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Research Article

PHYTOCHEMICAL INVESTIGATION AND ANTIOXIDANT ACTIVITIES OF CERTAIN *HAWORTHIA* AND *GASTERIA* SPECIES

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ABSTRACT

The cultivation of *Haworthia limifolia* Marloth, *Gasteria carinata* (Mill.) Duval and *Gasteria minima* Poelln is obviously increasing in Egypt as ornamental plants. Although they are widely used in folk medicine specially in South Africa, little is known about their phytochemical and pharmacological properties. Thus, this study was conducted to investigate their phytochemical and antioxidant properties. The preliminary phytochemical screening of their aerial parts revealed the presence of carbohydrates and/or glycosides, flavonoids, sterols, tannins and anthraquinones. The concentration of tannins was determined using modified vanillin- hydrochloric acid assay. HPLC analysis of phenolics and flavonoids resulted in the identification of 31 compounds involving 21 phenolic compounds and 10 flavonoids. *H. limifolia* Marloth extract showed higher concentration of phenolic compounds and flavonoids followed by *G. carinata* (Mill.) Duval then *G. minima* Poelln. Salicylic acid and vanillic acid were the major identified phenolic acids. The investigated species were rich in polysaccharides whose chromatographic investigation recorded the presence of seven saccharides, glucuronic acid, stachyose, galacturonic acid, sucrose, glucose, galactose, rhamnose and arabinose in the three species, but their quantitative composition differed among the species. Stachyose, was the highest detected sugar in the three-investigated species. Concerning sugar acids, galacturonic acid was detected in the three-investigated species in higher concentration than glucuronic acid. *H. limifolia* Marloth and *G. carinata* (Mill.) Duval showed DPPH scavenging activity (IC_{50} = 143.6 µg/ml and 249.1 µg/ml, respectively), while *G. minima* Poelln. showed weak antioxidant activity ($IC_{50} \geq$ 3000 µg/ml) and this difference in antioxidant activity is closely related to the phytochemical findings.

Keywords: *Haworthia limifolia*, *Gasteria carinata*, *Gasteria minima*, antioxidant.

INTRODUCTION

Haworthia and *Gasteria* (Haw.)[1] are ornamental small succulent plants native to South Africa. They belong to family Asphodelaceae, subfamily Asphodeloideae according to Mabberley classification system[2]. *Haworthia* includes 61 species[2,3], while *Gasteria* comprises 23 species according to Van Jaarsveld EJ [4].

Haworthia limifolia is often used in folk medicine as blood purifiers[5] and in the treatment of coughs, skin rashes, burns, sun burns, sores and gastro-intestinal ailments[6,7]. Its anti-tumor, anti-inflammatory, wound healing, antifungal and antibacterial activities were assessed by [5,6].

Gasteria is used in traditional medicine in the treatment of secondary fungal infections in HIV/AIDS patients and this was validated by the study of Otang *et al* on *Gasteria bicolor*[8].

Two isocoumarin glucosides and a chromone derivative were isolated from *Haworthia cymbiformis*[9]. While three dihydroanthracenones were isolated from the leaves of *Gasteria bicolor*[10].

Few phytochemical and biological data are available for both genera although they have promising pharmacological effects. Thus, we performed this study to investigate their phytochemical constituents using screening chemical tests, HPLC studies for the phenolic, flavonoid and polysaccharide content and to determine their antioxidant potential.

MATERIALS AND METHODS

Plant material and extraction

The aerial parts of *Haworthia limifolia* Marloth, *Gasteria carinata* (Mill.) Duval *and Gasteria minima* Poelln. were collected from Helal garden in Toukh, Egypt during January 2015. Their taxonomical identity was kindly confirmed by Dr. Mohammed El Gebaly (Consultant botanist-Orman Garden) and Dr. Ernst van Jaarsveld (South African National Biodiversity Institute) and a voucher specimen of each was kept in the herbarium of Faculty of Pharmacy, Cairo University (6-1-2015). The powdered air-dried aerial parts of the plants under investigation (200 g) were separately macerated in methanol at room temperature five times till exhaustion. The extracts were concentrated under reduced pressure using a Rotary evaporator (Buchi, Germany) giving 32.7, 39.2 and 27 gm *H. limifolia*

Marloth, *G. carinata* (Mill.) Duval *and G. minima* Poelln. methanolic extracts, respectively.

Chemicals

Standard tannic acid was obtained from E-Merck, Darmstadt, Germany. Authentic phenolic compounds and flavonoids for HPLC study were kindly supplied by Agricultural Research center, Food Technology Research Institute, Giza, Egypt. Authentic sugars for HPLC of mucilage content were purchased from Sigma Aldrich (Steinheim, Germany). 2,2-Diphenyl-1-picrylhydrazyine (DPPH) was obtained from Sigma Co (St. Louis, MO), while Ascorbic acid was purchased from E-Merck (Darmstadt, Germany).

Phytochemical screening

The presence of major phytoconstituents was detected using colored reactions of Dragendorff's and Mayer reagents for alkaloids, reagent of Liebermann Burchard for sterols and terpenes, ferric chloride for tannins, sodium hydroxide test for flavonoids, froth test for saponins, Borntrager's test for anthraquinones, Molisch test for carbohydrates and/or glycosides, Baljet test for cardiac glycosides[11-13].

Spectroscopic assay determination of tannin content

The tannin content in the plant samples was determined using modified vanillin- hydrochloric acid (MV-HCl) method described by Earp *et al*[14] and the absorbance was read at 500 nm using Jenway 6705 UV/Vis. spectrophotometer (Multicell changer jenway). The results were expressed as tannic acid equivalent (mg% methanolic extract) with reference to a pre-established standard calibration curve.

HPLC of phenolic compounds and flavonoids

The air dried powdered aerial parts (5 gm) of *H. limifolia* Marloth, *G. carinata* (Mill) Duval and *G. minima* Poelln. were separately macerated in methanol. The extract was evaporated under vacuum at 45°C and the phenolic compounds were determined by HPLC according to the method of[15], while the flavonoids were determined by HPLC according to the method of[16,17]. The extracts were filtered through an Acrodisc filter (0.2 µm) before HPLC analysis.

Chromatographic separation and determination were performed using HPLC Agilent 1200 series equipped with quaternary pump, auto sampler, solvent degasser, multiwave length detector (set at 330 nm, 280 nm for detection of flavonoid and phenolic compounds, respectively), Zobrax ODS (4.6 x 250 mm) column was used for fractionation. The column temperature was maintained at 35°C and gradient separation was performed using a mixture of methanol and acetonitrile (2:1) as a mobile phase at 1 ml/min flow rate. The authentic phenolic compounds and flavonoids were dissolved in the mobile phase and injected into HPLC. The concentration of the phenolic and flavonoid compounds was calculated using the retention time and peak area through data analysis of Agilent software.

HPLC determination of mucilage content

Extraction of polysaccharides

The polysaccharides were isolated from the fresh aerial parts of *H. limifolia*, *G. carinata* (Mill.) Duval and *G. minima* Poelln., by cutting each one of them into small pieces with size range (0.5-1 cm long × 0.5-1 cm wide). 250 g of each sample was extracted by hot extraction methods, with 1 L of boiling distilled water, then it was boiled for 15 minutes, filtered while hot through a muslin cloth. The aqueous extracts were then precipitated by addition of about 3 times ethyl alcohol and then dried in the oven at 40°C. The mucilage yield was calculated.

The mucilage samples (100 mg) of each plant under investigation were separately dissolved in 4M Trifluoro-acetic acid (100 ml), refluxed at 80°C for 8 hrs. The authentic reference sugars were mixed and dissolved in distilled water. The samples were filtered through 0.45 µm syringe filter and stored in vials to be used in HPLC investigation.

HPLC determination

Chromatographic separation of monosaccharides was performed using Agilent1260 infinity HPLC Series (Agilent, USA), equipped with Quaternary pump, Column used Phenomenex ® Rezex RCM- Monosaccharide, 300 mm x 7.8 mm operated at 80°C. The separation was achieved using Isocratic elution by HPLC grade water with flow rate 0.6 ml/min. The injected volume was 20 µL. Detection was achieved using Refractive Index (RI detector) operated at 40° C. While the chromatographic separation of organic sugars was achieved using HPLC knauer, Germany equipped with binary pump flow rate was set at 0.6 ml /min, UV detector set at 214 nm, column oven temperature kept constant at 65°c, the column used was Rezex@ column for organic acids analysis, mobile phase was 0.0065M H_2SO_4data integration by claritychrom software.

Determination of the in vitro antioxidant activity using DPPH assay

The effect of the extracts under investigation on DPPH radical was studied, employing the modified method described by Yamaguchi *et al*[18]. The absorbance of the samples, control (DPPH radical) and reference (ascorbic acid) was recorded at 515 nm continuously, until the absorbance stabilized (16 min), using UV-visible spectrophotometer (Milton Roy, Spectronic 1201).

The percentage inhibition (PI) of the DPPH radical was calculated according to the formula:

$$PI = [\frac{(Ac - At)}{Ac} \times 100]$$

Where A_C = Absorbance of the control at t = 0 min and A_t = absorbance of the sample + DPPH at t = 16 min[19].

IC_{50}, concentration of the tested sample in µg/ml that is required to scavenge 50% of free radicals, was calculated from the calibration curve and compared with ascorbic acid as a standard.

RESULTS

Phytochemical screening

The phytochemical results are represented in table (1) and they show a heterogeneity of chemical groups. The presence of carbohydrates and/or glycosides, flavonoids, sterols, tannins and anthraquinones suggest the rich chemical nature of the three plant extracts and that they have promising pharmacological activities.

Tannin content in the plant samples

The determination of total tannin contents revealed that the concentration of tannins was the highest in *H. limifolia* Marloth (8.04%) then in *G. minima* Poelln. (2.58%) and it was lowest *G. carinata* (Mill.) Duval (1.02%).

HPLC of phenolic compounds and flavonoids

Plant phenolics, including, flavonoids and phenolic acid, have protective effects against free radicals and reactive oxygen species counteracting the risk of cardiovascular diseases, cancer and cataract, and other degenerative diseases of aging[20].

According to the HPLC analysis of phenolics and flavonoids in the aerial parts of the plants under investigation, 31 phenolic compounds were identified Tables (2-3), involving 21 phenolic compounds and 10 flavonoids.

H. limifolia Marloth extract showed higher concentration of phenolic compounds and flavonoids followed by *G. carinata* (Mill.) Duval then *G. minima* Poelln. (8.334, 3.055 and 2.46

Concerning the flavonoids, quercitrin represented the major identified flavonoid in *H. limifolia* Marloth (0.888 g/100g), while hesperetin was the major identified one in *G. carinata* (Mill.) Duval (0.227 g/100g) and catechin (0.138 g/100g) was the major identified one in *G. minima* Poelln.

Yield of mucilage

Mucilage content from different plants showed wound healing properties[21], antinociceptive effect[22], antioxidant [23,24], hypoglycemic[25,26] and immunomodulatory activities[27].

It was obvious from table (4) and Figures (1-8) that the investigated species were rich in polysaccharides; *G. minima* Poelln. had the highest polysaccharide content, followed by *H. limifolia* Marloth and *G. carinata* (Mill.) Duval 1.01%, 0.541% and 0.44%, respectively. Glucose was detected *H. limifolia* Marloth and *G. carinata* (Mill.) Duval at a concentration of 0.02 and 0.04 g%. Galactose and rhamnose, having the same retention time, were detected either together or one of them only in *G. carinata* (Mill.) Duval (0.005 g%). While arabinose was detected only in *G. minima* Poelln. (0.03 g%). The disaccharide, sucrose, was detected in *G. carinata* (Mill.) Duval, *G. minima* Poelln. and *H. limifolia* Marloth (0.07, 0.067 and 0.01g %, respectively). The oligosaccharide, stachyose, was the highest detected sugar in the three-investigated species (3.59, 2.76 and 0.6 g % in *G. carinata* (Mill.) Duval, *H. limifolia* Marloth and *G. minima* Poelln., respectively). Concerning the sugar acids, galacturonic acid was

g/100g, respectively) and (2.547, 0.922 and 0.564 g/100g, respectively). Salicylic acid and vanillic acid were the major identified phenolic acids followed by catechol, pyrogallol, 3-OH Tyrosol, rosmarinic and chlorogenic acid. Salicylic acid was the highest identified phenolic acids in *H. limifolia* Marloth (5.667 g/100g), while vanillic acid was the highest one in *G. carinata* (Mill.) Duval and *G. minima* Poelln. (2.001 and 1.14 g/100g respectively). The major identified phenolic compounds in *H. limifolia* Marloth were salicylic acid, vanillic acid, α- coumaric, and rosmarinic acids. While the major identified phenolic compounds in *G. carinata* (Mill.) Duval were vanillic acid, salicylic acid and rosmarinic acid. The major identified phenolic compounds in *G. minima* Poelln. were vanillic acid, salicylic acid, catechol and 3-OH Tyrosol.

detected in the three-investigated species in higher concentration than glucuronic acid which was still present in high concentration. Galacturonic acid was detected in *G. carinata* (Mill.) Duval, *H. limifolia* Marloth and *G. minima* Poelln. at concentration of 1.6, 1.37 and 0.47 g %, respectively. While glucuronic acid concentration determined was 1.2, 0.91 and 0.35 g % in *G. carinata* (Mill.) Duval, *H. limifolia* Marloth and *G. minima* Poelln.

Antioxidant activity

Oxygen metabolism, which is a central key to life, is also associated with reactive oxygen species (ROS) production producing the "Oxygen Paradox". Many diseases, including cardiovascular and neurodegenerative diseases are caused by ROS[28]. Natural antioxidants could protect the human body from the destructive effects of free radicals and they can retard the progression of many chronic diseases[29].

The methanolic extract of the aerial parts of *H. limifolia* Marloth and *G. carinata* (Mill.) Duval displayed in vitro radical scavenging activities compared to the reference antioxidant ascorbic acid, showing relatively low IC_{50} values, where the activity of *H. limifolia* Marloth (IC_{50} = 143.6 µg/ml) exceeded that of *G. carinata* (Mill.) Duval (IC_{50} = 249.1 µg/ml). While *G. minima* Poelln. showed weak antioxidant activity $IC_{50} \geq 3000$ µg/ml under these experimental conditions (table 5,6).

Table 1: Preliminary phytochemical screening of the aerial parts of *H. limifolia* Marloth, *G. carinata* (Mill.) Duval, *G. minima* Poelln

Metabolites	*H. limifolia*	*G. carinata*	*G. minima*
Carbohydrates and/or glycosides	+	+	+
Tannins			
Condensed	+	+/-	+/-
Hydrolysable	-	-	-
Flavonoids			
Free	+/-	+	+/-
Combined	+/-	+/-	+/-
Saponins	-	-	-
Sterols and/or triterpenes	+	+	+
Alkaloids and/or nitrogenous bases	-	-	-
Anthraquinones			
Free	+/-	+	+
Combined	+	+	+/-
Cardiac glycosides	-	-	-

(+), (-) and (-/+) indicate the presence, absence and traces of the tested metabolites, respectively.

Table 2: Results of determination of Phenolic compounds using HPLC analysis of *H. limifolia* Marloth, *G. carinata* (Mill.) Duval, *G. minima* Poelln

Phenolic compounds			Test results of phenolic compounds (mg/100g)		
	Rt	RRt*	*H. limifolia*	*G. carinata*	*G. minima*
Gallic acid	7.06	0.67	17.31	62.51	9.19
Pyrogallol	7.17	0.68	67.26	127.38	114.27
4-Amino-benzoic acid	8.03	0.77	3.75	2.24	11.84
3-OH Tyrosol	8.31	0.79	53.79	46.97	127.93
Protocatechuic acid	8.48	0.81	39.85	43.62	30.38
Chlorogenic acid	9.17	0.87	79.96	41.62	87.33
Catechol	9.52	0.91	47.04	71.00	170.09
***p*-OH-benzoic acid**	10.01	0.95	62.73	61.54	24.18
Caffeic acid	10.37	0.99	12.73	16.02	33.46
Vanillic acid	10.50	1.00	1730.84	2000.98	1139.54
***p*-coumaric acid**	11.81	1.13	83.09	12.00	74.30
Ferulic acid	11.98	1.14	26.46	56.56	54.21
Iso-ferulic acid	12.33	1.18	14.05	18.94	29.10
Resveratrol	12.81	1.22	48.89	26.88	8.86
Rosmarinic acid	12.93	1.23	98.22	86.29	88.45
Ellagic acid	13.11	1.25	34.66	14.71	33.26
α-coumaric acid	13.52	1.29	161.63	62.70	53.42
3,4,5methoxy-cinnamic acid	14.20	1.35	45.00	52.01	61.95
Salicylic acid	14.52	1.38	5666.68	242.77	302.07
Cinnamic acid	15.29	1.46	40.00	8.24	5.96
Total			8333.96	3054.96	2459.79
Percentage of the total identified phenolic compounds (%)			15.71	20.48	19.19

*RRt: Retention time relative to vanillic acid.

Table 3: Results of determination of Flavonoids using HPLC analysis of *H. limifolia* Marloth, *G. carinata* (Mill.) Duval, *G. minima* Poelln

Flavonoids			Test results of flavonoids (mg/100g)		
	Rt	RRt*	*H. limifolia*	*G. carinata*	*G. minima*
Catechin	9.73	0.73	153.11	90.83	138.21
Luteolin	12.22	0.91	29.56	38.31	41.16
Naringin	12.34	0.92	80.01	111.43	82.17
Rutin	12.47	0.93	165.09	84.62	130.65
Hesperidin	12.61	0.94	95.50	87.09	47.12
Quercitrin	13.42	1.00	887.62	101.05	18.98
Quercetin	14.94	1.11	173.49	154.31	27.11
Hesperetin	15.68	1.17	646.47	227.04	52.93
Kaempferol	16.28	1.21	246.05	23.04	12.43
Apigenin	16.58	1.24	69.68	3.87	12.95
Total			2546.57	921.59	563.70
Percentage of the total identified flavonoids (%)			11.32	12.02	8.71

*RRt: Retention time relative to Quercitrin.

Table 4: HPLC analysis of the isolated saccharides of *H. limifolia* Marloth, *G. carinata* (Mill.) Duval, *G. minima* Poelln.

	Concentration (g %)		
	H. limifolia	*G. carinata*	*G. minima*
Stachyose	2.76	3.59	0.6
Sucrose	0.01	0.07	0.067
Glucose	0.02	0.04	-
Galactose and/ rhamnose (g/100g)	-	0.005	-
Arabinose	-	-	0.03
Glucuronic acid	0.91	1.2	0.35
Galacturonic acid	1.37	1.6	0.47

Table 5: The antioxidant potential of different concentrations of the methanolic extracts of *H. limifolia* Marloth, *G. carinata* (Mill.) Duval, *G. minima* Poelln. and ascorbic acid by DPPH radical scavenging assay

Sample conc. (µg)	DPPH scavenging %			
	H. limifolia	*G. carinata*	*G. minima*	Ascorbic acid
2560	81.16	80.63	46.56	92.48
1280	77.46	77.25	37.88	87.53
640	74.5	68.36	28.36	80.65
320	67.41	56.61	24.23	77.41
160	53.97	41.69	19.05	70.94
80	34.5	24.55	13.12	54.86
40	20.95	15.98	5.19	17.49
20	11.32	11.01	4.13	11.78

Table 6: IC_{50} of *H. limifolia* Marloth, *G. carinata* (Mill.) Duval, *G. minima* Poelln.

Sample	IC_{50} (µg/ml)
Haworthia limifolia	143.6
Gasteria carinata	249.1
Gasteria minima	>2560
Ascorbic acid	14.2

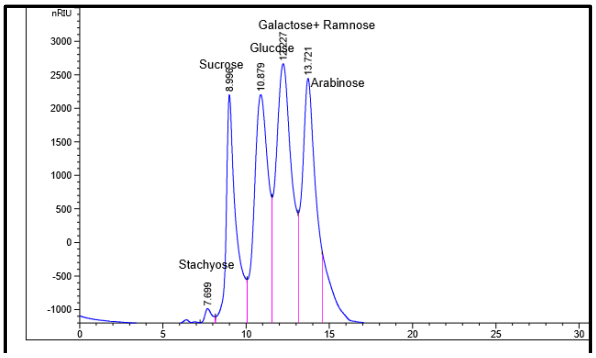

Fig. 1: Chromatogram of HPLC analysis of the mixture of the standard authentic sugars

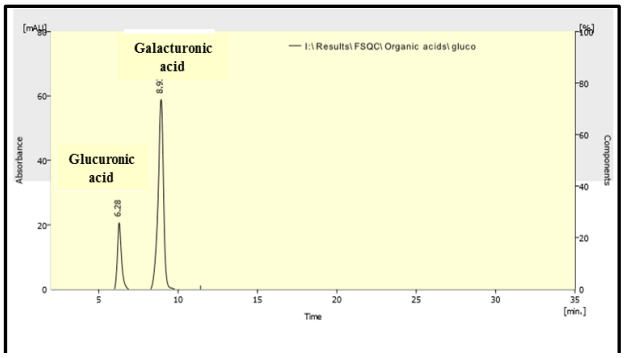

Fig. 2: Chromatogram of HPLC analysis of the mixture of the standard authentic sugar acids

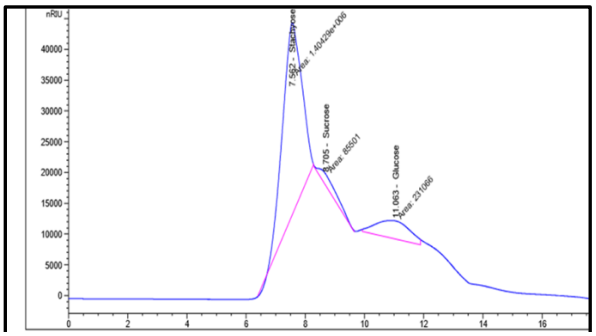

Fig. 3: Chromatogram of HPLC analysis of mucilage hydrolysate of the sugars of *H. limifolia* Marloth

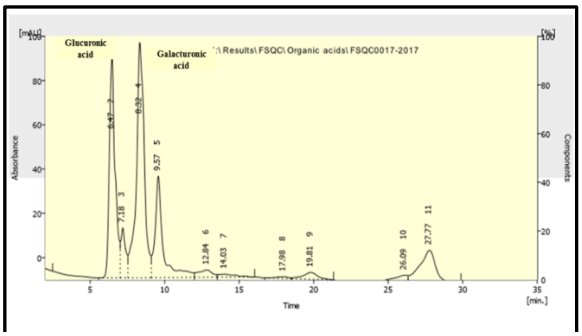

Fig. 4: Chromatogram of HPLC analysis of mucilage hydrolysate of the sugar acids of *H. limifolia* Marloth

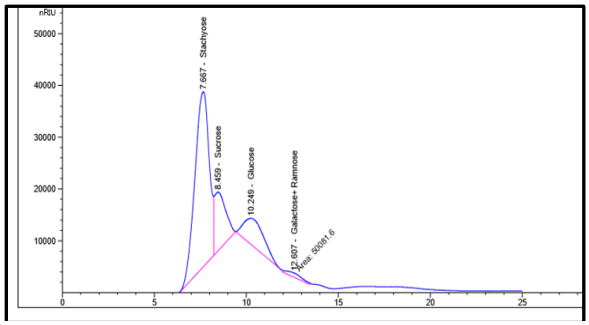

Fig. 5: Chromatogram of HPLC analysis of mucilage hydrolysate of the sugars of *G. carinata* (Mill.) Duval.

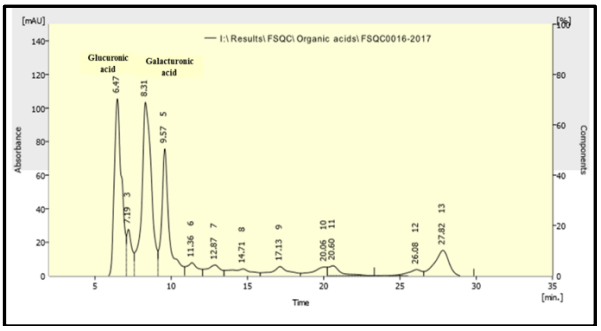

Fig. 6: Chromatogram of HPLC analysis of mucilage hydrolysate of the sugar acids of *G. carinata* (Mill.) Duval.

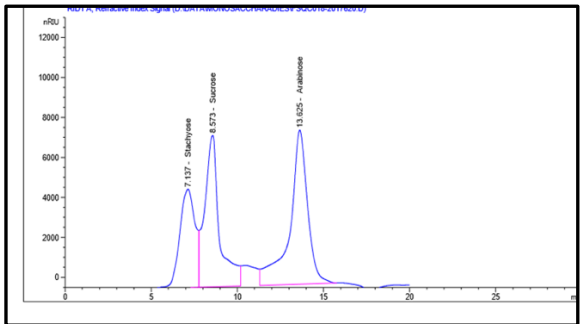

Fig. 7: Chromatogram of HPLC analysis of mucilage hydrolysate of the sugars of *G. minima* Poelln.

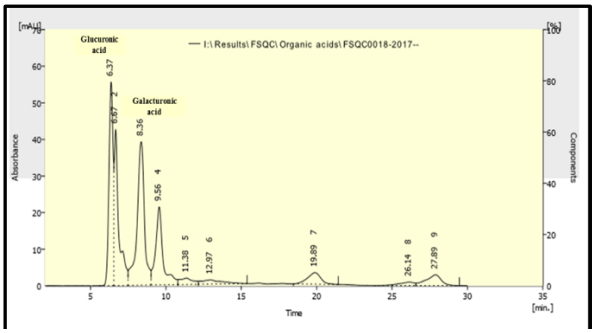

Fig. 8: Chromatogram of HPLC analysis of mucilage hydrolysate of the sugar acids of *G. minima* Poelln.

DISCUSSION

Phytochemical screening together with the HPLC results for the investigated plant extracts may explain the overall radical scavenging activity mainly the presence of phenolic compounds which contain a large number of double bonds and hydroxyl groups generating their antioxidant activity, specially flavonoids which have the ability to reduce free radicle formation and to scavenge free radicle, phenolic acids, tannins[30], anthraquinones and their polysaccharide contents specially the uronic acid conjugates [31,32].

Concerning the phenolic compounds, it is worth mentioning that their concentration in *H. limifolia* Marloth extract exceeds by about 3-fold their concentration in *G. carinata* (Mill.) Duval and *G. minima* Poelln. While the flavonoid content in *H. limifolia* Marloth extract exceeds by about 3-fold their concentration in *G. carinata* (Mill.) Duval and 4.5-fold their concentration in *G. minima* Poelln. and the tannin concentration in *H. limifolia* Marloth extract exceeds by about 3-fold their concentration in *G. minima* Poelln. and by about 8-fold their concentration in *G. carinata* (Mill.) Duval. The antioxidant activity in the three investigated extracts increases in a dose related relationship with the phenolic compounds and flavonoids.

CONCLUSION

This is the first characterization of the phenolic, flavonoid, polysaccharide and tannin content of *Haworthia limifolia* Marloth, *Gasteria carinata* (Mill.) Duval *and Gasteria minima* Poelln. In conclusion, the extracts of the aerial parts of *Haworthia limifolia* Marloth, *Gasteria carinata* (Mill.) Duval show good antioxidant effect and they can be used as a source for natural antioxidants.

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