SUPPLEMENTARY MATERIAL

Antioxidant activity optimization and GC-MS profile of aqueous extracts of *Vernonanthura patens* (Kunth) H. Rob. leaves

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Abstract

The temperature and extraction time of aqueous extracts of *Vernonanthura patens* (Kunth) H. Rob. (AEVP) leaves obtained by decoction were optimized for maximum recovery of DPPH radical scavenging activity, $ABTS^{+}$ inhibition activity, total phenolic content (TPC) and total flavonoids content (TFC) using response surface methodology (RSM). A central composite design (CCD) of 13 experimental runs was applied and second order polynomial models were used to describe the responses of the assessed extraction parameters. The optimized conditions: 79.79 °C and 126.23 minutes were found using the composite desirability function. The scavenging activity of assessed extracts could be correlated mostly to the presence of malic acid, succinic acid, α-ketoglutaric acid, citric acid, m-hydroxybenzoic acid, caffeic acid, inositol, and β-amyrin detected by gas chromatography-mass spectrometry (GC-MS). These results have not been reported and support the potential application of AEVP as natural source of antioxidants.

Keywords: *ABTS, catechin, DPPH, gallic acid, RSM, TEAC*

Experimental

Plant material

The genus *Vernonanthura* H. Rob. was established in order to differentiate a big number of species with morphological variations initially clustered in the genus *Vernonia* Schreb. Nonetheless, some species names are still considered synonym because they have not been completely examined, such as *Vernonanthura patens* H. Rob and *Vernonia patens* Kunth (Robinson 1992). In this research work , *V. patens* (Kunth) H. Rob. leaves in phenological stage of flowering were collected in October 2016 from Marcabelí, El Oro, Ecuador (3°47'6.43" S, 79°54'26.14" W). Preliminary analysis have been indicated high antioxidant activity in this period (Manzano et al. 2015). The collected plant material was authenticated by National Herbarium of Ecuador (QCNE) with voucher CIBE037. Then, it was dried 24 hours at 45°C in a convection oven (VWR Scientific Products, Atlanta, USA), it was grinding to a fine homogenous powder (500 µm) and it was stored at room temperature (25°C) before extraction process. Likewise, convection oven for 24 hours at 45°C, room temperature for 48 hours at 25°C and freeze dryer for 48 hours at -46°C were used individually for predictive model verification.

Standards and reagents

Aluminum chloride, (+)-catechin USP reference standard, Folin-Ciocalteu phenol reagent, galic acid, potassium persulfate and N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) were purchased from Sigma-Aldrich (Missouri, USA). Sodium nitrite and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) were purchased from Sigma (Missouri, USA). 2,2-diphenyl-1-picryl-hydrazyl (DPPH) was purchased from Aldrich (Missouri, USA). Saturated Alkanes Standard (C7 - C40) was purchased from Supelco (Bellefonte, USA). Ethanol, methanol and sodium hydroxide were purchased from Merck (Darmstadt, Germany). Sodium carbonate was purchased from Mallinckrodt (St. Louis, USA). Finally, Milli-Q grade water was prepared using a Direct-Q 3 with UV purification system Millipore (Massachusetts, USA).

Experimental design

The experiments were design according to central composite design (CCD) with a 2^2 factorial design consisting of 4 factorial points, 4 axial points and 5 center points (Table S1). The independent variables or factors defined were temperature (X_1) and extraction time (X_2). The response variables were DPPH radical scavenging activity (Y_1), ABTS radical cation

inhibition activity (Y_2), total phenolic content (Y_3) and total flavonoids content (Y_4). All dependent variables were used individually as colorimetric tests to quantify the potential of AEVP to neutralize free radicals. A total of 13 experimental runs at different levels of factors (temperature 40 – 90 °C and time 10 – 130 minutes) were used. The levels of the independent variables were based on the results of previous analysis (Manzano et al. 2015). All experiments were carried out 6 times. Each response variables (Y) was used to develop a mathematical model that explains the response to the extraction conditions employed using a second-degree polynomial equation (1) as given below:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 \qquad (1)$$

where β_0 is the model constant coefficient, β_i is the linear coefficient, β_{ij} is the interaction coefficient and β_{ii} is the quadratic coefficient.

Extraction

The aqueous extraction process was carried out in a water bath (VWR Scientific Products, Atlanta, USA), where dry sample was placed in water at different extraction conditions. Extraction was performed with solvent-to-solid ratio (1:10) and particle size within the ranges (180-710 μm) as indicated by the Pharmacopoeia for preparation of extracts and herbal tinctures from dry plant material (Sharapin 2000), in order to run the experiments the most similar possible to the traditional medicine. The aqueous extract obtained by decoction was filtered, it was dried and it was preserved at -17˚C prior to colorimetric assays.

DPPH radical scavenging activity

DPPH assay was done according to methodology previously described (Pérez-Jiménez et al. 2008). Briefly, 0.1 mL of sample (1250 μg/mL) was reacted with 3.9 mL of an ethanolic solution of DPPH (0.1mM) and the mixture was measured at 515 ηm using a Synergy HTX multi-mode microplate reader with UV-VIS detector (Biotek, Winooski, Vermont) after 30 minutes of incubation in dark. The radical scavenging activity was calculated according to equation (2), and was also expressed as μmol Trolox equivalents antioxidant capacity (TEAC) per g of extract.

$$\text{Radical Scavenging Activity (\%)} = \left[1 - \frac{Abs_{sample}}{Abs_{control}}\right] x\ 100 \qquad (2)$$

ABTS radical cation inhibition activity

ABTS assay was performed according to procedures previously described (Vasco et al. 2009). Rapidly, 10 µl of sample was mixed with 1 mL of ($ABTS^{+}$), then the mixture was incubated for 6 minutes and it was read to 734 ηm in a spectrophotometer UV-160A (Shimadzu, Kyoto, Japan). Note that ABTS radical cation ($ABTS^{+}$) was produced by reaction of 7 mM ABTS stock solution with 2.45 mM potassium peroxidisulfate and the mixture was kept to 4˚C for 18 h in dark before use. The radical inhibition activity was calculated according to equation (3), and was also expressed as µmol Trolox equivalents antioxidant capacity (TEAC) per g of extract.

$$\text{Radical Cation Inhibition Activity (\%)} = \left[1 - \frac{Abs_{sample}}{Abs_{control}}\right] x\ 100 \qquad (3)$$

Total phenolic content (TPC)

TPC analysis of aqueous extracts was performed using modified Folin-Ciocalteu method (Slinkard & Singleton 1977; Georgé et al. 2005). An aliquot of 0.5 mL of sample (1250 µg/mL) was mixed with 2.5 mL of Folin-Ciocalteu phenol reagent solution (10%), the mixture was shaken and was allowed to stand at room temperature for 2 minutes. Then, it was mixed with sodium carbonate (75 g/l) and it was incubated to 50°C for 15 minutes. Oasis HLB cartridges were employed as sample preparation technique to remove interfering components as vitamin C and reducing sugars. Absorbance was measured against the blank reagent at 760 ηm using a spectrophotometer UV-160A (Shimadzu, Kyoto, Japan). Gallic acid (10 - 90 mg/L) was used as the standard and results were expressed as mg of gallic acid equivalents per g of extract (mg GAE/g).

Total flavonoids content (TFC)

TFC analysis of aqueous extracts was done according to methodology previously defined (Al-Saeedi & Hossain 2015). An aliquot of 250 µL of sample was taken in a test tube with 1.25 mL of water and 75 µL of sodium nitrite (5%). The mixture was kept in a room temperature 6 minutes. Then 150 µL of aluminum chloride (10%) was added to each tube, was mixed and was kept in a room temperature for 5 minutes. Finally, the mixture was diluted with 500 µL of sodium hydroxide (1M) and 275 µL of water. Absorbance was measured against the blank reagent at 510 ηm using a Synergy HTX multi-mode microplate reader with UV-VIS detector

(Biotek, Winooski, Vermont). (+)-Catechin (25 – 300 mg/l) was used as the standard and results were expressed as mg of catechin equivalents per g of extract (mg CE/g).

Gas Chromatography – Mass Spectrometry (GC-MS)

Freeze dried samples were mixed with N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) and heated in a water bath to 80°C for 2 hours to allow the silylation of compounds prior to injection in GC-MS instrument (Saitta et al. 2002; Zuo et al. 2002). Compounds separation of derivatized samples (2 µL of reaction mixture) was performed in a gas chromatography-mass spectrometry equipment Agilent Technologies (7890A GC system and 5975C inert XL MSD with a triple axis detector). A capillary column DB-5MS (30 m × 0.25 mm) with phenyl dimethylpolysiloxane was used as the stationary phase (0.25-micron film thickness) and helium as the carrier gas (1.2 mL/min). The injection was done at 250°C with splitless mode. The MSD transfer line was 280°C and the detector temperature was 230°C. The oven temperature was maintained at 60°C for 2 minutes, then it was increased to 200°C at 15°C/min with a hold time of 5 minutes and it was increased to 300°C at 5°C/min with a final hold time of 5 minutes. Electron ionization of 70 eV was used and the data compounds were collected with the full scan mode (40-700 amu) in the quadrupole mass analyzer. Finally, compounds were identified by comparison of their retention index and mass spectra data of Wiley 9th with NIST 2011 MS Library

Statistical analysis

Response surface methodology (RSM) was performed to analyze the influence of defined independent variables on response variables using STATGRAPHICS Centurion XV (STATGRAPHICS Centurion, Warrenton, USA). The software was also used for model building, graph plotting and verification of predicted optimum values.

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Table S1. Regression coefficients and analysis of variance (ANOVA) of the predicted second-order polynomial model to the responses antioxidant activity, total phenolic content and total flavonoids content

	Regression coefficients [a]			
	Y_1	Y_2	Y_3	Y_4
Constant	49.3268	14.2054	-84.3414	143.0840
Linear				
X_1: Temperature (°C)	1.1179**	0.7223**	3.5628**	-2.8379*
X_2: Extraction time (min)	-0.2351	-0.0420	0.2310*	-0.9659
Quadratic				
X_1^2	-0.0069**	-0.0049	-0.0180	0.0177
X_2^2	0.0018**	-0.0021**	-0.0069	0.0022
Interaction				
X_1X_2	-0.0004	0.0058**	0.0155	0.0110*
R^2	0.6645	0.6553	0.4810	0.1653
Adjusted R^2	0.6412	0.6314	0.4449	0.1074
PRESS	961.50	3445.22	140024	21967
Analysis of variance				
Mean	84.4668	49.5469	110.5909	34.1024
Standard error	3.3709	6.3429	41.3464	16.2825
Coefficient of variation (%)	3.9908	12.8019	37.3868	47.7459
Mean Square	324.04**	1101.50**	22813.1**	756.13*
F-value (model)	28.52**	27.38**	13.34**	2.85*

[a] Y_1 = DPPH radical scavenging activity (%), Y_2 = ABTS radical cation inhibition activity (%), Y_3 = total phenolic content (mg GAE/g), Y_4 = total flavonoids content (mg CE/g); * $p < 0.05$; ** $p < 0.01$

Table S2. Experimental design and corresponding response values of the dependent variables to extraction conditions

Run	Independent variables		Response variables [a]							
	X_1	X_2	Y_1		Y_2		Y_3		Y_4	
	(°C)	(min)	Exp. [b]	Pred.	Exp. [b]	Pred.	Exp. [b]	Pred.	Exp. [b]	Pred.
1	40	70	73.49 ± 7.44	74.35	45.94 ± 2.91	38.21	67.11 ± 4.43	55.33	19.42 ± 3.69	31.97
2	47.5	28	84.30 ± 1.56	81.28	33.40 ± 3.30	42.38	92.11 ± 3.60	65.98	28.61 ± 6.51	37.65
3	47.5	70	72.83 ± 3.27	78.04	47.54 ± 3.18	43.47	35.27 ± 2.31	78.39	55.62 ± 4.37	28.11
4	47.5	112	84.42 ± 0.98	81.09	29.37 ± 0.41	37.05	69.62 ± 4.11	66.46	21.14 ± 2.69	26.32
5	65	10	89.44 ± 2.11	90.60	50.96 ± 2.83	43.63	64.08 ± 2.99	82.75	42.82 ± 3.15	31.31
6	65	28	87.64 ± 1.34	87.16	46.10 ± 2.48	48.24	105.23 ± 3.34	100.44	28.57 ± 3.78	28.32
7	65	70	85.74 ± 1.71	83.65	49.02 ± 3.49	53.63	79.85 ± 4.37	124.32	21.12 ± 3.38	26.89
8	65	112	86.99 ± 1.46	86.44	53.87 ± 3.58	51.49	227.89 ± 5.71	123.85	44.88 ± 3.02	33.20
9	65	130	87.95 ± 0.56	89.57	50.61 ± 2.09	48.27	60.23 ± 3.21	116.20	36.37 ± 3.94	38.27
10	82.5	28	87.86 ± 2.30	88.84	48.81 ± 4.47	51.11	142.43 ± 2.64	123.85	19.26 ± 1.28	29.86
11	82.5	70	84.96 ± 0.70	85.06	66.29 ± 3.35	60.78	178.95 ± 3.32	159.19	9.64 ± 2.27	36.53
12	82.5	112	86.90 ± 1.70	87.59	61.92 ± 6.53	62.92	165.79 ± 10.51	170.19	44.21 ± 8.79	50.94
13	90	70	85.55 ± 1.50	84.38	60.28 ± 3.50	62.92	149.10 ± 9.20	170.75	71.68 ± 4.42	43.98

[a] Y_1 = DPPH radical scavenging activity (%), Y_2 = ABTS radical cation inhibition activity (%), Y_3 = total phenolic content (mg GAE/g), Y_4 = total flavonoids content (mg CE/g); [b] Mean values (n=6) ± standard deviation.

Table S3. Experimental data of the verification of predicted models

	Response variables [a]			
	Y_1 (%) [b]	**Y_2 (%)** [b]	**Y_1 (µmol Trolox / g)** [b]	**Y_2 (µmol Trolox / g)** [b]
Predicted values	89.57	60.30	738.13	1538.39
Convection oven	87.14 ± 1.13 A	50.68 ± 5.43 A	549.80 ± 25.95 A	1020.68 ± 99.07 B
Room temperature	89.33 ± 3.55 A	36.60 ± 2.50 B	566.62 ± 32.88 A	1491.75 ± 99.53 A
Freeze dryer	89.14 ± 0.71 A	33.53 ± 2.39 B	570.67 ± 16.20 A	1373.63 ± 77.35 A

[a] Y_1 = DPPH radical scavenging activity, Y_2 = ABTS radical cation inhibition activity; [b] Mean values (n=6) ± standard deviation; Mean values with a common letter in the same column are not significantly different according to Tukey Test (p>0,05).

Table S4. Compounds detected in aqueous extracts of *V. patens* (Kunth) H. Rob leaves dried by convection oven

Peak	Retention time (minutes)	Compound	Molecular formula	Peak Area (%) [a]	Retention index (estimated) [b]	Retention index (reference) [c]
1	9.08	L-threonine	$C_4H_9NO_3$	0.11 ± 0.02	1354.42	1357.00
2	9.82	Malic acid	$C_4H_6O_5$	0.70 ± 0.22	1435.81	1390.00
3	10.42	L-threonic acid	$C_4H_8O_5$	0.69 ± 0.27	1504.89	1518.00
4	11.47	L-asparagine	$C_4H_8N_2O_3$	0.79 ± 0.39	1632.82	1538.00
5	16.92	Caffeic acid	$C_9H_8O_4$	0.41 ± 0.12	2140.45	2144.00
6	17.40	Heptadecanoic acid	$C_{17}H_{34}O_2$	0.61 ± 0.26	2107.31	2087.00
7	18.00	cis-11-Octadecenoic acid	$C_{18}H_{34}O_2$	1.03 ± 0.60	2145.90	2194.00
8	23.58	2 α-Mannobiose	$C_{12}H_{22}O_{11}$	0.29 ± 0.17	2504.01	2598.00
9	30.40	Hexacosanoic acid	$C_{26}H_{52}O_2$	0.15 ± 0.03	2991.56	2981.00
10	34.17	β-Amyrin	$C_{30}H_{50}O$	0.14 ± 0.05	3299.42	3320.40

[a] Mean values (n=6) ± standard deviation; [b] Estimated values in capillary column DB-5MS; [c] Reference values estimated in non-polar capillary column.

Table S5. Compounds detected in aqueous extracts of *V. patens* (Kunth) H. Rob leaves dried at room temperature

Peak	Retention time (minutes)	Compound	Molecular formula	Peak area (%) [a]	Retention index (estimated) [b]	Retention index (reference) [c]
1	8.34	Succinic acid	$C_4H_6O_4$	0.27 ± 0.07	1390.72	1314.00
2	9.98	Malic acid	$C_4H_6O_5$	0.48 ± 0.11	1454.46	1390.00
3	10.68	m-Hydroxybenzoic acid	$C_7H_6O_3$	0.13 ± 0.03	1536.80	1559.00
4	10.96	α-Ketoglutaric acid	$C_5H_6O_5$	0.35 ± 0.13	1571.27	1538.00
5	12.80	Citric acid	$C_6H_8O_7$	0.45 ± 0.19	1773.65	1839.00
6	17.25	Caffeic acid	$C_9H_8O_4$	0.52 ± 0.10	2097.22	2144.00
7	17.39	Heptadecanoic acid	$C_{17}H_{34}O_2$	0.31 ± 0.04	2106.41	2087.00
8	26.47	2-Monostearin	$C_{21}H_{42}O_4$	0.21 ± 0.04	2700.14	2772.00
9	30.38	Hexacosanoic acid	$C_{26}H_{52}O_2$	0.17 ± 0.02	2989.85	2981.00
10	34.14	*β*-Amyrin	$C_{30}H_{50}O$	0.24 ± 0.09	3296.95	3320.40

[a] Mean values (n=6) ± standard deviation; [b] Estimated values in capillary column DB-5MS; [c] Reference values estimated in non-polar capillary column.

Table S6. Compounds detected in aqueous extracts of *V. patens* (Kunth) H. Rob leaves dehydrated by freeze drying

Peak	Retention time (minutes)	Compound	Molecular formula	Peak area (%) [a]	Retention index (estimated) [b]	Retention index (reference) [c]
1	8.37	Succinic acid	$C_4H_6O_4$	0.21 ± 0.07	1392.00	1314.00
2	9.79	Malic acid	$C_4H_6O_5$	0.56 ± 0.09	1432.38	1390.00
3	10.69	m-Hydroxybenzoic acid	$C_7H_6O_3$	0.17 ± 0.07	1537.65	1559.00
4	10.97	α-Ketoglutaric acid	$C_5H_6O_5$	0.43 ± 0.10	1572.13	1538.00
5	12.63	Fructose	$C_6H_{12}O_6$	1.19 ± 0.27	1756.47	1805.10
6	12.81	Citric acid	$C_6H_8O_7$	0.65 ± 0.27	1774.42	1839.00
7	15.01	Inositol	$C_6H_{12}O_6$	1.34 ± 0.24	1946.03	1848.00
8	17.25	Caffeic acid	$C_9H_8O_4$	0.23 ± 0.05	2097.22	2144.00
9	17.39	Heptadecanoic acid	$C_{17}H_{34}O_2$	0.24 ± 0.01	2106.86	2136.40
10	18.51	cis-11-Octadecenoic acid	$C_{18}H_{34}O_2$	1.08 ± 0.14	2178.27	2194.00
11	26.48	2-Monostearin	$C_{21}H_{42}O_4$	0.20 ± 0.01	2700.64	2772.00
12	27.39	Squalene	$C_{30}H_{50}$	0.14 ± 0.06	2765.65	2797.00
13	30.39	Hexacosanoic acid	$C_{26}H_{52}O_2$	0.14 ± 0.03	2991.01	2981.00
14	34.16	*β*-Amyrin	$C_{30}H_{50}O$	0.12 ± 0.02	3298.77	3320.40

[a] Mean values (n=6) ± standard deviation; [b] Estimated values in capillary column DB-5MS; [c] Reference values estimated in non-polar capillary column.

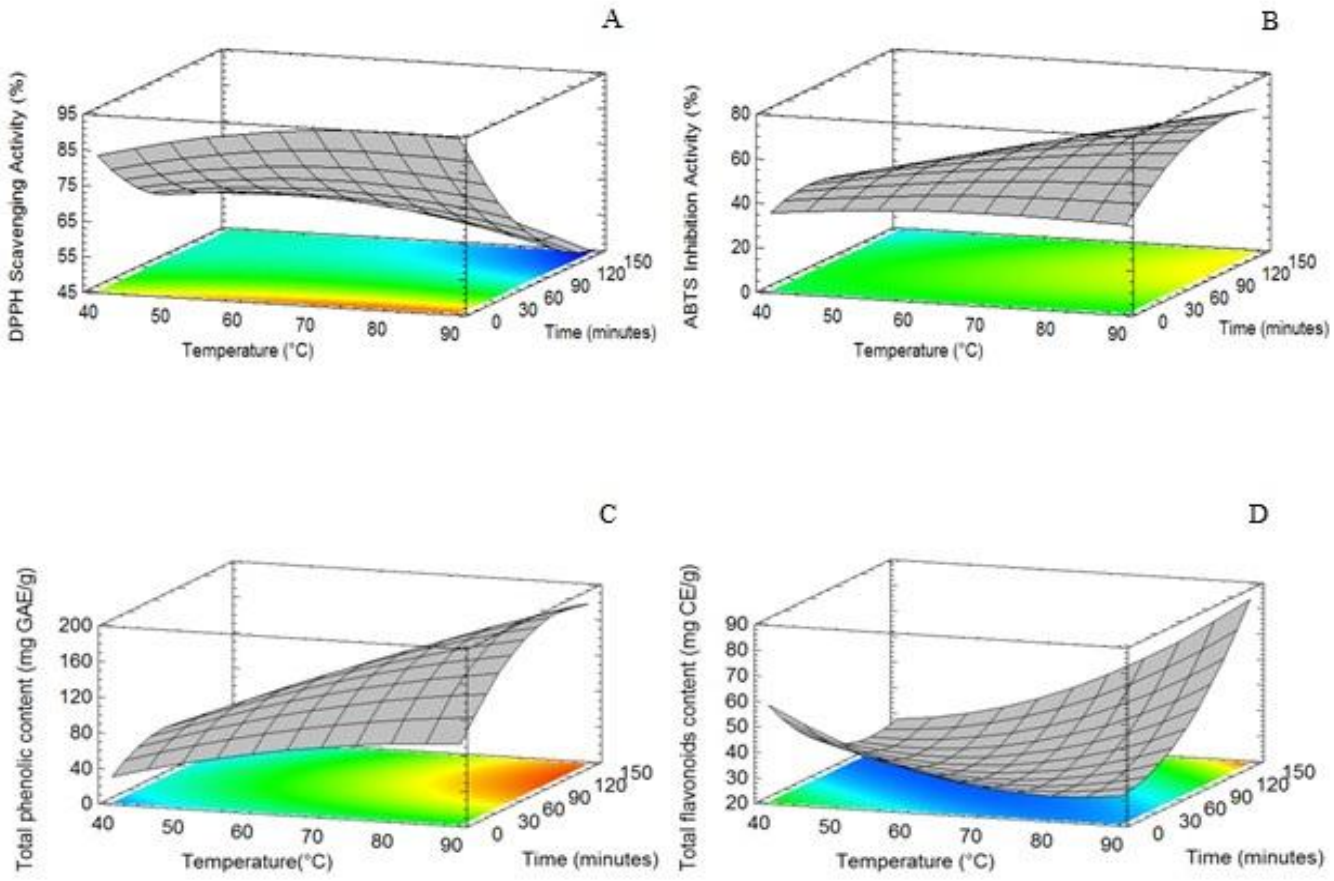


Figure S1. Response surface and contour plots for the effect of dependent variables on response: (A) DPPH radical scavenging activity, (B) ABTS+ inhibition activity, (C) total phenolic content, and (D) total flavonoids content.

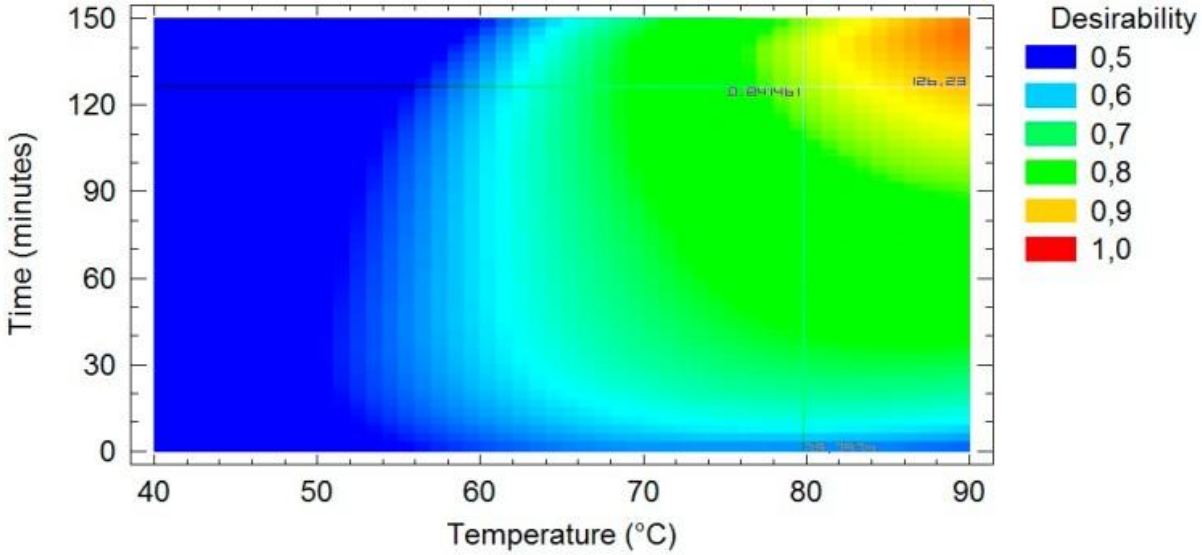
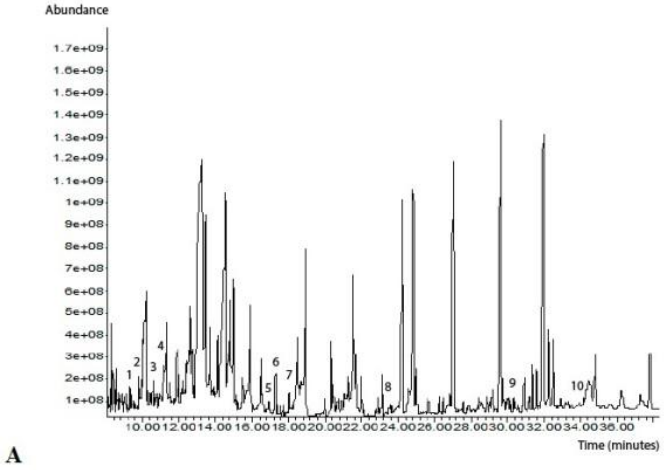


Figure S2. Contour graph of desirability function.



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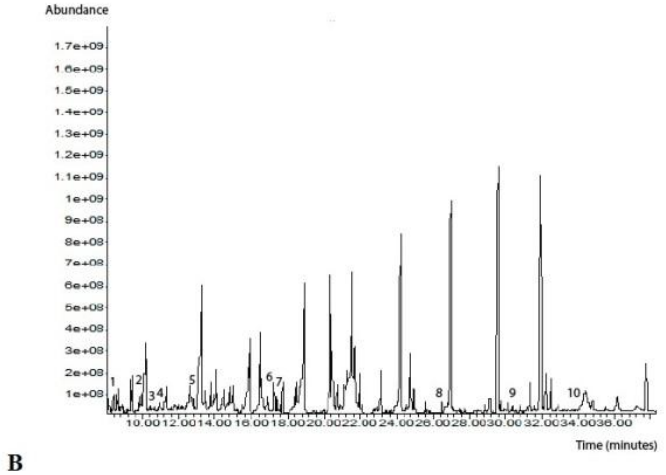

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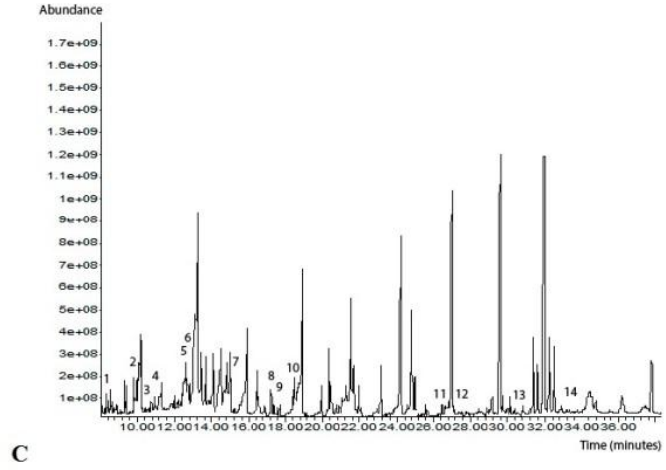

C

Figure S3. Chromatographic profiles of aqueous extracts of *V. patens* (Kunth) H. Rob. leaves dried by convection oven (A), room temperature (B), and freeze dryer (C).