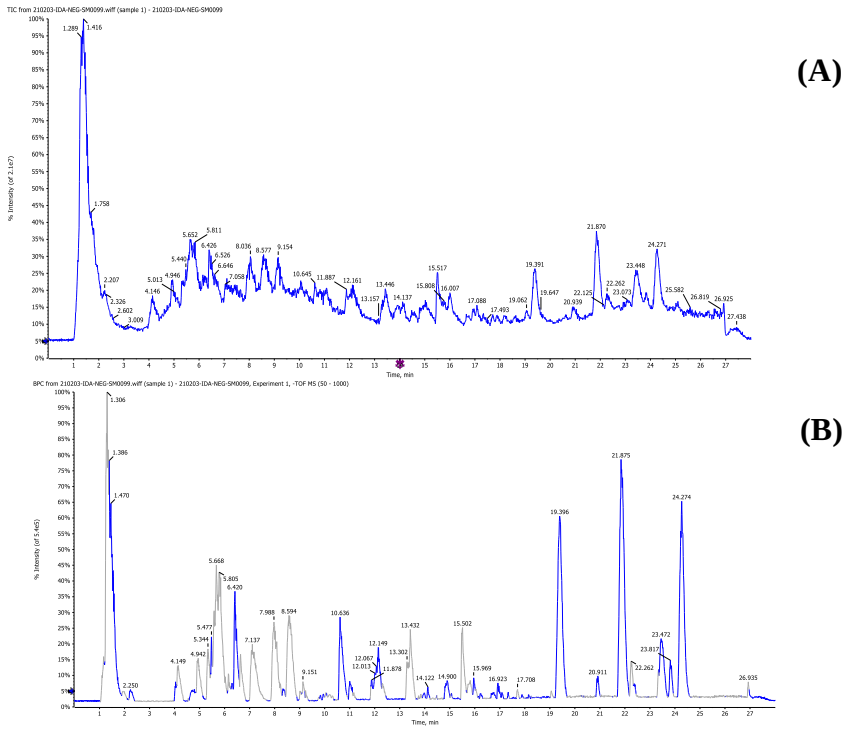


Fig.1. Negative ion mode TIC (A) and BPC (B) MS chromatograms of UPLC-ESI-qTOF-HRMS/MS for the MeOH-soluble portion (MSP) of *C. variegatum Zanzibar (Pictum spot)*

Identification of the compounds (1–114) was achieved by comparing retention time (R_t) values, monoisotopic masses of the molecular and some selective fragment ions, and their relative abundances with the available scientific literature and the open-source software of a library database mentioned above(Figures 1S-114S), section 2.5 [17], [23], [28]. The Respect negative database, which consists of 1573 records, was used as a reference database [29]. The relative concentration of each compound was determined by calculating the peak area in the MS chromatograms, as presented in Table 3 and Figure 1. It is noteworthy that most ofthe detected polyphenols belong to the flavonoid category, including various subgroups such as flavones, flavonols, flavanones, flavanonols, and chalcones, together with some of their mono-/di-(*O*- or *C*-)-glycosides at C-3, C-7, C-8 or C-4' with only one acylated by 3-*O*-*p*-coumaroyl moiety. Some phenolic acids and two coumarins are also presented together with alkaloids and other natural product types listed in Table 3.

Table 3. Negative UPLC-ESI-qTOF-HRMS/MS identification of the metabolites in the MSP of *C. variegatum Zanzibar (Pictum spot)*

Peak NO.	Rt/min.	$[M–H]^-$ / Exp.	M.F.	MS/MS fragments (m/z)	Area	Tentative identification	Error ppm
Polyphenols							
flavonoids							
1.	3.867	447.1703	$C_{21}H_{20}O_{11}$	385.04355:36, 447.10312:214 447.26141:107	62700	Quercitrin	5.2
2.	5.951	445.1638	$C_{21}H_{18}O_{11}$	293.08087:71, 445.16382:71	56505	Baicalein 7-*O*-glucuronide	-26.5
3.	1.189	477.1547	$C_{22}H_{22}O_{12}$	133.01608:71 341.11984:36 370.97839:36 373.03787:71, 445.13083:36, 477.0437:108, 477.10233:180	41389	Isorhamnetin 3-*O*-glucoside	-6.9
4.	6.308	461.1238	$C_{21}H_{18}O_{12}$	149.05548:36 153.01997:71 191.06337:36 307.12692:36 315.0762:107, 392.88751:36 413.81714:72 415.16287:218 461.07681:293 461.10715:1043	160241	Kaempferol 3-glucuronide	-2.3
5.	10.017	417.1566	$C_{20}H_{18}O_{10}$	137.01886:36, 166.03241:71 181.05165:179, 280.92606:36, 311.05249:36, 371.24789:71 387.11911:71 402.1236:71, 417.15656:822, 417.19983:74	459881	Kaempferol 3-*O*-α-L-arabinoside	0.8
6.	5.633	431.1593	$C_{21}H_{20}O_{10}$	101.02187:71 113.02946:54, 119.03705:232 131.04147:54 149.04176:36, 153.09169:36, 161.04419:179, 179.05638:965 223.12741:71 225.05991:357, 385.18771:271, 431.15085:114 431.19189:2701	2712963	Kaempferol 3-*O*-α-L-rhamnoside	4
7.	7.212	507.1061	$C_{23}H_{24}O_{13}$	218.95297:71 354.91867:71 461.25922:36 507.10611:143	19572	Syringetin 3-*O*-galactoside	0.9
8.	1.061	448.9352	$C_{21}H_{22}O_{11}$	448.93515:71	26841	Okanin 4'-*O*-glucoside	2
9.	7.407	577.1591	$C_{27}H_{30}O_{14}$	174.08485:36 237.08644:75 239.06523:75 269.04782:959 415.16898:36, 541.18359:73 577.15607:1515	498491	Rhoifolin	-1.4
10.	6.513	609.1405 $[M–2H]^-$	$C_{27}H_{31}O_{16}$	300.02289:71 301.03165:36 556.07715:18 563.13031:54 563.24091:36, 609.1405:1107	124917	Delphinidin 3-O-(6''-O-α-rhamnopyranosyl-β-glucopyranoside)	-0.3
11.	4.433	609.1289	$C_{27}H_{30}O_{16}$	447.07867:36 473.71768:36 495.10355:36 563.1593:74 586.14093:36, 609.12885:296 609.28223:38	69877	Luteolin 7,3'-di-*O*-glucoside	0
12.	26.592	591.1906	$C_{28}H_{32}O_{14}$	112.98903:12 180.9762:10 248.95349:17 250.96544:12 279.23425:10 316.93707:17 318.93924:14 318.95438:33 384.93033:10 386.92807:12 386.94476:24 ,446.16928:17 452.91315:10 453.92368:12 454.92929:41, 489.15768:12, 504.17484:31, 522.92395:14 523.33423:12, 589.29572:17 589.31628:14 590.88422:12 590.90826:14, 591.22418:2491	604427	Acacetin 7-*O*-rutinoside	1.2

13.	11.225	611.1994	$C_{28}H_{36}O_{15}$	565.28455:71 611.1994:179	44325	Neohesperidin dihydrochalcone	-0.8
14.	4.919	593.1454	$C_{27}H_{30}O_{15}$	297.07632:107, 353.06555:751, 383.06927:333 395.07919:143 413.08701:143 455.09976:107, 473.09973:607 485.10696:73 503.10214:255, 503.11798:252, 575.14099:179, 591.20551:143 593.01324:180 593.1474:6959	4046339	Kaempferol 7-neohesperidoside	1
15.	5.498	593.1863	$C_{28}H_{34}O_{14}$	161.04602:36 209.08107:54 227.09016:89 341.10992:36 431.14795:36, 457.06128:54 503.12842:36 547.17322:36 547.21289:36 593.03839:36 593.14502:109 593.15875:238 593.18628:352 593.20007:314	77483	Isosakuranetin 7-*O*-neohesperidoside	-0.2
16.	4.956	593.1474	$C_{30}H_{26}O_{13}$	297.07632:107, 353.06555:751 383.06927:333 395.07919:143 413.08701:143 455.09976:107, 473.09973:607, 503.10214:255, 503.11798:252, 575.14099:179, 591.20551:143 593.01324:180 593.1474:6959	56301	Kaempferol 3-*O*-(6-*p*-coumaroyl)-glucoside	-39.4
17.	6.636	431.086	$C_{21}H_{20}O_{10}$	269.04346:179, 281.05228:250 282.05478:107, 283.06381:713 295.07483:116, 311.05826:2976 323.05649:291 323.07935:145 325.07529:107, 341.03729:294, 341.06598:1621 353.05878:107 383.0816:145, 413.07687:146 413.08261:217 429.71558:164 431.05756:107 431.09863:5306	3444532	Apigenin 8-*C*-glucoside	3.3
18.	6.050	447.0968	$C_{21}H_{20}O_{11}$	327.04602:250 353.16946:36 357.06604:143 401.12152:73 413.14197:73, 447.09677:706	286762	Luteolin 8-*C*-glucoside	-42.4
19.	7.187	415.1112	$C_{21}H_{20}O_{9}$	192.04716:36 207.0706:107 278.92542:71 415.11176:71 415.18658:107	52353	Daidzein 8-C-glucoside	-0.3
20.	6.408	577.1548	$C_{27}H_{30}O_{14}$	59.01639:107, 269.04724:107 282.05624:214 293.04666:1711, 295.05688:113 310.05667:108 311.05975:328 314.03864:107, 323.05292:433 335.05832:143, 337.07275:109, 341.07013:293, 353.0683:179, 395.08768:227 413.08133:1652, 413.08423:2032, 431.10608:110, 457.10574:960, 559.13745:143 575.26294:251 576.80872:96 577.15479:8652	7851975	Vitexin 2"-*O*-rhamnoside	2.9
21.	9.990	303.1474	$C_{15}H_{12}O_{7}$	190.94823:107 258.94391:107 284.99863:71 303.14743:143	9013	(+-)-Taxifolin	44.5
22.	3.060	299.0809	$C_{16}H_{12}O_{6}$	137.02002:86 157.04805:7 239.05974:14 253.07399:14, 299.07291:122 299.10956:21	47273	3,5,7-Trihydroxy-4'-methoxyflavone	-0.4
23.	1.428	315.0738	$C_{16}H_{12}O_{7}$	83.01669:36 102.95147:73 108.02106:438 109.02747:110 123.04733:71, 133.01793:107 135.03239:109 151.03456:107 152.01164:1053, 153.0146:520 163.03969:107 165.02045:71 315.05127:123 315.07385:2851	1924908	5,7,4'-Trihydroxy-3'-methoxyflavonol	0.8
24.	11.758	283.0564	$C_{16}H_{12}O_{5}$	253.1105:12 268.10742:95 283.12939:286 283.27203:24	62988	Acacetin	39.1
25.	16.786	269.0872	$C_{15}H_{10}O_{5}$	171.08308:109 269.0831:73 269.15494:404	103829	Apigenin	0.7
26.	9.130	301.1443	$C_{16}H_{14}O_{6}$	171.05313:36 255.32474:73 271.12985:107 299.13031:109 301.05344:73, 301.1441:750 301.19559:38	142503	Hesperetin	0.2
27.	12.312	285.0595	$C_{15}H_{10}O_{6}$	186.06366:71 187.07455:143 199.07211:71 255.10374:71 269.1174:214, 270.05429:179 285.08228:197 285.14905:4804	1888739	Luteolin	2.1

28.	8.207	271.1953	$C_{15}H_{12}O_5$	227.15813:36 271.10419:179	40365	Naringenin	0.9
phenolic acids and their derivatives							
29.	3.919	359.1416	$C_{18}H_{16}O_8$	195.11145:12 197.0338:24 239.0643:36 319.06152:12 359.09656:167, 359.21967:24	48542	Rosmarinic acid	-0.4
30.	1.365	153.0178	$C_7H_6O_4$	81.03513:71 108.02235:219 109.02876:797 153.01782:323	441578	3,4-Dihydroxybenzoic acid	5.6
31.	7.113	167.0352	$C_8H_8O_4$	66.01006:36 86.00443:36 95.0125:143 98.00111:36 107.01364:111, 108.01977:1004 111.00835:183 124.0192:775 135.00792:222, 139.01361:148 152.0135:1900 167.03516:5004	9654869	5-Methoxysalicylic acid	0.5
32.	3.301	137.0238	$C_7H_6O_3$	65.03806:36 93.03739:330 137.02383:197	181590	*p*-Hydroxybenzoic acid	-0.4
33.	4.703	137.0241	$C_7H_6O_3$	95.01244:36 119.01072:109 122.99416:36 136.01573:511 137.02406:2109	990700	Salicylic acid	0.4
34.	2.274	193.0497	$C_{10}H_{10}O_4$	106.06429:36 133.03404:73 134.03781:323 149.05684:72 149.06374:179, 178.02547:289 193.04968:322	165030	3-(4-Hydroxy-3-methoxyphenyl)prop-2-enoic acid	51.2
35.	1.981	163.0377	$C_9H_8O_3$	93.03328:147 118.67332:74 119.04905:1434 163.03775:322	252271	3-(4-Hydroxyphenyl)prop-2-enoic acid, *p*-Coumaric acid	5
36.	1.478	317.0896	$C_{18}H_{22}O_5$	181.07999:71 317.04935:107 317.07196:143 317.08957:179	93087	Zearalenone	-12.8
37.	11.013	207.0662	$C_{11}H_{12}O_4$	105.03298:143 133.02145:338 133.0296:1293149.02112:107 164.04601:214 177.00983:146 177.01921:635,192.04092:4219 193.04633:143 207.06616:2409	2054437	Sinapyl aldehyde	3
38.	5.548	183.0003	$C_8H_8O_5$	183.00731:71 183.03023:71	8804	3,4-Dihydroxymandelicacid	-1.4
coumarins							
39.	10.627	177.0551	$C_9H_6O_4$	77.03918:71,89.03964:52 93.03374:43 ,105.03456:390 116.02539:38, 116.66373:52 117.03473:5498, 118.04229:1609 133.02812:257 134.03514:262 135.03938:12144.56873:64 145.02931:4240, 146.03119:36 147.04335:67, 162.03174:1199, 176.49174:112, 177.05511:7002	6858963	Daphnetin	5.8
40.	16.749	339.0883	$C_{15}H_{16}O_9$	119.05129:71 183.01729:217 338.13062:79 339.19888:2801	1618907	Esculin	-9.6
stilbene							
41.	8.709	405.1425	$C_{20}H_{22}O_9$	135.0491:36 153.02013:71 390.07938:36, 405.11212:179 405.18036:71	148404	*E*-3,4,5'-Trihydroxy-3'-glucopyranosylstilbene	-0.8
***N*-containing compounds**							
42.	6.163	455.1912	$C_{17}H_{21}N_4O_9P$	112.9875:36 455.19122:143	52597	Riboflavin 5′-monophosphate sodium salt hydrate	-3.6
43.	6.798	136.0126	$C_7H_7NO_2$	136.01256:107	167104	*ortho*-Aminobenzoic acid	-0.1
44.	2.325	182.0452	$C_8H_9NO_4$	120.04367:71 122.72794:36 138.05591:357 182.04517:214	118115	4-Pyridoxic acid	-14.5
45.	7.344	174.0534	$C_{10}H_9NO_2$	116.04514:71 128.05157:214 130.06526:214 144.0461:107 156.05243:108, 174.05336:474	182404	*β*-Indoleacetic acid	-0.9
46.	1.227	308.1201	$C_{11}H_{19}NO_9$	128.03514:71 193.05966:107 218.06525:71 308.09036:71 308.12009:179	63887	*N*-Acetylneuraminate	0.3
47.	2.237	307.1382	$C_9H_{13}N_2O_8P$	145.08949:71 172.02341:36 307.13821:179	56973	2'-Deoxyuridine-5'-monophosphate	-1.5
48.	4.588	467.0768	$C_9H_{15}N_2O_{14}P_3$	180.97017:36 421.21088:71 467.07684:214	20823	2'-Deoxyuridine-5'-triphosphate sodium salt	-37.2
49.	2.122	322.0888	$C_9H_{14}N_3O_8P$	71.01141:36 161.04562:71 179.05791:143 322.112:214	500012	Cytidine-5'-monophosphate	-0.1
50.	8.083	241.0932	$C_{10}H_{14}N_2O_5$	161.06694:36 241.09323:71	260382	Thymidine	6.6
51.	9.914	401.1078	$C_{10}H_{16}N_2O_{11}P_2$	112.98695:107 219.0612:36 248.95512:107 288.94382:71 295.07416:71, 333.1947:71 337.07574:71 354.9263:71 356.94345:108 369.10944:107 399.86401:75 401.09921:73 401.12466:487 401.14163:879	142479	Thymidine 5'-diphosphate	19.5

52.	7.571	549.1909	$C_{15}H_{24}N_2O_{16}P_2$	339.07285:107 405.11456:71 549.13739:107 549.16382:214	44498	UDP-β-L-rhamnose	0.7
53.	1.753	243.0668	$C_9H_{12}N_2O_6$	82.03754:36 200.05919:107 243.0668:179	118861	Uridine	-0.6
54.	6.436	403.1388	$C_9H_{14}N_2O_{12}P_2$	403.14905:107	76218	Uridine 5'-diphosphate	9.8
55.	5.236	579.1252	$C_{15}H_{22}N_2O_{18}P_2$	302.07623:36 514.15277:72 579.0777:72 579.12524:366	250071	Uridine 5'-diphosphoglucuronic acid	12.5
56.	1.339	323.0684	$C_9H_{13}N_2O_9P$	57.03558:36 73.02735:36 143.07072:71 161.05087:71 323.04535:73, 323.13675:524	155336	Uridine 5'-monophosphate	-2.4
57.	3.386	346.118	$C_{10}H_{14}N_5O_7P$	161.06363:18 179.05231:71 346.11798:54	85334	2'-Deoxyguanosine 5'-monophosphate	0.1
58.	9.626	251.1684	$C_{10}H_{12}N_4O_4$	251.15987:143	58980	2'-Deoxyinosine	-2
59.	1.566	422.1266	$C_{11}H_{21}NO_{10}S_3$	354.18356:36 422.12662:71	13834	3-(Methylsulfinyl)propyl-glucosinolate	-4.2
60.	10.750	436.1488	$C_{12}H_{23}NO_{10}S_3$	122.06233:18, 227.08142:54 408.15063:36, 436.10062:72 436.15372:496	60157	4-Methylsulfinylbutyl-glucosinolate	6.1
61.	1.917	134.0459	$C_5H_5N_5$	92.03053:36 107.03667:107 134.04593:536	142261	Adenine	0.9
62.	3.009	346.1075	$C_{10}H_{14}N_5O_7P$	89.0246:9 113.02319:4 161.0439:13 170.94205:4 179.05042:27 210.9146:9, 278.10666:9 278.90335:13 327.91501:4 346.10751:27 346.13641:18	34636	Adenosine 3'-monophosphate	0.7
63.	9.403	157.1315	$C_4H_6N_4O_3$	157.1315:71	47118	Allantoin	6.3
64.	12.589	243.1238	$C_6H_{13}O_8P$	243.19691:179	46955	α-L-(-)-Fucose 1-phosphate bis(cyclohexylammonium) salt	-4.9
65.	2.390	408.1656	$C_{14}H_{19}NO_9S_2$	340.07159:18, 408.14127:36	16313	Benzylglucosinolate	7.8
66.	2.572	306.1266	$C_{10}H_{17}N_3O_6S$	89.02154:12, 175.03752:12 306.12662:36	21883	Glutathione	0.9
67.	4.034	344.0608	$C_{10}H_{12}N_5O_7P$	176.0448:107, 344.06082:71	28779	Guanosine 3',5'-cyclic monophosphate	0.8
68.	1.214	267.0934	$C_{10}H_{12}N_4O_5$	71.01974:36 103.00158:71 103.04745:36 113.02315:71 115.00632:107, 129.0231:36 133.01784:474 141.01385:36 163.063:36 223.02582:74 231.03584:72 267.06799:981	313496	Inosine	0.1
69.	22.682	743.1703	$C_{21}H_{29}N_7O_{17}P_3$	233.04564:36 539.1579:71 697.43036:71 743.12799:71 743.17035:107	34014	NADP+	-0.7
70.	8.897	151.04	$C_5H_4N_4O_2$	92.02804:179 97.25856:36 136.01572:430 151.03807:833	366334	Oxypurinol	6.6
71.	8.647	424.1471	$C_{14}H_{19}NO_{10}S_2$	218.05112:71 362.16629:36 378.18198:36 424.14709:215	24773	*p*-Hydroxybenzylglucosinolate	0.8
72.	7.886	303.1576	$C_{10}H_{13}N_2O_7P$	190.96025:71 303.15759:286	9675	Thymidine-3',5'-cyclic monophosphate sodium salt	-6.9
73.	11.878	383.2527	$C_{14}H_{20}N_6O_5S$	383.25275:71	31463	S-Adenosyl-L-homocysteine	-0.7
74.	1.579	283.1032	$C_{10}H_{12}N_4O_6$	151.02902:71 168.00725:36 237.09886:257 265.10706:36 283.10324:334	1946100	Xanthosine	10.5
75.	1.352	363.1744	$C_{10}H_{13}N_4O_9P$	327.13553:214 363.08844:107 363.11267:357	327591	Xanthosine 5'-monophosphate	-1.3
76.	7.113	124.0117	$C_2H_7NO_3S$	123.01171:36	50814	Taurine	14.3
77.	1.227	128.0319	$C_5H_7NO_3$	128.03194:393	353570	L-5-Oxoproline	0.7
78.	8.360	144.044	$C_7H_{15}NO_2$	65.99708:20 114.03431:36 115.03951:36 116.04912:123 126.03557:168, 131.0394:20 142.03027:71, 143.58478:66 144.04549:4169	1616155	L-β-Homoisoleucine	-8.3
79.	4.931	275.1468	$C_{11}H_{20}N_2O_6$	118.9568:36 137.0257:179 201.12366:71 257.14221:107 275.14682:107	44276	L-Saccharopine	5.8
80.	3.258	203.0852	$C_{11}H_{12}N_2O_2$	116.05076:197 130.0696:36 157.08575:18 159.09425:71 203.08517:413	226206	L-Tryptophan	8.5
81.	11.435	174.0586	$C_6H_9NO_5$	66.7117:12, 115.04426:36 129.0248:12, 142.02713:71 144.04572:24, 159.03029:71 174.05858:420	102116	*N*-Acetylaspartic acid	-0.3

82.	5.436	146.0718	$C_5H_9NO_4$	91.0309:36, 146.07184:179	123507	N-Acetyl-DL-serine	-0.9
83.	7.728	195.9099	$C_9H_{11}NO_4$	195.90994:107	30162	3,4-Dihydroxy-L-phenylalanine	-16.5
84.	2.772	159.0994	$C_6H_{12}N_2O_3$	71.00535:18 113.10708:18 114.99252:71 158.97829:36 159.09941:143	52792	D-Ala-D-ala	1.1
85.	2.287	164.071	$C_9H_{11}NO_2$	72.00761:71 91.06179:36 147.04462:431 164.07101:433	337425	L-(-)-Phenylalanine	6.5
86.	4.039	176.0603	$C_6H_{11}NO_3S$	106.04252:36 133.04387:36 176.0448:214	132269	N-Formyl-L-methionine	0.9
87.	1.803	130.0858	$C_5H_9NO_3$	61.98452:36 130.0842:107 130.0858:107 130.08742:36 130.08902:36, 130.09064:36 130.1003:36	135961	*trans*-4-Hydroxy-L-proline	-2.9
88.	6.358	171.0975	$C_7H_{12}N_2O_3$	127.1134:71 171.09752:107	35876	Glycyl-L-proline	-0.2
organic acid derivatives							
Terpenoidal acids							
89.	6.991	407.1549	$C_{24}H_{40}O_5$	259.05548:36 263.16125:107 331.11008:36 407.09589:72 407.17569:216, 407.19565:362	112193	Cholic acid	-11.9
90.	4.765	391.1677	$C_{24}H_{40}O_4$	145.03235:36 179.06375:36 291.1568:36 391.11987:215 391.16177:107	183289	Sodium Deoxycholate	1.1
Fatty acids							
91.	19.391	277.2157	$C_{18}H_{30}O_2$	59.01248:311, 68.11813:51, 77.16438:7, 179.17975:7, 182.12981:24 , 233.22525:100, 259.20828:88, 275.19931:83 276.32983:131, 277.2157:6902	20257560	γ-Linolenic acid	-0.4
92.	2.481	201.1115	$C_{10}H_{18}O_4$	133.02283:36 183.0986:71 201.11153:250	40730	Sebacic acid	10.5
93.	4.753	173.1189	$C_8H_{14}O_4$	127.11807:71 173.11893:539	165996	Suberic acid	0.2
Simple organic acids							
94.	1.778	128.0993	$C_5H_6O_4$	84.99095:36 84.99226:71 84.99355:36 85.02611:36 85.03132:36, 128.93506:36 129.02328:36, 129.09229:36	227268	Citraconic acid	-0.4
95.	1.202	191.0534	$C_6H_8O_7$	72.99487:36 87.00783:179 101.02725:73 111.01406:370 115.00026:73, 115.01086:36 129.0231:144 133.01295:435 171.03067:36 173.04253:107 191.05336:876	539833	Citric acid	0.4
96.	1.164	133.0129	$C_4H_6O_5$	59.01307:228 71.0126:1599 72.99004:291 89.02589:267 114.64006:112, 115.00481:1958 119.00438:81, 133.01295:2696	3843364	D-(+)-Malic acid	-0.1
97.	1.227	165.0542	$C_9H_{10}O_3$	73.02957:73 75.01024:289 87.00752:521 99.00986:254 101.02551:73, 105.0193:519, 129.01796:179 147.01851:72 147.02878:287 164.83315:149 165.03993:1615	1518844	D-3-Phenyllactic acid	3.1
98.	8.145	345.1287	$C_{19}H_{22}O_6$	301.14676:143 345.12872:477 345.2258:107	82435	Gibberelin A3	0.6
99.	2.534	331.1921	$C_{19}H_{24}O_5$	153.02155:107 331.09909:143	225136	Gibberellin A4	0.7
100.	1.227	195.0506	$C_6H_{12}O_7$	57.03556:116, 59.01295:355 71.01486:170 72.99233:152 75.00919:1844, 85.0284:197 87.0077:379 89.02443:223 97.99655:1899.00864:466 129.01816:1867, 159.02826:286, 160.84123:693, 177.03935:370 194.42978:71195.05064:4331	4003543	Gluconic acid	-3.9
101.	8.988	193.0506	$C_6H_{10}O_7$	133.02666:218 133.03317:433 134.03531:364137.02621:108 161.01053:109 161.02487:398 161.34943:36178.03012:251	835088	D-(+)-Galacturonic acid	42.4

				193.05061:1963			
102.	1.214	105.0225	$C_3H_6O_4$	72.99607:71 75.01298:36 105.02252:107	64995	Glyceric acid	0
103.	1.227	149.0465	$C_4H_6O_6$	85.02451:36 131.03131:71 149.04646:250	504243	L-(+)-Tartric acid	0.6
104.	1.164	115.0039	$C_4H_4O_4$	71.00923:36 71.01042:71 71.01161:36 71.0128:71 71.01399:36, 71.01637:36 71.01875:36 115.00194:36 115.00345:36	591538	Maleic acid	-3.6
105.	1.994	131.0694	$C_5H_8O_4$	86.9917:36	77175	Methylsuccinic acid	10.3
106.	1.150	117.0373	$C_4H_6O_4$	55.02174:110 73.02982:868 99.01152:220 117.02041:548	475042	Succinic acid	-0.9
Sugars and polyhydric alcohols							
107.	1.403	341.1097	$C_{12}H_{22}O_{11}$	59.01395:517 59.03349:37 71.01241:331,81.03123:3685.03224:71, 89.0177:117, 89.0217:867 99.00718:36 101.0228:257, 107.04245:36, 113.01997:323 119.03349:671 131.03777:112 143.04031:143, 149.04646:252 149.0706:71 159.0247:72 161.04553:400 179.05594:1715, 193.06349:71 249.09555:36 340.0228:114 341.10971:3551	14344156	Sucrose	-1.5
108.	1.566	179.0803	$C_6H_{12}O_6$	58.005:109 59.01297:525 66.03612:111, 71.01605:444 83.01653:36 85.02969:107 87.01163:146, 89.02174:333, 95.01899:36, 101.0214:71 107.05999:107 110.99155:36 113.02596:143 119.035:107 161.04539:143 179.05386:252 179.06709:256 179.08032:362	544716	D-(-)-Tagatose	24.4
109.	3.232	503.1681	$C_{18}H_{32}O_{16}$	143.03668:36 197.07343:54 197.08333:89, 503.16495:89, 503.16812:197	59204	D-(+)-Raffinose	-31.3
110.	11.616	341.1461	$C_{12}H_{22}O_{11}$	183.01233:36 290.18124:12 295.14902:36 296.92059:24 324.90619:12, 340.99188:24, 341.1275:36 341.17969:274	38910	D-(+)-Trehalose	48.1
111.	1.753	181.0745	$C_6H_{14}O_6$	59.0141:393, 71.01255:214 87.00777:71, 89.0245:179, 101.02577:214, 112.98405:286 131.033:107, 149.03793:71 163.05933:323, 179.05032:75 181.07446:1877	288803	L-Iditol	0.7
112.	1.403	181.0726	$C_6H_{14}O_6$	58.00827:182 59.01301:594, 69.03645:146, 71.01255:525, 73.02981:322, 85.02587:107, 89.02317:562, 101.02436:595, 113.0201:71, 119.0259:145 119.03515:181 121.04996:71 149.05347:107 163.06116:322 180.49147:76 181.07259:2422	6270212	Mannitol	7.1
113.	6.965	261.078	$C_6H_{15}O_9P$	219.07208:36 234.08829:71 243.11136:36 261.07797:323	45786	Sorbitol 6-phosphate	3.5
Others							
114.	5.249	171.1357	$C_3H_9O_6P$	171.10268:143	38591	Glycerol 2-phosphate	-6

3.3. Investigation of antioxidant activity

The different biological actions of secondary metabolites produced by plants have garnered significant interest in recent times. It's considered that using plants for medication depends on fewer side effects that have been recorded. In this work, we assessed the antioxidant and free radical scavenging properties of the MSP for different eleven *C. variegatum* cultivars using a variety of validated *in vitro* antioxidant tests.

3.3.1. DPPH radical scavenging activity

Results of the DPPH radical scavenging activity of eleven clone cultivars of *C. variegatum*(MSP) are presented in Fig. 2, which clearly indicate that all investigated cultivars exhibited DPPH radical scavenging activity. *C. variegatum Zanzibar* recorded the highest radical activity with 67.57±0.41%, coming after *C. variegatum Spirale*, which achieved 42.36±0.24%, while *C. variegatum* Oak leaf recorded the lowest DPPH radical inhibition activity with 20.62±0.34% at a concentration of 500 µg /ml. The radical scavenging activity of the other cultivars ranged from 24.37±0.36% for cultivar Gold Dust to 40.35±0.32% for *Eleanor Roosevelt* at the same concentration. Previous results [30] supported our results, they recorded that extracts from *C. variegatum* demonstrated efficient DPPH free radicals. The IC_{50} values of the extracts were found to be 40.93 µg/ml and 73.16 µg/ml. Similar results were obtained by Anim et al. [31], who found that the extracts of stem bark and leaf *C. variegatum*'s possess strong antioxidant capacity in a dose-dependent manner. Each of the examined samples had a concentration-dependent pattern of free radical scavenging ability, with a rise in antioxidant activity with higher concentrations. Another study [32] determined the DPPH radical's scavenging of *C. variegatum* leaf extracts and fractions in a concentration-dependent manner ranging from 1 to 500 µg/ml. It reported that, according to the IC_{50}, the extracts and fractions had a moderate level of antiradical potential; however, it was still less than that of the standard antioxidant Ascorbic acid.

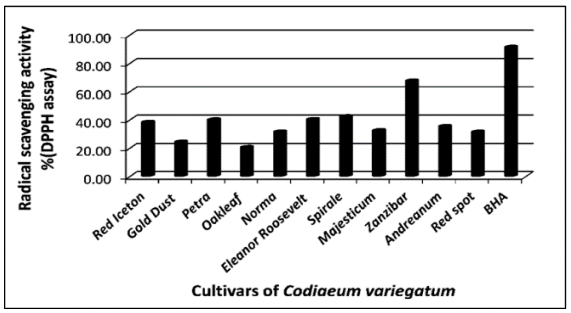


Figure (2) DPPH Radical scavenging activity (%) of theMSP (500 µg/ml)of eleven clone cultivars of *C. variegatum* . Values are means of three replicates.

3.3.2. Reducing power capability

There has previously been evidence of a direct relationship between some plant extracts' reducing power and antioxidant activity. Previous studies [33], reported that the presence of reductones, which demonstrated antioxidant action by disrupting the free radical chain and contributing a hydrogen atom, is typically linked to the reducing characteristics. High absorbance in the reducing power technique suggests that the extracts have a great ability to donate hydrogen atoms [34]. As displayed in Fig. 3, at a concentration of 500 ug/ml, *C.variegatum* clone cultivars resulted in good reducing power ability, which was identified through the increase in absorbance at 700nm. By extension, *C. variegatum Zanzibar* listed the highest reducing power capacity with a 1.019±0.023 absorbance reading. Also, the cultivar *Red Iceton* recorded 0.842±0.027, reducing power activity. Convergent results were obtained with the cultivars *Petra*, *Spirale*, *Majesticum*, *Andreanum*, *Eleanor Roosevelt*, *Red Spot*, and *Norma* by 0.692±0.013, 0.646±0.013, 0.638±0.010, 0.631± 0.017, 0.612±0.014, 0.608±0.014, and 0.604±0.024, respectively. The results of reducing power capacity are relatively similar to previous findings[30], investigated the reducing power ability of *C. variegatum* extracts and confirmed that they have good reducing properties. Reducing properties are usually associated with the presence of reductones, which have been shown to exhibit antioxidant effects by breaking the chain of free radicals and providing a hydrogen atom.

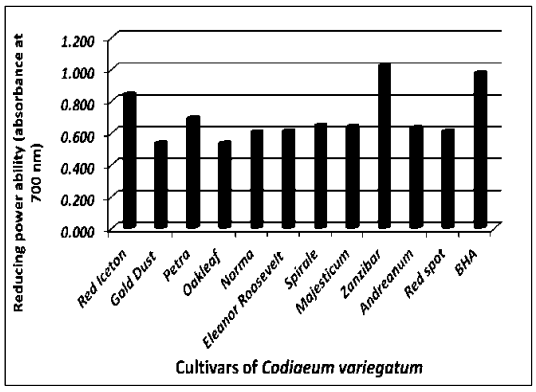


Figure (3) Reducing power ability of the MSP (500 µg/ml) for different eleven *C. variegatum* cultivars. Values are means of three replicates.

3.3.3. ABTS radical scavenging activity

The ABTS assay depends on grape skin antioxidants' capacity for reducing the ABTS green-blue cation radical by an electron transfer process that can be seen as a discoloration [35]. Concerning the results of ABTS radical scavenging activity, it is shown in Fig. 4, that the cultivars *Zanzibar* and *Red Iceton have* the strongest ABTS radical scavenging activity by 86.77±0.17 and 76.38±0.27% at a lower concentration (150 µg/ml), which is considered to be near the radical scavenging activity of the synthetic antioxidant BHA, which was 88.42±0.24% at 100 µg/ml. Referring to the rest of the cultivars, *Norma, Eleanor Roosevelt, Andreanum, Petra, Red Spot,* and *Majesticum* recorded high ABTS radical scavenging activity with 76.42±0.23, 74.43±0.18, 73.62± 0.23, 73.54±0.26, 72.13±0.20, and 70.42±0.19, respectively, at a higher concentration (500 µg/ml).

The results clearly reflect the high antioxidant activity of most of the studied cultivars.

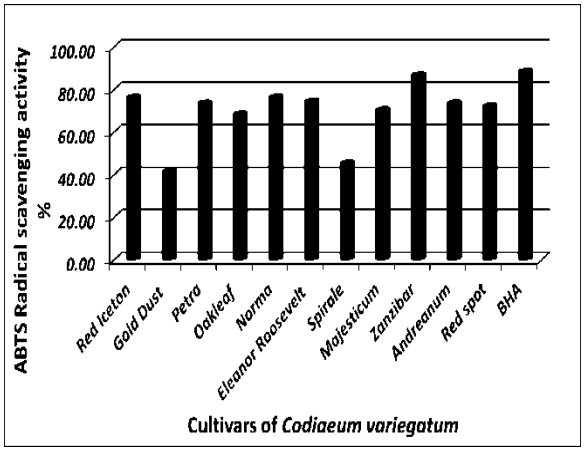


Figure (4) ABTS Radical scavenging activity (%) of theMSP for different eleven *C. variegatum* cultivars. Values are means of three replicates. (150 µg/ml was used for *Zanzibar* and *Red Iceton* cultivars, while 500 µg/ml for the other cultivars).

3.3.4. Metal chelating capacity

According to our knowledge and studies, not all plant extracts possess metal chelating ability. The ability of the plant extract may depend on the active constituents of the plant, which seems to be the main reason for this metal-chelating activity. Figure 5 illustrates the ability of the MSP for the different investigated cultivars of *C. variegatum* to chelate metals, which highly recommends their antioxidant activity. Cultivar *Zanzibar* exhibited the highest metal chelating activity, 61.76 ± 0.35%; second came *Petra*, recording 54.74±0.20%; and the cultivar *Red Iceton* achieved 53.15±0.18% metal chelating capacity at 500µg/ml. Concerning the metal chelating activity of the standard antioxidant BHT, it recorded 85.36±0.22% at a concentration of 100 µg/ml.

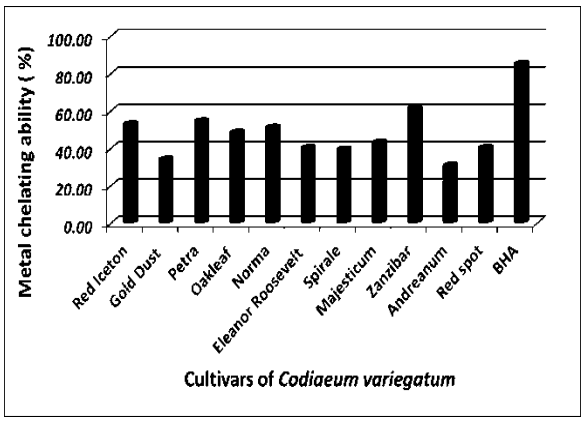
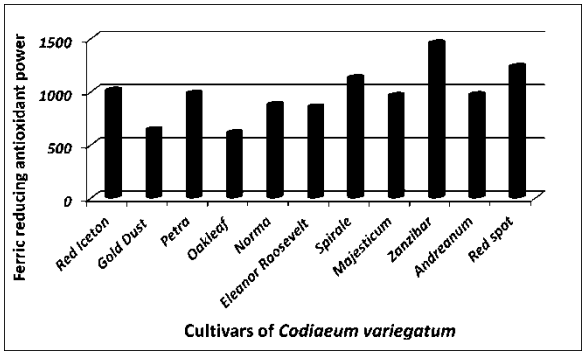


Figure (5) Metal chelating activity (%) of the MSP (500 µg/ml) for different eleven *C. variegatum* cultivars. Values are means of three replicates.

3.3.5. Ferric reducing antioxidant power

Similar to the ABTS radical scavenging activity experiment, a ferric-reducing antioxidant power assay was employed with the MSP of eleven clone cultivars of *C. variegatum*and displayed in Figure 6.

A concentration of 150 µg/ml was used for *Zanzibar* and *Red Iceton* cultivars, while 500 µg/ml was applied for the other cultivars. As expected, the same pattern was found; thus, cultivar *Zanzibar* recorded high FRAP values, achieving 1459±16.52 µmol Trolox/100 g, while cultivar *Red Iceton* ferric reducing antioxidant power was found to be 1014±10.07 µmol Trolox/100 g. All the investigated cultivars exhibited good ferric reducing power, especially cultivars *Red Spot* and *Spirale* with 1238±12.53 and 1133±9.29 µmol Trolox/100 g at the higher concentration (500 µg/ml). Similar results proved that *Croton caudatus* FRAP radical-scavenging activity increased in proportion to concentration until reaching a maximum for ethanol, chloroform, and aqueous extracts; suppression of FRAP radical was seen at a concentration of 2000 µg/ml. The ethanol extract had the most impact and was found to scavenge FRAP radicals more effectively than the other two extracts. The ethanol *Croton caudatus* extract FRAP inhibitory activity was 3230±27.83 mg of Ascorbic acid equivalent and 1588±13.68 mg of Trolox equivalent. In contrast, the aqueous and chloroform extracts showed 3020±10.0 mg and 995±10.0 mg of Ascorbic acid equivalent and 1484±4.91mg and 489±4.91 mg of Trolox equivalent,



respectively [36].

Figure (6) Ferric reducing power ability of the MSP for different eleven *C. variegatum* cultivars. Values are means of three replicates. (150 µg/ml was used for *Zanzibar* and *Red Iceton* cultivars, while 500 µg/ml for the other cultivars).

3.4. Effect on the viability of EAC cells

Ehrlich ascites carcinoma cells were employed for the *in vitro* antitumor activity assay. According to this cell line is known as an undifferentiated carcinoma. It was initially hyperdiploid and has high transplantable capacity, no regression, rapid proliferation, a shorter life span, 100% malignancy, and no tumor specific

transcription factor [37]. The results recorded in Figure 7 summarize the effect of the investigated MSP of the different eleven *C. variegatum* cultivars on the viability of EAC cells as an indication of their antitumor activity .The obtained results followed a similar pattern as all antioxidant activity results, where the cultivar *Zanzibar* recorded the highest antitumor efficacy on the viability of EAC cells with 63.55±0.33% dead cells, followed by the cultivars *Red Iceton*, *Eleanor Roosevelt*, and *Petra* by 48.79±0.21, 45.58± 0.20, and 44.38± 0.27 % dead cells at 500 µg/ml. The standard antitumor drug effect on the viability of EAC cells was found to be 90.64±0.39 at 100 µg/ml. All other studied varieties exhibited fairlygood inhibition of the viability of tumor cells. In a previous study on *C. variegatum Petra* [38], it was demonstrated that the cytotoxicity of this plant's leaves on human Caucasian breast adenocarcinoma (MCF7), hepatocellular carcinoma (HepG2), colon cell line (HCT116), and lung carcinoma cell line (A549), with activities ranging from 17.3% to 98%. Also, another study studied the antitumor activity of *C. variegatum* leaf and stem bark extracts and reported that both plant sections exhibited varying degrees of cytotoxicity against MCF-7 cells [31]. *C. variegatum's* stem bark was found to be the most cytotoxic, with an IC_{50} of 35.55 ± 1.50 µg/ml. Regarding the leukaemia (Jurkat) and prostate (PC 3) cancer cell lines, a comparable pattern was noted. The stem bark showed an IC_{50} of 59.71 ± 12.20 µg/ml and 52.54 ± 1.88 µg/ml, respectively, whereas the leaf recorded 62.03 ± 8.49 µg/ml and 211.20 ± 77.09 µg/ml. This implies that both the leaf and stem bark of *C. variegatum* contain active metabolites that may cause this cytotoxic effect.

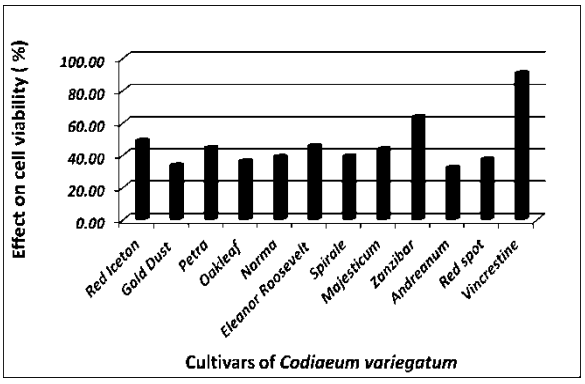


Figure (7) Effect of the MSP (500 µg/ml) for different eleven *C. variegatum* cultivars on the viability of EAC cells compared with the standard drug Vincristine. Values are means of three separate readings.

4. Discussion

4.1. Phytochemical analyses

4.1.1. UPLC-QTOF-ESI-HRMS/MS

A valuable output LC/MS/MS data was presented in the form of TIC and BPC for the total run (Fig. $1_{A, B}$), XIC, and MS^2-two stage selective fragmentation spectra of each constitutive metabolite (Figs. 1S–114S) in the MSP for the aq. alcoholic extract of *C. variegatum Zanzibar (Pictum spot)* along with the important identification parameters in Table 3. Such information represented how much the investigated plant extract rich in different types of secondary metabolites and should be enabled for the structural identification of a total of 114 metabolites. Table 3 summarizes the R_t, observed monoisotopic *m*/*z* masses of molecular $[M–H]^–$, some selective fragments and/or adduct ions together with molecular formulas (MFs), and errors (ppm) values for the characterized metabolites. Matching of the above-mentioned parameters and data with the corresponding convenient library database (MoNA-Mass Bank of North America) and reported literature for each metabolite confirmed its identification. Accordingly, the recorded HRMS/MS data would be enough for accurate identification of the metabolites structures, even their stereo-structural features in most cases [17], [39]–[43].According to their chemical structures, the identified metabolites are sorted into 41 polyphenols as 28 flavonoids-based metabolites (Figs. 1S–28S), including 2 chalcones (Figs. 8S, 13S) and 1 anthocyanin (Fig. 10S), 10 phenolic acids and their derivatives (Figs. 29S, 38S), 2 coumarins (Figs. 39S, 40S), 1 stilbene (Fig. 41S), 47*N*-containing compounds e.g. alkaloids, amino acid derivatives, organic bases and some of their salts (Figs. 42S–88S), 18 organic acid derivatives (Figs. 89S–106S), and 7 sugars/polyhydric alcohols (Figs. 107S–113S). The polyphenols and *N*-containing metabolites constituted 35.96 and 41.23%, respectively of the total LC-MS peaks identified, as the major constituents from *C. variegatum Zanzibar (Pictum spot)* (Figs. 1S–114S), Table 3. The MS/MS fragmentation patterns using controlled CID energy produced always $[M–H]^–$, together with diagnostic product fragment ions, unambiguously more than enough for establishing the accurate structures by the aid of the comparison of the relative abundances of their monoisotopic peaks with the library database. Although the identification of the metabolites depends only on the automatic matching of the R_t-values and other library database information with the experimental values, however, it can be followed up and recommended through some characteristic common fragments or fragmentation pathways for each class. This can be explained in the case of flavonoid glycosides and diagnosing if their structures are *O*- or *C*-glycosides, and the types and number of present glycoside moieties (Figs.1S-114S, Table 3). For mono-*O*-glycosides (Figs. 1S–8S, Table 3), MS^2 spectrum of quercitrin revealed a quasi-molecular ion peak at *m/z* 447.0898 $[M–H]^–$ together with two fragment ions at 301.0292 $[M–H–146]^–$and its oxidative one at 300.0346 $[M–2H–146]^–$ corresponding to the loss of rhamnoside moiety followed by oxidation that was indicative to quercetin as an aglycone(Fig. 1S) [44]. As an example, for di-*O*-glycosides (Figs. 9S–15S, Table 3), the molecular ion peak at *m/z* 577.1575 $[M–H]^–$cleaved to269.0459 $[M–H–308]^–$ of apigenin aglycone (Fig. 9S)to

diagnose the loss of rhamnoside (146) alongside glucoside (162) units. The consistency of R_t and full fragmentation pattern in library databases and literature led to the accurate structural characters of apigenin 7-*O*-rhamnosyl-(1→2)-glucoside (rhoifolin) [45].For the anthocyanin-type flavonoids, delphinidin 3-*O*-β-rutinoside displayed a molecular ion at *m/z* 609.1456 $[M–2H]^-$as a base peak alongside an intrinsic fragment ion at 300.0286 $[M–3H–308]^-$on high CID fragmentation energy, corresponding to the loss of a di-O-glycoside moiety and delphinidin aglycone(Fig.10S). Finally, the full experimental data was in complete accordance with the library database and previously reported data of delphinidin 3-*O*-(6''-O-α-rhamnopyranosyl-β-glucopyranoside) [46].

Unlike *O*-type, *C*-type glycosides behave in quite different fragmentation pathways through cross-ring cleavages of the glycoside moiety producing two product fragments as$[M–H–90]^-$ and $[M–H–120]^-$ because of the high stability and resistance of glycosidic linkages to CID fragmentation energy. It is also of high interest that the relative abundances of such two fragments to each other are diagnostic for the connectivity position of the glycoside moiety to the aglycone (C-6 or C-8, the most common positions). It was noticed that in this concern if the second fragment recorded a higher abundance than the first one, this means the structure under investigation is of C-8 isomer, and vice-versa for the reverse probability. This concept clarifies that with some simple information from HRMS/MS many stereochemical properties could be concluded (Table 3). Figure 17S Exhibited two MS^2 fragments at 341.0670 $[M–H–90]^-$ and 311.0568 $[M–H–120]^-$ due to the losses of cross ring cleavages of $C_3H_6O_3$and $C_4H_8O_4$.Because of the higher abundance of the second fragment, it was confirmed as an 8-C-glycosidic positional isomer. Moreover, the observation of the molecular ion at *m/z* 431.0978 $[M–H]^-$ and the fragment ion at *m/z* 283.0615 $[M–H–120\text{-}CO]^-$ together with the above-mentioned documents, this metabolite was confirmed as apigenin 8-*C*-glucoside (Table 3)[47]. Similarly, luteolin 8-*C*-glucopyranoside demonstrated a fragmentation pattern based on the cross-ring cleavages of the hexose unit to release357.0644 $[M–H–90]^-$ (lower %) and 327.0559 $[M\text{-}H–120]^-$ (higher %) for 8-*C*-glycosidic positional isomer(Fig. 18S)together with the molecular ion at 447.0957 $[M–H]^-$, aglycone fragment ion at *m/z* 284.0254 $[M–2H–162]^-$ and 429.0711 $[M–H–18]^-$that was fully agreed with the reported data and database for luteolin 8-*C*-glucopyranoside(Table 3) [47]. Accordingly, the fragmentation process of glycosides starts with stepwise cleavage from the outer to the inner of the individual sugar moieties depending on CID energy to generate the corresponding aglycones.

Concerning different types of flavonoidal aglycones (Figs. 21S–28S), the identification normally being simpler than their glycosides because they gave simple MS^2 spectra with a low probable number of fragments due to their relatively higher stability than corresponding glycosides. Normally they start with removing H, OH, and CO, and then going with the specific Retro-Diels-Alder Reaction (RDA) cleavage**.** Isorhamnetin MS^2 spectrum (Fig. 23S) showed three major mass ions identified as the molecular ion at *m/z* 315.0724 $[M–H]^-$as the base peak, together with two informative RDA-fragments of C-ring cleavage at 108.0214 and 152.0124 amu [48].

Concerning MS^2 spectra of the second investigated polyphenolics type, i.e., phenolic acids (Figs. 29S–38S), are commonly reported to demonstrate the molecular ions ($[M–H]^-$) agreed with their *calcd.* monoisotopic masses with some labile intrinsic MS^2 characteristic fragments due to the loss of neutral molecules, e.g., decarboxylation ($–CO_2$, 44 amu) and dehydration ($–H_2O$, 18 amu) along with releasing of 15 amu corresponding to Me-group if the methoxy derivatives included. In the MSP of *C. variegatum Zanzibar (Pictum spot)* rosmarinic acid was identified, where its MS^2 spectrum (Fig. 29S) displayed the molecular ion at *m/z* 359.2083 $[M–H]^-$ corresponding to the MF, $C_{18}H_{15}O_8$ and a base peak fragment at 197.0437 [M–H–162] due to the loss of caffyl moiety along with a fragment at 161.0 as di-dehydrated ions of caffeate $[179–H_2O]^-$ and salvianate $[197\text{-}2H_2O]^-$ anions, respectively. This was in turn confirmed by full agreement with the conventional database and reported literature [49]. As well, Figure30S exhibited a molecular ion peak at 153.0186 $[M–H]^-$ of 3,4-dihydroxybenzoic acid together with a base peak decarboxylation ion at 109.0299 $[M–H–44]^-$ and a lower abundant dehydration fragment ion at 81.0353$[M–H–18]^-$. Another example of ESI-MS/MS of phenolic acid identified in the MSP sample was represented for 5-methoxysalicylic acid, where its MS/MS spectrum (Fig. 31S) gave a molecular ion peak at *m/z* 167.0358 $[M–H]^-$ and two intrinsic fragment ions at *m/z* 152.0130 $[M–H–15]^-$ and 108.0214 $[M–H–15–44]^-$ that were assignable to demethylation and decarboxylation [50]. Other two examples for hydroxyphenolic acids identified in this plant were *p*-hydroxybenzoic and ***(E)-p***-coumaric acids that displayed molecular ion peaks at *m/z*137.0232 and 163.0397$[M–H]^-$, together with characteristic decarboxylation fragment ions at *m/z*93.0344and 119.0503 $[M–H–44]^-$as the base peaks (Figs. 32S, 35S) [51], [52]. The 3rd. type-phenolics detected in MSP of *C. variegatum Zanzibar (Pictum spot)* agreed well in their monoisotopic masses of MWs and fragments and accurate MFs with the corresponding data in the library database and literature for 7,8-dihydroxycoumarin (daphnetin) and 6,7-dihydroxycoumarin 6-*O*-glucoside (esculin),(Figs. 39S, 40S, Table 3). In literature,

coumarins are characterized by successive loss of CO and /or CO_2 [53], [54]. The first one was tentatively identified as daphnetin that showed a molecular ion peak at *m/z* 177.0557 $[M–H]^–$ in the MS^2 spectrum (**Fig**. 39S), *calcd*. 177.0188 for a MF of $C_9H_6O_4$ and MW 178.0266. In addition, two characteristic fragments were produced at *m/z* 145.0297 $[M–H–2xOH]^–$ followed by 117.0349 $[M–H–2xOH–CO]^–$. According to its MS^2 spectrometric data, the 2nd. coumarin was identified as 6,7-hydroxycoumarin glucoside based on the molecular ion at *m/z* 339.1989$[M-H]^–$ *calcd*. 339.0716 for MF $C_{15}H_{16}O_9$ and MW 340.0794 (Fig. 40S). In a similar concept, the structures of all remaining metabolites, regardless of their natural product class, were established depending on their own characteristic MS/MS fragmentation pattern and the consistency of the output data in MS^2-spectra (Figs. 41S–114S), and Table 3 with their library databases and literature and can be systematically explained like previously discussed metabolites.

4.2. Biological investigations

All our obtained biological results indicate the superiority of the cultivar *C. variegatum Zanzibar (Pictum spot)*, which led us to define its secondary metabolites to explain the main reason for these antioxidant and antitumor activities. The output data by LC/MS system identification of the metabolite structures as polyphenols such as flavonoids, chalcones, and anthocyanin, phenolic acids and their derivatives, coumarins, stilbene, *N*-containing compounds, e.g. alkaloids, organic bases and some of their salts, amino acid derivatives, and organic acid derivatives. The obtained results of LC/MS system identification led us to assume the responsibility of these secondary metabolites for the resulting biological effects. The antioxidant activity may be caused by chelating metal ions or contributing electrons or a hydrogen atom from the free hydroxyls, oxidants are suppressed, protecting biological systems from harmful effects caused by radicals [35]. A previous study [55] confirmed that flavonoids have antioxidant activity because they may scavenge free radicals, bind metal ions like iron and copper, and limit the activity of the enzymes that produce free radicals. The capacity to chelate metals is crucial because it lowers their concentration, which catalyzes the peroxidation of lipids. Additionally, because they lower the redox potential and stabilize the oxidized metal ions, metal-chelating compounds are regarded as secondary antioxidants [56]. The plants with high phenol content are known to have potent antioxidant and anticancer effects [57]. The catechol moiety contributes to metal binding, as seen by the more dramatic bathochromic shift that occurs when Cu binds to quercetin as opposed to kaempferol's capacity to chelate [58]. The human body's excess metal ions are chelated by flavonoids, demonstrating their bioavailability. Flavonoids' ability to chelate metals is crucial for both binding extra aluminum and detoxifying other heavy metals like Cr, Sn, Cd, and Pb. The poisonous metal ions that produce the complexes are efficiently chelated by the chelating agents [59]. The ability to chelate Fe^{2+} ions that have phenolic ring-attached -OH and -COOH groups. Resveratrol was found to bind Fe^{2+} ions on their -OH groups at Meta locations in another successful investigation [60]. Endogenous and exogenous chemicals are components of antioxidant defences. Many of these substances can scavenge free radicals directly; however, doing so requires permanently changing the scavenging molecule's chemical structure. Others are capable to react as chelators, restricting the participation of transition metal ions in electron transfer processes such as the production of catalytic radicals identical to Fenton and subsequent peroxidation processes. Coumarins are a class of chemicals linked to inhibition of ROS-producing enzymes, scavenging, and chelating antiradical activity [61]. Plant-derived products exhibit cytotoxic and anticancer actions by either inducing apoptosis or inhibiting neovascularization [62]. Numerous tumor cells encourage oxidative stress and have a pro-oxidant state. By generating mutations, activating redox signaling, and increasing pro-survival factors like NF-κB and AP-1, this enhances the cancer cells' ability to survive [63]. The most remarkable biological role of phenolic compounds is to keep the body's levels of oxidative stress below a critical limit. Similar investigations were recorded by [64], who confirmed that alkaloids, anthraquinones, flavanoids, terpenes, steroids, phenol, saponins, tannins, phlobatannin, and cardenolide, which were present in *C. variegatum*, could be responsible for the strong cytotoxicity in brain shrimp lethality bioassays. The cytotoxicity of the plant extract may also be caused by a high ellagic acid concentration, according to the HPLC examination of the *C. variegatum* extracts. On the other hand, rutin hydrate, (-)-epicatechin, and *p*-coumaric acid are plant phenolics that likewise have strong antioxidant and tumor cell-inhibiting properties. As a result, using certain plant extracts may allow those substances to work synergistically [30].

5. Conclusion

This study confirmed the high efficacy of the output data by the LC/MS/MS system, as a fast precise analytical tool for sorting and identification of 114 metabolites based on their MS^1/MS^2-two stage selective fragmentation spectra of each constitutive metabolite in the MSP for the aqueous alcoholic extract of *C. variegatum Zanzibar (Pictum spot)*. Such information i.e., R_t observed monoisotopic *m/z* masses of molecular $[M–H]^–$, some selective fragments and/or adduct ions together with molecular formulas (MFs) and errors (ppm) values would be enough for accurate identification of the metabolites' structures, even their stereo-structural features in most cases. According to their chemical structures, the identified metabolites are

sorted into 41 polyphenols as 28 flavonoids-based metabolites, including 2 chalcones and 1 anthocyanin, 10 phenolic acids and their derivatives, 2 coumarins, 1 stilbene, 47 *N*-containing compounds e.g., alkaloids, organic bases and some of their salts, amino acid derivatives, 18 organic acid derivatives, and 7 sugars/polyhydric alcohols. The polyphenols and *N*-containing metabolites constituted 35.96 and 41.23%, respectively of the total LC-MS peaks identified as the major constituents of *C. variegatum Zanzibar (Pictum spot).* The antioxidant and antitumor activities of all studied cultivars confirmed the superiority of the cultivar *C. variegatum Zanzibar (Pictum spot)* as an effective antioxidant and antitumor-safe agent. The study highly recommends the use of *C. variegatum Zanzibar (Pictum spot)* in pharmaceutical and food applications to prevent free radical-mediated disease, after performing a confirmative *in vivo*safety study.

Authors' contribution

R.T.R., A.M.Y.M., H.A.A.T, and M.M. conceived and designed the experiments, searched for information,and M.M. carried out structural elucidation. R.T.R. and A.M.Y.M. performed the extraction and chromatographic examination. R.T.R., H.A.A.T., and M.M. drafted the original paper. H.A.A.T: Design, evaluate, Data curation, and write the biological assessment part.All authors contributed to the manuscript: revision, reading, and approval of the final version.

Ethical Approval

The used procedures were carried out in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals and approved by the Ethical Committee at the Faculty of Science, Port Saied University, and the Departmental Committee for Research and Ethics (PSU.Sci.6).

Conflicts of Interest

There are no conflicts to declare

Data availability statement

All data about this study is presented in this published article and its supplementary attached files.

List of abbreviations

UPLC/ESI-qTOF-HRMS/MS: Ultra-performance Liquid Chromatography/Electrospray Ionization-Quadrupole Time-of-Flight-High-resolution Mass spectrometry/ Mass spectrometry; HRESI-MS: High-resolution ElectrosprayIonization-Mass spectrometry; DPPH: 2,2-Diphenyl-1-picrylhydrazyl; ABTS: (2,2'-azino-bis (3- ethylbenzothiazoline-6-sulfonic acid)); Trolox: 6-hydroxy -2,5,7,8- tetramethyl -3,4-dihydrochromene -2- carboxylic acid, BHA: butylatedhydroxyanisole

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