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COMPARISON OF PHENOLIC PROFILE, CYTOTOXICITY AND ANTIOXIDANT ACTIVITIES OF *ROSMARINUS OFFICINALIS* L. STEM COLLECTED FROM EGYPT AND LIBYA

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ABSTRACT

Rosmarinus officinalis L. (Rosemary); woody herb plant with fragrant evergreen needle-like leaves. It has a potential pharmaceutical and economic impact. It is used as flavoring agent in cooking and used in industry as a natural antioxidant for food conservation. It has been reported to have diverse biological activities such as antioxidant, antimicrobial, antitumor, anti-HIV, hypoglycemic, hypolipidemic, hypotensive, anti-atherosclerotic, anti-thrombotic, hepatoprotective, and hypocholesterolemic effects and anti-inflammation. HPLC/PDA/MS/MS profile of Egyptian and Libyan rosemary led to identification of 65 phenolic compounds including phenolic acids, rosmarinic acid and its dimers and glycosides. In addition to lignans, phenolics diterpenes and flavonoids including aglycones, glycosides and methoxylated flavonoids. Moreover, our study showed that stems of both Egyptian and Libyan rosemary possess antioxidant and anticancer activities more than the leaves. The Libyan rosemary stems showed antioxidant and anticancer activities more than the Egyptian species. We can conclude that whole rosemary plant should be used in both pharmaceutical and food industries.

KEYWORDS

Egyptian Rosemary, Libyan Rosemary, ORAC, Cytotoxicity and HPLC/MS/MS.

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INTRODUCTON

Rosmarinus officinalis L. (Rosemary); woody herb belongs to the Family *Lamiaceae* with fragrant evergreen needle-like leaves. It has a potential pharmaceutical and economical impact. It is used as flavoring agent in cooking and used in food industry

as a natural antioxidant for food conservation (Bai *et al.* 2010)¹. It has been used in folk medicine to treat epilepsy, headaches, poor circulation and in other ailments. It has been reported to have diverse biological activities such as antioxidant, antimicrobial, antitumor, anti-HIV, hypoglycemic, hypolipidemic, hypotensive, anti-atherosclerotic, anti-thrombotic, hepatoprotective, and hypocholesterolemic effects and anti-inflammation (Yu *et al.* 2013², Abutbul *et al.* 2004³ and Hassani *et al.* 2016)⁴. Phytochemical investigation of Rosemary leaves showed the presence of Rosmarinic acid, their derivatives in addition to phenolic diterpenes like carnosic acid, flavonoids like flavones and phenolic acids (Almela *et al.* 2006⁵, Bai *et al.* 2010¹ and Borrás-Linares *et al.* 2014)⁶. On the other hand, stem of rosemary is useless and acts as byproduct. Fortunately, stem of this plant has lack of information. It is only investigated using analytical HPLC against standard compounds and was shown to contain carnosic acid, carnosol and rosmarinic acid and flavones; eriocitrin, luteolin 3'-O -D-glucuronide, hesperidin, diosmin and it showed antioxidant activity (Del Baño *et al.* 2003⁷ and 2004)⁸. Cancer acts as an important cause of mortality nowadays. So there is an urgent need for new therapeutic approaches. *Rosmarinus officinalis* Leaves has been reported to possess antitumor and antioxidant activities both *in vitro* and in animal studies. These activities were attributed to its major components, such as rosmarinic acid, carnosol, carnosic acid and ursolic acid. Moreover, the U.S. Food and Drug Administration and the European Food and Safety Authority, consider that demonstration of rosemary extract is safe for human health (González-Vallinas *et al.* 2015)⁹. Thus, our study is concerned with exploring the phenolic profile of aq. Methanol stem extracts of Egyptian and Libyan rosemary stem and leaves, investigation of antioxidant and cytotoxic activities of both aiming to find an economical benefit of the rosemary stems which are useless.

MATERIAL AND METHODS

Plant materials

Plant material were collected from the Green mountain (Elgabl Alakhdar) voucher specimens were deposited at the herbarium of faculty of science,

Benghazi University, Libya and the other plant material were purchased from a local support.

Extraction

Rosemary leaves and stems from Egyptian and Libyan sources (50g) were homogenized in a MeOH-H₂O (3: 1) mixture (three extractions each with 0.5 litres). The obtained extract was filtered and dried under reduced pressure to give a yield of 5 g (EL), 5.2g (LL), 2.5 g (ES) and 2.6 (LS) dried extract, respectively.

HPLC-PDA-MS/MS

The extract was analyzed by HPLC-PDA-MS/MS using a Thermo Finnigan LC system (Thermo Electron Corporation, Austin, TX, USA). A Zorbax Eclipse XDB-C18, Rapid resolution, 4.6 × 150mm, 3.5µm column was used (Agilent, Santa Clara, CA, USA). A gradient consists of water and acetonitrile (ACN), each having 0.1% formic acid, was applied and acetonitrile was increased from 5 to 30% within 60min in 1mL/min flow rate and a 1:1 split before the ESI source (Marrez *et al.* 2017)¹⁰. The sample was injected using autosampler. LCQ-Duo ion trap having a Thermo Quest ESI source was used for MS analysis. Xcalibur software (Xcalibur™ 2.0.7, Thermo Scientific, Waltham, MA, USA) was used to control the system. MS operating parameters in the negative mode were used as described in (Sobeh *et al.* 2017)¹¹.

Biological assays

Oxygen radical absorbance capacity (ORAC assay)

Reactive oxygen species, ROS are generated by the thermal degradation of 2, 2'-azobis (2-amidinopropane) dihydrochloride (AAPH) and quench the signal of the fluorescent probe fluorescein. The subsequent addition of antioxidants reduces the quenching by preventing the oxidation of the fluorochrome (Lucas-Abellán *et al.* 2008)¹². A vitamin E derivative, 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox), was used as positive control. Tested samples were dissolved in phosphate buffered saline (10mM, pH 7.4) and investigated for their antioxidant capacity. Experiments were done in black 96-well plates. In each well of a 96-well Plate 150µl fluorescein (final concentration: 2.5nM), 25µl Trolox (final concentrations: 0.78 - 25µM) or 25µl tested samples were pipetted in quadruplicate. Plate was allowed to

equilibrate at 37°C for 30min. After this time, fluorescence measurements (Ex. 485nm, Em. 520nm) were taken every 90 s; first to determine the background signal. After three cycles 25µl AAPH (final concentration: 60mM) were added manually in each well with a multi-channel-pipette. This was done as quickly as possible since the ROS generator displays immediate activity after addition. Fluorescence measurements were continued for 90min. Half-life time of fluorescein was determined using MS Excel software.

Cytotoxicity assay (NRU)

Non tumorigenic HaCaT keratinocytes was obtained from the Vaccera (Giza, Egypt). Cells were cultured in RPMI 1640 medium (Bio Whittaker, Lonza, Verviers, Belgium) supplemented with 8 % fetal bovine serum (Sigma Aldrich, Taufkirchen, Germany) and antibiotics (100U/ml penicillin/100µg/ml streptomycin; Sigma Aldrich, Taufkirchen, Germany) at 95% humidity, 5% CO₂ and 37°C. HaCaT cells were subcultured twice a week and regularly tested for mycoplasma. Cytotoxicity of test samples against the four cell lines was investigated using the neutral red uptake (NRU) assay (Lindl *et al.* 1989)¹³.

After 24 h cultivation in 96 well plates (3 or 8 x 10³ cells/well) medium was removed and cells were exposed for 72 h to various concentrations (max. 500µg/ml) of test samples. After removal of the medium wells were washed with HBSS (Hanks Balanced Salt Solution, PAA). Cells were then incubated for 3 h with 100µl 3-amino-7-dimethylamino-2-methylphenazine hydrochloride (neutral red, Merck, Darmstadt, Germany, stock solution 3.3µg/ml, working solution 33ng/ml). Medium was removed and wells were washed twice with HBSS. Afterwards cells were lysed with 100µl of 1% acetic acid in 50% EtOH. Finally, after 45min optical density was measured at 450nm in a plate reader (Fluostar Omega, BMG Labtech, offenburg, Germany). The IC₅₀ values were defined from obtained dose-response curves and expressed in mean ± SD. All compounds were tested in duplicate. Etoposide (Alexis Biochemicals, ≥ 98 % purity) was used as positive control.

RESULTS AND DISCUSSION

Phenolic profiling of rosemary stem extracts

HPLC/PDA/MS/MS analysis of both Egyptian and Libyan rosemary stems which fortunately showed higher cytotoxicity than leaves extract led to identification 65 phenolic metabolites. These metabolites are shown in Table No.1 and Figure No.1 were identified by comparing its Mass/Mass fragmentation results with literature.

Cytotoxic activity

Rosemary leaves were reported for its potent cytotoxic and antioxidant activities. Rosemary stems were reported for its antioxidant only. Fortunately, our investigation showed that stems more active as anticancer than leaves this is due to presence of novel compounds such as dicaffeic acid derivatives which are not reported in leaves, in addition to presence of cinnamic acid derivatives.

In order to get information about the biological activities of the Egyptian and Libyan Rosemary, both stem and leaves aqueous methanolic extracts were tested for radical scavenging activity by DPPH and by ORAC assays and for cytotoxicity on HaCaT keratinocytes, colorectal adenocarcinoma cell line HCT-116, breast cancer MCF- 7 and liver carcinoma cell line Hep G2 by neutral red assay (NRU). The ED₅₀ of the extract for radical scavenging activity in DPPH assay was given in table () with the highest antioxidant activity of the Libyan stem extract with ED₅₀ values of 7.41 ± 0.57µg/ml and 6.21 ± 1.56µg/ml in the DPPH and ORAC assay, respectively. The latter value is lower than that of the positive control Trolox which had an ED₅₀ of 27.0 50± 13.41µg/ml. The IC₅₀ values for cytotoxicity of both extracts were given in table (). The vehicle in which the test samples were dissolved had no influence on measured parameter. Using Etoposide (positive control for cytotoxicity) viability of the tumorigenic tested cell lines were reduced to 40 to 60%.

The Libyan leaves extract showed the highest cytotoxicity against hepatocellular carcinoma cell lines (Hep G2) with IC₅₀ of 29.34±2.67µg/ml compared to a much lower cytotoxic activity against normal cell line HaCaT. The Libyan stem extract showed the highest cytotoxic activity against all tested cell lines with a much more pronounced activity against breast cancer cell line (MCF-7) with

IC₅₀ of 12.5±2.33µg/ml which is nearly similar to the positive control Etoposide followed by hepatocellular carcinoma cell lines (Hep G2) with IC₅₀ of 15.34±1.11µg/ml. On the other hand, the Libyan stem extract showed a lowest cytotoxicity

on the normal HaCaT –keratinocytes with IC₅₀ 520±6.69µg/ml.

Table No.1: Phenolic profile of Egyptian and Libyan stem

S.No	R _t	UV	[M-H] ⁻	Mass Fragments	Proposed structure	ER	LR	References
1	1.53	---	665	503, 383, 343, 341, 249	Caffeoyl trihexoside	+	+	-----
2	2.82	---	197	135, 179	Danshensu	+	+	Don et al. 2006 ¹⁴
3	3.23	279	305	97, 225	Gallocatechin	+	+	Borrás-Linares et al. 2014 ⁶
4	4.29	---	353	179, 191	Neochlorogenic acid	+	+	An et al. 2013 ¹⁵
5	4.87	---	359	161, 179, 197	Danshensu hexoside	+	-	-----
6	5.37	---	377	137, 161, 179, 359	Hydroxyrosmarinic acid	+	+	-----
7	5.73	---	353	179, 191	chlorogenic acid	+	+	An et al. 2013 ¹⁵
8	6.24	---	357	179, 203, 269, 295, 313	Dicaffeic acid I	+	+	-----
9	6.63	---	357	179, 203, 269, 295, 313	Dicaffeic acid II	+	-	-----
10	7.68	---	353	179, 191	Cryptochlorogenic acid	+	+	An et al. 2013 ¹⁵
11	8.13	---	333	161, 179, 197, 223, 315	Danshensu derivative	-	+	-----
12	8.31	---	539	179, 297, 359, 495	Yunnaneic acid D	+	-	Liu et al. 2007 ¹⁶
13	10.98	---	539	179, 297, 359, 495	Yunnaneic acid D isomer	+	-	Liu et al. 2007 ¹⁶
14	12.11	---	717	537, 519, 359, 179	Salvianolic acid E	+	+	Liu et al. 2007 ¹⁶
15	13.18	---	717	537, 519, 339	Salvianolic acid B	+	+	Liu et al. 2007 ¹⁶
16	14.67	---	179	135, 161	Caffeic acid	+	+	Don et al. 2006 ¹⁴
17	16.37	---	493	295, 313	Salvianolic acid A	+	+	Liu et al. 2007 ¹⁶
18	16.93	---	493	295, 313	Salvianolic acid A isomer	+	+	Liu et al. 2007 ¹⁶
19	17.73	---	521	359	Rosmarinic acid Hexoside	+	+	-----
20	20.80	---	359	161, 179, 197, 223	Cis-rosmarinic acid	+	+	Martins et al. 2015 ¹⁷
21	21.88	---	359	161, 179, 197, 223	Trans-rosmarinic acid	+	+	Martins et al. 2015 ¹⁷
22	22.26	---	719	359, 539, 701	Sagerinic acid	+	+	-----
23	24.09	---	537	341, 519	Lithospermic acid	-	+	Don et al. 2006 ¹⁴
24	28.42	---	461	285	Scutellarin hexouronic acid	+	+	-----
25	28.57	---	771	301, 463, 609	Hesperetin dihexoside rhamnoside	-	+	-----

26	28.74	---	461	285	Isocutellarin hexouronic acid	+	-	-----
27	29.72	---	503	285, 399, 443	luteolin-3'-O-(O-acetyl)- β -D-glucuronide	+	-	Borrás-Linares <i>et al.</i> 2014 ⁶
28	30.95	---	595	287, 433	Eriodictyl galactosyl rhamnoside	+	+	-----
29	32.01	---	595	287, 433	Eriodictyl glucosyl rhamnoside	+	+	-----
30	32.56	---	503	285, 399, 443	luteolin-3'-O-(O-acetyl)- β -D-glucuronide	+	-	Borrás-Linares <i>et al.</i> 2014 ⁶
31	32.75	---	311	283, 267	2(3,4-Dihydroxyphenyl)-7-hydroxy-5-benzene propanoic acid	-	+	Liu <i>et al.</i> 2007 ¹⁶
32	35.08	---	593	285, 447	Scutellarin hexosyl rhamnoside	+	-	-----
33	36.25	---	579	271, 417	Naringenin hexosyl rhamnoside	+	+	-----
34	36.27	---	477	284, 300, 315	6-methoxy luteolin hexoside	+	-	Borrás-Linares <i>et al.</i> 2014 ⁶
35	37.31	---	477	315	6-methoxy luteolin hexoside II (Nepitrin)	+	+	Borrás-Linares <i>et al.</i> 2014 ⁶
36	38.81	---	609	301	Hesperetin galactosyl rhamnoside	+	+	Mena <i>et al.</i> 2016 ¹⁸
37	39.09	---	609	301	Hesperetin glucosyl rhamnoside	+	+	Mena <i>et al.</i> 2016 ¹⁸
38	39.97	---	607	284,299, 445	Hispidulin hexosyl rhamnoside	+	+	Mena <i>et al.</i> 2016 ¹⁸
39	41.84	---	665	503, 397, 302	Luteolin 3'-O-(O-acetyl)-glucuronide-7-O-hexoside	-	+	-----
40	41.88	---	461	283, 299	Hispidulin hexoside	+	-	Mena <i>et al.</i> 2016 ¹⁸
41	43.85	---	435	273	Phloridzin	-	+	-----
42	44.01	---	639	300, 315, 477	Nepitrin caffeoyl hexoside	+	+	Bai <i>et al.</i> 2010 ¹
43	45.88	---	651	387, 519	Medioresinol acetyl pentoside	+	+	Hossain <i>et al.</i> 2010 ¹⁹ and Mena <i>et al.</i> 2016 ¹⁸
44	46.93	---	357	161, 179, 277, 231, 295, 313	Dehydrorosmarinic acid I	+	+	-----
45	48.12	---	357	161, 179, 231, 277, 295, 313	Dehydrorosmarinic acid II	+	+	-----
46	49.37	---	475	313	Cirsimaritin hexoside	-	+	-----
47	49.71	---	623	300, 315, 477	Nepitrin coumaroyl hexoside	+	+	Bai <i>et al.</i> 2010 ¹
48	51.56	---	491	179, 197, 293, 311	Salvianolic acid C	+	+	Liu <i>et al.</i> 2007 ¹⁶
49	53.99	---	551/505	269, 343	Rosmadiol hexoside	-	+	-----
50	54.35	---	607	284,299,341,	Hispidulin coumaroyl	+	-	Bai <i>et al.</i> 2010 ¹

				461	hexoside			
51	56.81	---	461	283, 299	Hispidulin hexoside	+	+	-----
52	59.74	---	299	179, 284	Hispidulin	+	+	-----
53	60.12	---	301	151, 257, 284	Hesperitin	+	+	-----
54	62.86	---	345	283, 300	Rosmanol	+	+	Mena <i>et al</i> 2016 ¹⁸
55	64.00	---	345	283, 300	Epirosmanol	+	+	Mena <i>et al</i> 2016 ¹⁸
56	66.66	---	343	315, 299	Rosmadiol	+	+	-----
57	68.14	---	313	283, 298	Cirsimaritin	+	+	Borrás-Linares <i>et al.</i> 2014 ⁶
58	70.85	---	343	315, 299	Rosmadiol isomer	+	+	-----
59	74.29	---	359	283, 329	Epirosmanol methyl ether	+	+	Mena <i>et al</i> 2016 ¹⁸
60	76.50	---	359	283, 329	Rosmanol methyl ether	+	+	Mena <i>et al.</i> 2016 ¹⁸
61	76.97	---	343	299, 315	Rosmanol quinone	+	+	-----
62	77.41	---	315	135, 215, 271, 287, 297	Rosmaridiphenol	-	+	Kontogianni <i>et al.</i> 2013 ²⁰
63	79.87	---	329	285, 299, 315	Carnosol isomer	-	+	Ivanović <i>et al.</i> 2009 ²¹
64	82.86	---	345	286, 301	Methyl carnosate	+	+	Hohmann <i>et al.</i> 1999 ²²
65	84.09	---	329	-----	Carnosol	-	+	Borrás-Linares <i>et al.</i> 2014 ⁶
66	85.15	---	297	283, 269	4'-Methoxy tectochrysin	+	+	Mena <i>et al.</i> 2016 ¹⁸

Table No.2: Cytotoxic activities

S.No	Organ	Cytotoxicity [IC50 (µg/ml)]
1	Leaves lybia (colon)	45.24 ±2,67
2	Stem lybia (colon)	29,92 ±3.54
3	Leaves Egypt (colon)	50,07 ± 1, 83
4	Stem Egypt (colon)	35,09 ± 2, 63
5	Leaves Egypt (breast)	53,34±4, 56

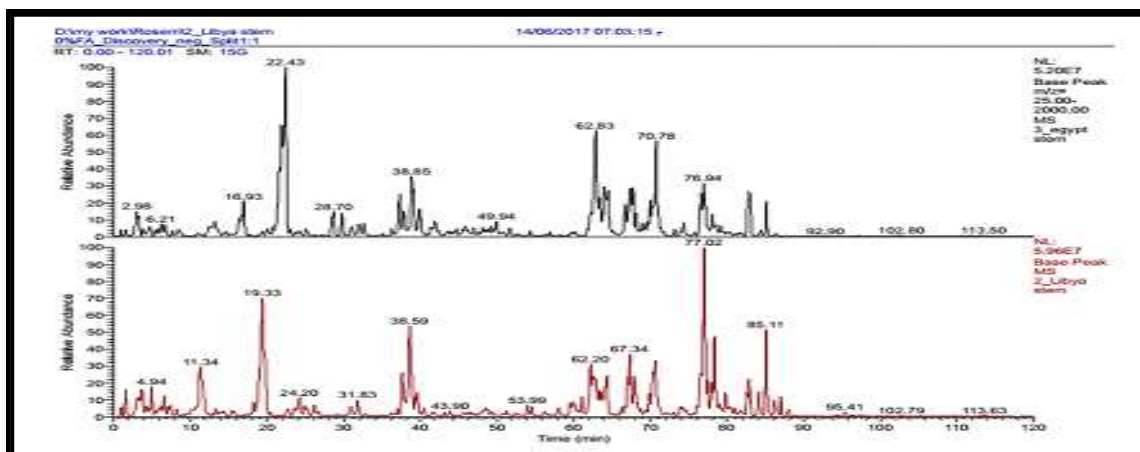
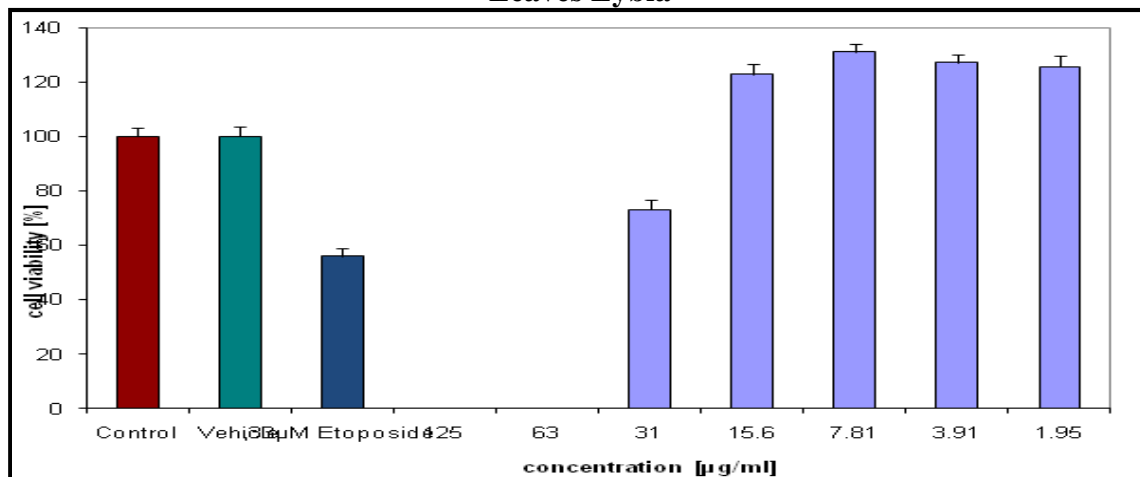
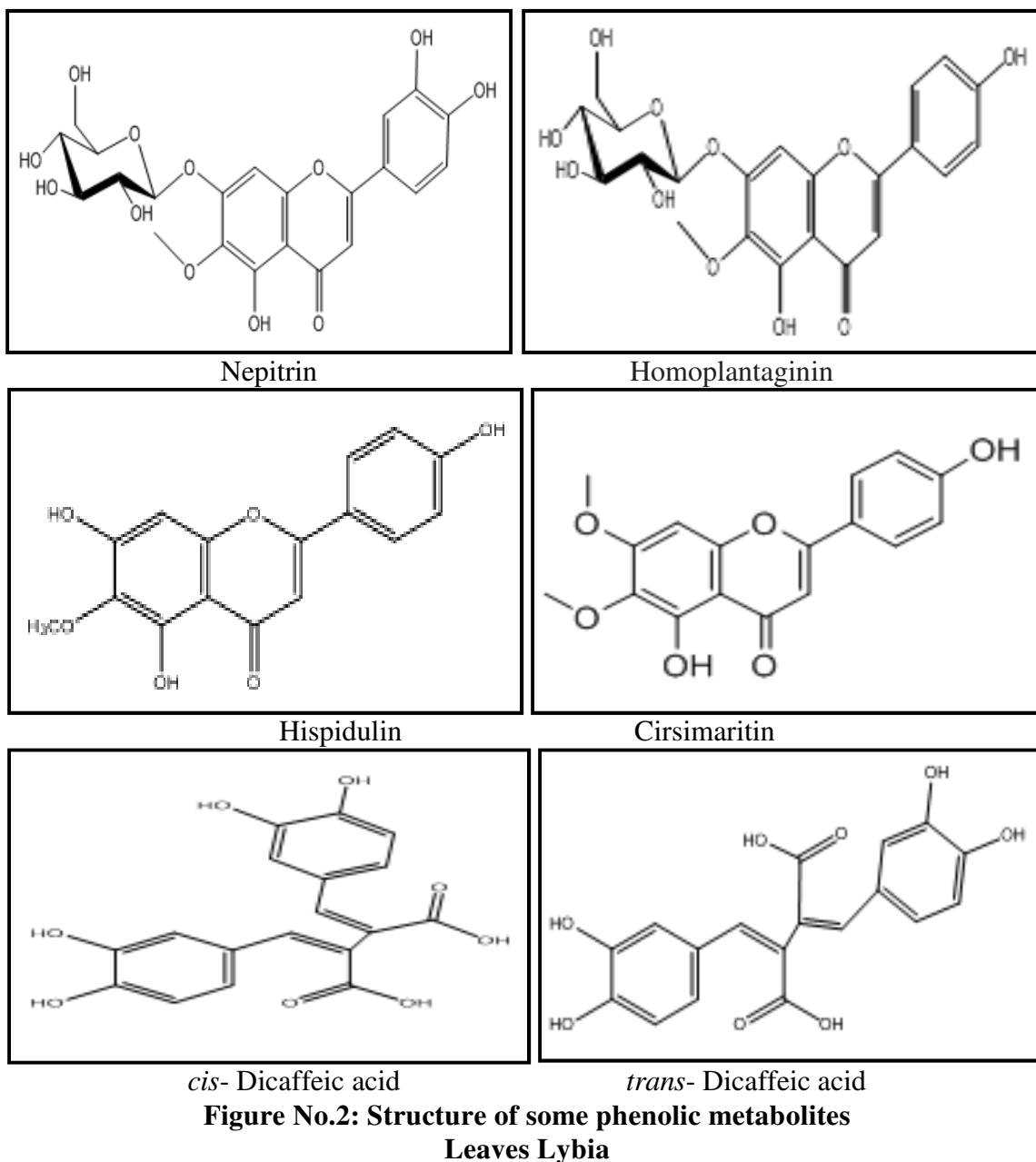


Figure No.1: Base peak chromatogram of Egyptian and Libyan rosemary Stem



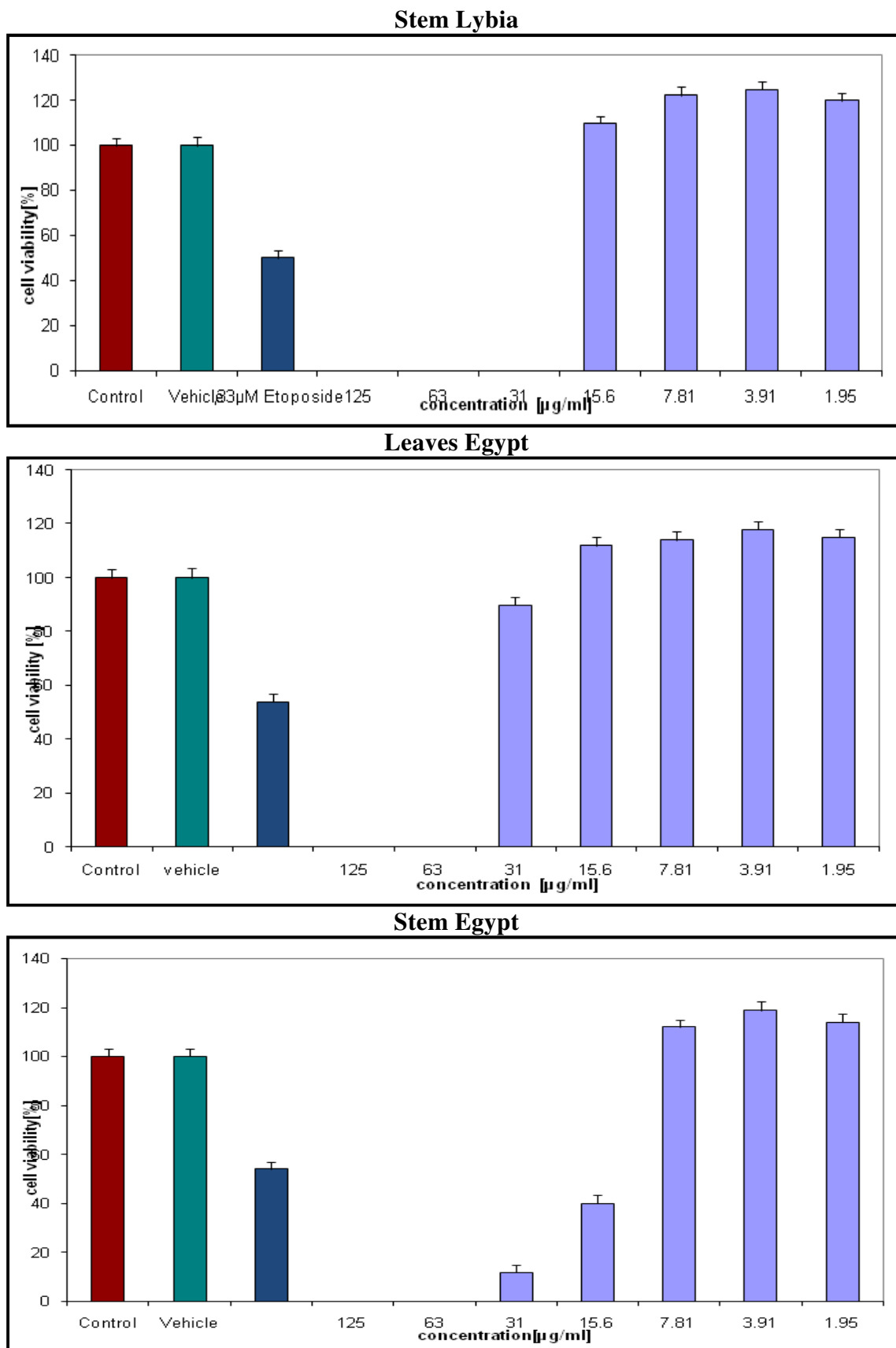


Figure No.3: Anticancer NRU assay result

CONCLUSION

Rosemary stems is more active than leaves so it should be used in pharmaceutical and food industries as preservative from oxidation and chemo preventive or aiding agent for cancer therapy.

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CONFLICT INTEREST

Authors declare that there is no conflict of interests.

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