RJC

RASĀYAN J. Chem.
Vol. 15 | No. 2 |1469-1480| April - June | 2022
ISSN: 0974-1496 | e-ISSN: 0976-0083 | CODEN: RJCABP
http://www.rasayanjournal.com
http://www.rasayanjournal.co.in

A COMPREHENSIVE ANALYTICAL APPROACH FOR QUALITY EVALUATION OF FLAVONOID-RICH EXTRACT OF *Glycyrrhiza glabra* (GutGard®)

Vineet Kumar Singh[1, 2, ✉], Rojison Koshy[1, 2], Deepak Mundkinajeddu[2] and Jothie Richard Edwin[2]

[1]Manipal College of Pharmaceutical Sciences, Manipal Academy of Higher Education, Manipal, Karnataka, India, 576104.

[2]Research and Development Centre, Natural Remedies Pvt Ltd, Bangalore, Karnataka, India, 560100.

✉Corresponding Author: vineet@naturalremedy.com

ABSTRACT

Quality evaluation of botanical preparations is still evolving globally due to the complex, variable, and unknown phytochemical compositions of herbs. Accordingly, the quality of commercially available products needs to be better defined and controlled to ensure consistent health benefits and safety for consumers. This study aims to develop a comprehensive analytical methodology involving both phytochemical and biological evaluations towards achieving a meaningful quality control of commercial batches of a flavonoid-rich extract (GutGard®) derived from *Glycyrrhiza glabra*. Nine different commercial batches of the extract were analyzed to establish the chromatographic fingerprint of flavonoids as well as biological consistency using *in-vitro* assays for evaluating antioxidant and anti-inflammatory activity. A total of 53 peaks were assigned using MS/MS as the "common peaks" and nine peaks as "characteristic peaks" in the fingerprint of all the nine batch samples. Quantitative determination of the latter was achieved with a validated HPLC method. The finding revealed that all the examined samples were enriched with flavonoids, although with varied contents. The biological assays complemented the phytochemical analysis by way of providing a range of IC_{50} values that represent the overall chemistry of the extract including both the known and unknown constituents.

Keywords: Chromatographic fingerprint; flavonoid; quality control; LC-MS/MS; similarity analysis.

RASĀYAN *J. Chem.*, Vol.15, No.2, 2022

INTRODUCTION

Glycyrrhiza glabra Linn. (*G. glabra* L.; Family: Fabaceae), also known as licorice, is a well-known medicinal plant native to the Mediterranean and certain parts of Asia with a long history of use for its unique and diverse pharmacological effects. It has been traditionally used for the management of digestive problems such as peptic ulcers, acid reflux, and functional dyspepsia.[1-3] *G. glabra* has been shown to be useful in the treatment of peptic ulcers.[4] The flavonoid elements of licorice such as isoflavans (glabridin, hispaglabridin A and B, 4ʹ-O-methylglabridin), chalcones (isoliquiritigenin), and isoflavones (formononetin) have been linked to its overall activity.[5-10] Quality control of crude drugs and their extracts are quite complex as their composition might vary depending on numerous factors like genetic variability, soil, geography, growing conditions, harvesting processes, extract processing, and ecological conditions (insect feeding and microbial infections). Due to the variability of the plant material and contamination or intended adulteration with other *Glycyrrhiza* species, verification of ground licorice samples and standardization of extracts are difficult. In recent years because of their pharmacological activities, licorice flavonoids have increased and sparked an interest. On the other hand, quality control of licorice is typically based on the analysis of glycyrrhizin and glabridin.[11-12] For quality control purposes, LC-UV analysis of flavonoids such as liquiritin, isoliquiritigenin, and liquiritigenin has also been used.[13] The overall efficacy of the herbal drug is not dependent on a single or a few molecules.[14] Herbal medicines are thought to work by combining the effects of numerous components in a synergistic way.[15] As a result, the biological activity and therapeutic efficacy of herbal medicine are determined by the overall content of active components.[16] These complexities of natural products have made a case for using bioassays for the quality control, as a

http://doi.org/10.31788/RJC.2022.1526831
This work is licensed under a CC BY 4.0 license.

complementary method for the classical chemical approaches based on chromatographic (HPLC, TLC), spectroscopic (NMR, Mass), and hyphenated (LCMS and GCMS) methods.[17-19] Although many phytochemicals have been reported in the scientific literature, the applicability of the phytochemical knowledge for developing meaningful analytical methods that can raise the bar on quality control of *G. glabra* based products is lacking. Hence, this study is undertaken to develop a comprehensive analytical approach for a commercially manufactured extract derived from *G. glabra*. The approach involves qualitative and quantitative analysis of flavonoids with LC-MS/MS and HPLC respectively and utilizes *in-vitro* bioassays towards achieving biological standardization.

EXPERIMENTAL

Chemicals and Reagent

The reference compounds viz., liquiritin, isoliquiritin, liquiritigenin, isoliquiritigenin, glabridin, glabrol, 4'-O-methylglabridin, hispaglabridin A and B were isolated in-house from *G. glabra* by using chromatographic techniques and their chemical structures were precisely identified by ultraviolet, infrared, mass spectrometry, ^{1}H and ^{13}C-NMR spectroscopy by comparing with corresponding spectral data available in the literature.[20-22] The purity of each compound was found to be >98% by the area normalization method with HPLC/UV. Methanol and acetonitrile (LC-MS grade) were procured from Biosolve (India) whereas water (analytical grade) and formic acid were procured from Rankem (India). ABTS [2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)] (A1888, Sigma, USA or 11557, Fluka USA, store at room temperature), 96 well flat clear plates (Cat. no. 980040, Tarsons), gallic acid [3,4,5-trihydroxy benzoic acid] (G7384, Sigma, USA, store at room temperature), DPPH (2, 2-diphenyl-1-picryl-hydrazyl) (D9132, Sigma Aldrich), phosphate buffer saline (P3813, Sigma Aldrich, USA, store at room temperature), Methanol (HPLC grade, M0275, Rankem, India), ammonium persulphate (A0550, Rankem, India, store at room temperature), DMSO (Rankem, #D-0170), J774A.1 cell line murine macrophage (ATCC, #TIB-67™) DMEM (Gibco, #12100-046).

Plant Material

G. glabra used in the present study was verified by the National Institute of Science Communication and Information Resources (NISCAIR) and then a voucher specimen (Batch number-RD/21868) was submitted to our herbarium. The flavonoid-rich extract used in this study (GutGard®) was manufactured as per the process described previously.[23] Nine different batches of GutGard® were used in this study for evaluation of chemical and biological consistency using LC-MS/MS, HPLC, and *in-vitro* bioassays as described in the subsequent sections.

Standard Preparation

The nine reference standards (liquiritin, isoliquiritin, liquiritigenin, isoliquiritigenin, glabridin, glabrol, 4'-O-methylglabridin, hispaglabridin A, and hispaglabridin B) stock solutions (1mg/ml) were prepared by using methanol as solvent. The calibration curves were plotted using a series of concentrations ranging from 1.0 to 100ppm obtained by diluting the combined standard solution with methanol. All solutions were sonicated before injection and kept at 4°C until use.

Sample Preparation

In a 50 ml volumetric flask, 350mg of sample was carefully weighed. Approximately, 25 ml of methanol was added, heated gently on a water bath, cooled to room temperature and the volume was increased to 50 ml with methanol. Before use, the solution was well mixed and filtered through a 0.45μm filter membrane. For analysis, 5µL of each sample solution was injected into the HPLC-PDA-MS^{n} system.

Chromatographic Separation

A Shimadzu Prominence 20AD system with a binary pump solvent supply system, an online degasser-DGU-20A3R, SIL-20AC auto-sampler with sample cooler, a column oven-CTO-10ASVP, CBM-20Alite System Controller, SPD-M20A PDA detector, and an analytical workstation was used for LC analysis. A Merck Hibar® Purospher® STAR C18 column (4.6 x 100 mm, 3µm) was used for chromatographic separation. Water (0.1 percent formic acid v/v, A) and acetonitrile (0.1 percent formic acid v/v, B) was used in the mobile phase. Before use, the solution was filtered through a 0.45 µ membrane filter and degassed.

The gradient elution method employed was as follows: 0 min, 5 percent B; next 5 min, 20 percent B; next 5 min, 25 percent B; next 3 min, 30 percent B; next 7 min, 35 percent B; next 3 min, 38 percent B; next 7 min, 43 percent B; next 5 min, 50 percent B; next 10 min, 65 percent B; next 10 min, 85 percent B; next 10 min, 100 percent B; next 5 min, equilibration period of 100 percent B; next 5min, solvent B decreased to 5 percent followed by an equilibration period of 5 percent for the last 5 min. The extract chromatograms were obtained at 280 nm at a flow rate of 0.4 ml/min. The sample injection volume was 5μL and UV spectra were acquired from 190 to 400nm.

Mass Spectrometry

Mass spectral data were obtained on a Thermo Scientific LCQ Fleet (San Jose, CA, USA) coupled with an ESI interface. Negative ion mode was used with the ion source. The following MS parameters were used: collision gas, helium (He); sheath gas (N_2), 22 arbitrary units; auxiliary units (N_2), five arbitrary units; ion spray voltage, -4.5 kV; capillary temperature, 300°C; capillary voltage, -5 V; tube lens offset voltage, -40 V. In MS scan mode, full scan data acquisition was captured from m/z 50-2000. Xcalibur 2.07 was used for data collecting, data interpretation, and peak determination (Thermo Scientific).

Similarity Analysis and Principal Component Analysis (PCA)

Based on HPLC-PDA data, the similarity between the *G. glabra* samples of different batches was calculated using the software named XLSTAT Base, Version 18.06. All the nine marker peaks in the chromatograms of the nine batches were calculated using the Pearson correlation coefficient, and then the similarities among the nine batches were calculated. Higher similarities usually implied closer relationships in geography, variety, and chemical composition. PCA is the unsupervised algorithm, which is used to unfold the unseen structures in the data. PCA permits the interpretation, of relationships among different samples as well as among dissimilar variables. PCA was carried out based on the contents of nine quantified flavonoids. PCA is an unsupervised approach that is used to reveal the previously overlooked data. PCA was carried out on the contents of nine flavonoids that were quantified.

In-vitro Activities

ABTS Radical Scavenging Activity

The extract samples, ABTS radical scavenging activity was measured using the method described by Auddy (2003) with some modifications.[24] In a nutshell, the complete reaction mixture, which includes 10mM PBS (pH7.4) with varied quantities of samples, and ABTS radical solution (0.238 mM), was mixed and immediately measured at 734nm using a VersaMax microplate reader (Molecular Devices, USA). As a reference, gallic acid was used as a standard. The experiment was carried out in triplicates and IC_{50} was measured using log-probit analysis, the data were represented in μg/mL and the percentage inhibition was computed as follows-

$$\% \text{ Inhibition} = \frac{\text{Absorbance (control)} - \text{Absorbance (test)}}{\text{Absorbance (control)}} \times 100$$

DPPH Radical Scavenging Activity

The extract samples, DPPH radical scavenging activity was measured spectroscopically, as described by Auddy (2003) with some modifications.[24] In a nutshell, the whole reaction mixture consisted of methanol, vehicle buffer, positive control, various concentrations of the test solution, and DPPH at a final concentration of 0.132mM. For 20 minutes, the reaction mixture was incubated at 25°C. The absorbance was measured at 510 nm using a micro-well plate reader after incubation (Molecular devices VersaMax microplate reader). Without the test samples, a control reaction was performed. As a reference, gallic acid was used as a standard. The experiment was carried out in triplicates and IC_{50} was measured using GraphPad Prism, the data were represented in μg/mL and the percentage inhibition was computed as follows-

$$\% \text{ Inhibition} = \frac{\text{Absorbance (control)} - \text{Absorbance (test)}}{\text{Absorbance (control)}} \times 100$$

IL-6 inhibition Assay and Nitric Oxide Radical Scavenging Activity

The effect of extract samples or compounds on LPS-induced NO release in J774A.1 murine macrophage was assessed using the IL-6 inhibition assay and nitric oxide radical scavenging activity technique reported

by Chandrasekaran (2010) with some modifications.[25] Every 96 well plates were labeled with a code system that identified the test substance, test phase, dose levels, and cell line details. The murine monocytic macrophage cell lines were adjusted to $5x10^5$ cells/mL in DMEM supplemented with FBS (10 percent), and a 96-well culture plate was suspended with 200µL of the cell suspension and cultured overnight at 37°C in a humified 5 percent carbon dioxide incubator. The adhering cells were rinsed three times with phosphate-buffered saline (PBS) after a 16-hour incubation period. The cells were exposed to test items for one hour before being cultured with LPS (1g/mL) for 24 hours. The medium was collected after 24 hours of incubation for IL-6 estimation and nitrite measurement. The IL-6 levels were tested using a sandwich ELISA according to the manufacturer's instructions (BD Bioscience), and the nitrite concentration was quantified using the Griess reaction as an indicator of nitric oxide generation. Graph pad Prism statistical software was used for statistical analysis, which includes a one-way analysis of variance. Differences with a p-value <0.05 were deemed statistically significant. The Mean ± SD of the three replicates per treatment group is used to calculate the results.

RESULTS AND DISCUSSION

Optimization of Chromatographic Conditions

To obtain a good resolution, we optimized the separation conditions such as mobile phase gradient and column. Longer retention time and better peak morphologies were achieved by using acidified mobile phases of acetonitrile and water (0.1 percent v/v formic acid). To maximize the separation conditions, reverse-phase HPLC columns from various manufacturers were tested during preparatory work. A reverse-phase column, Merck Hibar® Purospher® STAR C18(4.6 x 100mm, 3µm), provided the optimum separation efficiency and peak form. The detection wavelength, 280nm was chosen based on the UV max of the nine separated flavonoids, which resulted in adequate detector response with little interference from other chemicals present in the samples.

Analysis of Chromatographic Fingerprints

Chromatographic peaks found in all nine samples with good resolution were assigned as 'common peaks' and there were 53 common peaks in the chromatographic fingerprints. Glabridin (peak No 40) at retention time 46.43 min was found to be the major peak and was chosen as the reference peak. In the nine samples, the relative retention times (RRT) of 53 peaks for peak 40 were calculated. The content of total flavonoids in the extract was calculated as glabridin by quantifying the compounds corresponding to the 53 peaks. In addition, similarity analysis was performed using an assay of the nine standards for the nine different batches of *G. glabra* by using the Pearson correlation coefficient method. As mentioned in Table-1, the least similar values of these nine samples were 0.924 whereas the majority of the values were >0.98, indicating that the *G. glabra* extract samples produced in different batches had comparable chemical compositions and these chromatograms may be considered as a typical HPLC fingerprint (Fig.-1).

Nine commercial samples (N=9) were subjected to PCA biplot, which had eight factors and each factor corresponds to one dimension. In this biplot, the first two factors themselves allow us to represent 79.09% of the initial variability of the entire data. Present commercial samples fall under four different quadrants i.e., batch 3, 4, 5, and 9; 7 and 8; are near to each other therefore they have a close relationship with each other while batches 1, 2, and 6 were distinct from each other as they are located far from each other in their respective quadrant. With this biplot, PCA helps us to quickly visualize and analyze the correlations between the multiple variables (Fig.-3).

Qualitative Identification of Common Peaks in *G. glabra*

HPLC-PDA/ESI-MS^n was used for qualitative identification of 53 common peaks in *G. glabra*. By comparing with the ESI-MS^n data and HPLC retention time of standard flavonoids, the nine peaks corresponding to the standards viz., liquiritin, isoliquiritin, liquiritigenin, isoliquiritigenin, glabridin, glabrol, 4ˈ-O-methylglabridin, hispaglabridin A, and hispaglabridin B were identified. Another 44 peaks were discovered based on their fragmentation pattern as well as with their online UV spectra and confirmed the HPLC elution order for the identified flavonoids from the previously published literature (refer to Table -2).

Table-1: Similarity indexes of the nine batches of *Glycyrrhiza glabra*

	NR-GG-01	NR-GG-02	NR-GG-03	NR-GG-04	NR-GG-05	NR-GG-06	NR-GG-07	NR-GG-08	NR-GG-09	Reference
NR-GG-01	1									
NR-GG-02	0.968	1								
NR-GG-03	0.989	0.924	1							
NR-GG-04	0.989	0.926	0.999	1						
NR-GG-05	0.989	0.927	0.999	1.000	1					
NR-GG-06	0.989	0.938	0.988	0.990	0.988	1				
NR-GG-07	0.994	0.946	0.994	0.993	0.993	0.980	1			
NR-GG-08	0.995	0.952	0.992	0.992	0.992	0.979	1.000	1		
NR-GG-09	0.985	0.927	0.996	0.997	0.997	0.983	0.993	0.991	1	
Reference	0.993	0.983	0.992	0.996	0.991	0.982	0.994	0.990	0.993	1

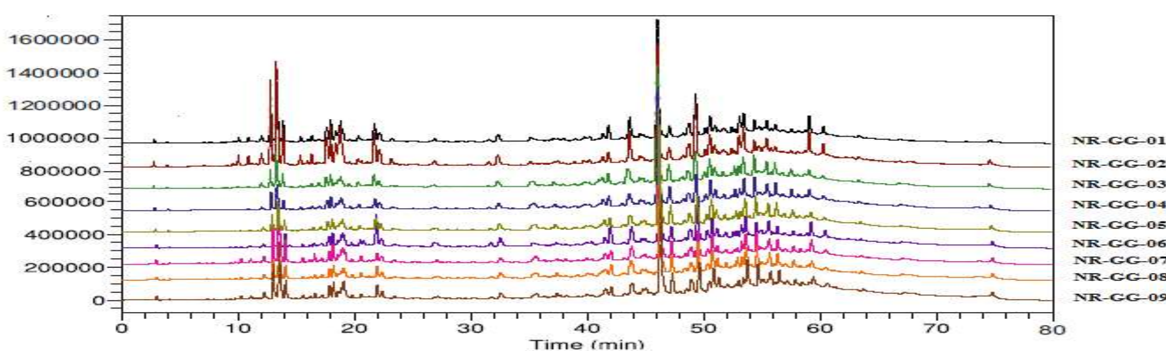

Fig.-1: HPLC fingerprint chromatograms of the nine batches of *Glycyrrhiza glabra* obtained at 280 nm

These 53 identified peaks were further categorized into individual classes of flavonoids such as nine peaks as flavones, fifteen peaks as flavanones, thirteen peaks as chalcones, one peak as isoflavene, seven peaks as isoflavones, six peaks as isoflavans, and two peaks as benzenoids. These flavonoids were found to be consistent in all the nine commercial batches of GutGard®.

Quantitative Determination of Characteristic Peaks in The Extract

Nine markers isolated in-house from “common peaks” were chosen for the quantitative determination of the individual flavonoids. The total flavonoid content of the extract was calculated by adding all the 53 peaks and calculating them as glabridin. HPLC profiles of *G. glabra* extract and standard substances recorded at 280 nm are displayed in Fig.-2a and 2b, respectively. Method validation parameters of the quantitative determination of nine flavonoids are given in Table-3. Out of the nine flavonoids, the content of glabridin was the highest (>3.5%) and isoliquiritigenin the least (~0.02%), in different batches of the extract analyzed. The total content of nine flavonoids in GutGard® ranges from 6.13% to 8.17% while the total flavonoids content in *G. glabra* ranges from 10.63% to 14.44%.

In-vitro Assays Details

ABTS and DPPH Radical Scavenging Assay Details

The results of the ABTS and DPPH methods for determining the free radical scavenging capacity of extracts and pure compounds are shown in Tables-4 and 5, respectively. The extract’s IC_{50} values in the ABTS and

DPPH assays were 17-28 µg/mL and 28-72 µg/mL, respectively when tested at a concentration between 5-200 µg/mL. In the ABTS assay, pure substances such as isoliquiritigenin, glabridin, 4ˈ-O-methylglabridin and hispaglabridin A showed positive findings, however only glabridin, and Hispaglabridin A responded in the DPPH assay at concentrations of 1.25-20 µg/mL.

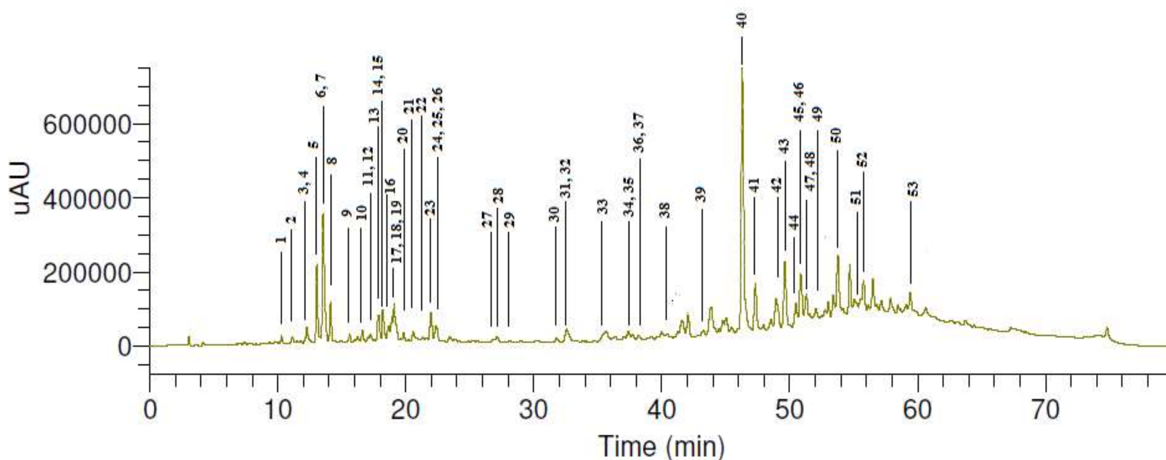

Fig.-2a: Chromatograms of the representative *Glycyrrhiza glabra* fingerprint

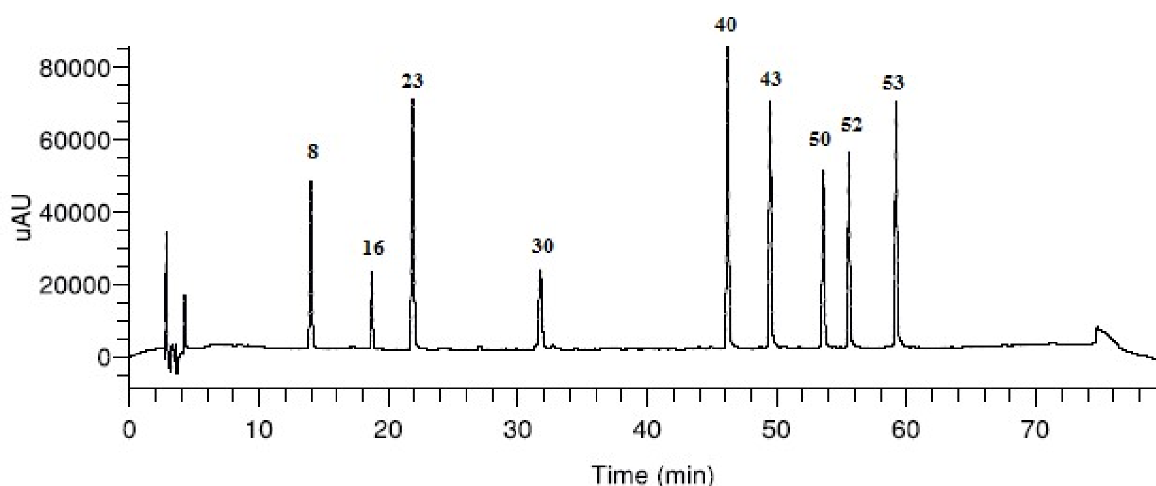

Fig.-2b: Chromatograms of standard mixture compounds including (8) liquiritin, (16) isoliquiritin, (23) liquiritigenin, (30) isoliquiritigenin, (40) glabridin, (43) glabrol, (50) 4ˈ-O-methylglabridin, (52) Hispaglabridin A and (53) Hispaglabridin B

Table-2: Characterization of 53 identified flavonoids in *Glycyrrhiza glabra* acetone extract by HPLC-PDA–ESI-MS/MS

Peak No.	RT (min.)	UV max. (nm)	Identification	Aglycone class	Mass of compound	$[M-H]^-$ (m/z)	MS^n ions	$[M+H]^+$(m/z)	MS^n ions	Reference
1.	10.44	230, 272, 334	Vicenin II	Flavone	594	593	461, 387, 353, 266, 165, 137	595	474, 371	26
2.	11.25	232, 272, 328	Schaftoside/ Isoschaftoside	Flavone	564	563	517, 472, 433, 383, 310, 266, 199, 165	565	445, 370, 206, 191, 109	27
3.	12.24	232, 253, 329	7-o-Apioglucosyl-7,4'-	Flavone	548	547	503, 451, 390, 298, 254, 174	549	520, 454, 396, 322, 238, 184	28

			dihydroxyflavone							
4.	12.41	231, 271, 335	Isoviolanthin	Flavone	578	577	565, 436, 397, 283, 228	579	566, 546, 483, 433, 365, 315, 226	27
5.	13.16	234, 269, 314	Naringenin-7-O-(2-β-D-apiofuranosyl)-β-D-glucopyranoside	Flavanone	550	549	429, 255, 135	551	418, 389, 257, 137	27
6.	13.42	237, 275, 311	Liquiritin Apioside	Flavanone	550	549	417, 255, 135	551	418, 257, 137	27
7.	13.66	233, 271, 313	Liquiritin Apioside isomer	Flavanone	550	549	417, 255, 135	551	418, 257, 137	27
8.	14.28	236, 276, 311	Liquiritin	Flavanone	418	417	255, 135, 119	419	257, 137	29
9.	15.77	231, 283, 327	6'-Hydroxy isoliquiritin apioside	Chalcone	566	565	271, 151, 119	567	445, 373, 273, 257	27
10.	16.74	231, 282, 324	Choerospondin	Flavanone	434	433	299, 271, 151	435	301, 273	29
11.	17.2	235, 279, 334	2',7-dihydroxy-4'-methoxyisoflavone	Isoflavene	270	-	-	271	257, 207, 143, 139	30
12.	17.37	219, 233, 255, 365	3,3',4,4'-Tetrahydroxy-2-methoxychalone	Chalcone	302	301	269, 161	303	273	26
13.	17.93	213, 236, 362	IsoliquiritinApioside	Chalcone	550	549	255, 135	551	257, 137	27
14.	18.09	223, 249, 295, 370	Formonetin-7-o-apiosyl-(1->2)-glucoside	Isoflavone	562	561	549, 445, 403, 329, 267, 171, 139	563	551,269, 167	31
15.	18.31	241, 297, 307, 369, 383	Licuraside	Chalcone	550	549	255, 135	551	257, 137	27
16.	18.87	234, 356	Isoliquiritin	Chalcone	418	417	255, 135	419	257	29
17.	19.2	235, 282, 313	Licorice glycoside D_2/D_1	Flavanone	696	695	255, 135	-	-	29
18.	19.31	223, 239, 249	Licorice glycoside C_2	Flavanone	726	725	255, 135	-	-	27
19.	19.42	248, 296, 371	Neoisoliquiritin	Chalcone	418	417	255, 135	-	-	27
20.	19.93	234, 330	4',7-dihydroxy flavone	Flavone	254	253	135, 117	-	-	32
21.	20.77	234, 357	Licochalcone B	Chalcone	286	285	270, 150	-	-	27

22.	21.5	237, 284, 326	Licorice glycoside E	Flavanone	693	692	685, 565, 515, 441, 369, 267, 187	-	-	29
23.	22.12	235, 275, 311	Liquiritigenin	Flavanone	256	255	153, 135, 119	-	-	27
24.	22.35	235, 324, 365	Licorice glycoside A	Chalcone	726	725	531, 255, 119	-	-	27
25.	22.52	216, 237, 284	Calycosin (3'-hydroxy formononetin)	Isoflavone	284	283	268, 255, 175	-	-	26
26.	22.68	238, 259, 309, 319	3', 4'-Dihydroxy-7-methoxyisoflavone	Isoflavone	284	283	268, 137	285	257, 122	27
27.	26.99	237, 269, 283, 359	Echinatin	Chalcone	270	269	237, 161	-	-	27
28.	27.38	235, 288, 327	Naringenin	Flavanone	272	271	151	-	-	27
29.	28.23	240, 265, 283, 324	Genkwanin	Flavone	284	283	268, 255, 239	-	-	33
30.	31.91	216, 241, 369	Isoliquiritigenin	Chalcone	256	255	153, 135, 119	-	-	27
31.	32.69	216, 242, 299	Formononetin	Isoflavone	268	267	252, 223,	-	-	27
32.	33.54	237, 262, 276	Isoformonetin	Isoflavone	268	267	252, 223,	-	-	27
33.	35.84	239, 276, 349	3,3',4,4'-tetrahydroxy-2'-methoxy-5-prenylchalcone	Chalcone	370	369	337, 311, 285, 191	371	339	34
34.	37.79	237, 281, 319	Kanzonol D	Flavone	322	321	266, 255, 235	323	271, 267, 203, 137, 123	27
35.	37.96	236, 261, 287, 328	Licoflavone A	Flavone	322	321	266, 177, 167	323	271, 137	27
36.	38.46	240, 265, 283, 342	Licochalcone D	Chalcone	354	353	339, 245, 165, 150	355	323, 219, 113	27
37.	38.52	240, 264, 332	Licoisoflavone A	Isoflavone	354	353	337, 323, 245, 201, 177	355	339, 323, 283, 187, 179, 149	27
38.	40.68	237, 276, 318	Abyssinone II	Flavanone	324	323	245, 187, 177	325	311, 269, 199, 137	35
39.	43.51	240, 261, 316	Licoisoflavone B	Isoflavone	352	351	283, 265	-	-	27
40.	46.43	240, 277	Glabridin	Isoflavan	324	323	201, 135	-	-	29
41.	47.09	216, 237, 280, 320	3-hydroxy glabrol	Flavanone	408	407	307, 231,203, 177, 163	-	-	36
42.	49.61	237, 291, 326	Licoflavone B	Flavone	390	389	187, 175	-	-	27
43.	49.8	238, 282	Glabrol	Flavanone	392	391	337, 325, 187	-	-	29

44.	50.41	236, 282	Glyinflanin A	Benzenoid	408	407	391, 325, 245, 229, 161	409	393, 337, 316, 189	29
45.	50.72	236, 282	Kanzonol Y	Chalcone	410	409	391, 235, 177, 147	411	392, 323, 265, 253, 175	37
46.	50.87	236, 279	Kanzonol Z	Flavanone	406	405	389, 321, 286, 185, 128	406	399, 323, 264, 184	38
47.	51.38	236, 281	Kanzonol H	Isoflavan	424	423	405, 393, 336, 287, 229, 193, 177, 128	-	-	36
48.	51.41	236, 280	Kanzonol X	Isoflavan	394	393	367, 308, 271, 215, 177	395	337, 321, 279, 260, 191, 184	36
49.	52.19	236, 281	Glyinflanin A isomer	Benzenoid	408	407	339, 311, 201, 135	409	316, 189	29
50.	53.82	236, 280, 320	4-O-Methyl glabridin	Isoflavan	338	337	201, 177		-	22
51.	55.63	236, 280	Lespedezaflav anone	flavanone	408		-	409	391, 389, 297, 167	35
52.	55.72	236, 280	Hispaglabridin A	Isoflavan	392	391	351, 339, 265, 201	393	371, 189, 135	22
53.	59.44	235, 280	Hispaglabridin B	Isoflavan	390	389	349, 333, 217, 201	391	375, 189, 147	22

Table-3: Validation parameters for nine reference analytes

Peak No.	Markers	Calibration curve	Linear range (µg/ml)	R2	Interday precision (RSD %)	Intraday precision (RSD %)	Recovery (%) (n=3)	Stability up to 48 hrs
8	Liquiritin	y = 180164x + 114082	2.10 to 135	0.9999	<2.5%	<2.5%	90-110%	Variation observed is <5.0%
16	Isoliquiritin	y = 75782x - 166816	1.62 to 104	0.9899				
23	Liquiritigenin	y = 298060x + 246621	2.04 to 131	0.9992				
30	Isoliquiritigenin	y = 78610x - 111574	2.04 to 131	0.9854				
40	Glabridin	y = 207950x - 25139	1.59 to 102	0.9998				
43	Glabrol	y = 73864x - 22309	1.80 to 115	0.9998				
50	4'-O-Methyl glabridin	y = 183188x - 61138	2.10 to 135	0.9999				
52	Hispaglabridin A	y = 93476x - 70901	1.76 to 113	0.9978				
53	Hispaglabridin B	y = 140891x - 28254	1.25 to 80	0.9988				

Gutgard's Effect on LPS Induced Nitric Oxide and IL-6 Production in J774A.1 Cells

The effect of Gutgard extracts on LPS-induced nitric oxide (NO) and interleukin-6 (IL-6) production was studied, and considerable concentration-dependent suppression of IL6 and NO release was reported at a concentration ranging from 2.5-40 µg/mL. IL6 and NO IC_{50} values for the extract were in the range of 17-43 µg/mL and 15-30 µg/mL, respectively. Pure compounds were also tested for the same at a concentration between 1.25-20 µg/mL and detailed results were captured in Tables-4 and 5, respectively.

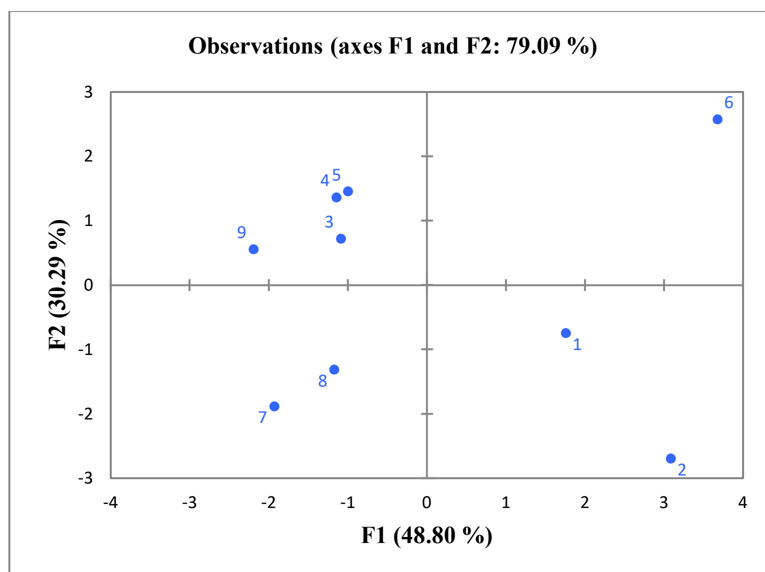

Fig.-3: PCA biplot of nine *Glycyrrhiza glabra* commercial batches with respect to nine reference analytes

Table-4: ABTS, DPPH, IL-6, and NO IC_{50} values of *Glycyrrhiza glabra* batches

	IC_{50} (μg/mL)			
Commercial Batches	ABTS Scavenging	DPPH Scavenging	IL-6 inhibition	NO Scavenging
NR-GG-01	21	71.7	40	30
NR-GG-02	28	28	43	20
NR-GG-03	20	51.04	20	17
NR-GG-04	17	41.24	20	15
NR-GG-05	19	35.21	19	15
NR-GG-06	18	51.69	19	15
NR-GG-07	19	57.17	17	16
NR-GG-08	25	49.05	34	15
NR-GG-09	22	48.05	24	17

Table-5: ABTS, DPPH, IL-6, and NO IC_{50} values of pure compounds isolated from *Glycyrrhiza glabra*

	IC_{50} (μg/mL)			
Commercial Batches	ABTS Scavenging	DPPH Scavenging	IL-6 inhibition	NO Scavenging
Liquiritin	NA	NA	10	>20
4'-O-Methylglabridin	14	NA	8	6
Isoliquiritigenin	6	NA	4	<1.25
Hispaglabridin B	NA	NA	NA	NA
Glabridin	5	19.97	6	4
Hispaglabridin A	4	108.27	NA	5
Glabrol	NA	NA	7	4
Liquiritigenin	NA	NA	NA	11
IsoLiquiritin	NA	NA	NA	1.3

NA- not active up to the tested concentration

CONCLUSION

In conclusion, the present phytochemical analysis with the aid of HPLC-PDA-ESIMS/MS identified 53 flavonoids in a flavonoid-rich extract of licorice. The nine different batches of the extract had a similar chromatographic pattern with all 53 flavonoid peaks being present in all the batches indicating qualitative phytochemical consistency. Quantitative analysis indicated that glabridin was the major flavonoid present in the extract while isoliquiritigenin was the lowest. PCA helped us to analyze the correlations between the

multiple variables, which enabled us to conclude the similarity among the various batches produced at different intervals. Biological assays revealed that the individual flavonoids have significantly different IC_{50} values indicating that each compound behaves differently in each assay. In other words, as expected, the biological activities of different flavonoids can vary. Thus, quantitative phytochemical differences of batches can contribute to the range of IC_{50} values seen with different batches. The IC_{50} values can be a good additional indicator of the consistency of commercial batches as the IC_{50} values of the total extract are contributed by multiple constituents of the extract, both known and the unknowns. Since it is virtually impossible to do a 100% chemical characterization of the extracts, an approach that combines both phytochemical and biological assays can be a better option than phytochemical assays alone. This comprehensive analytical approach can support the process of quality assurance of the plant extract in conjunction with controls over the quality of the herb and manufacturing process.

REFERENCES

1. D. J. McKenna, K. Jones, K. Hughes, Botanical Medicines: the desk reference for major herbal supplements, 2nd edition, Haworth Herbal Press, New York, 2002.; *Complimentary Therapies in Nursing and Midwifery*, **9(2)**, 99(2003), https://doi.org/10.1016/S1353-6117(03)00016-7
2. F. Borrelli, and A. A. Izzo, *Phytotherapy Research*, **14(8)**, 581(2000), https://doi.org/10.1002/1099-1573(200012)14:8%3C581::AID-PTR776%3E3.0.CO;2-S
3. P. A. Bafna, and R. Balaraman, *Phytomedicine*, **12(4)**, 264(2005), https://doi.org/10.1016/j.phymed.2003.12.009
4. M. T. Khayyal, M. A. el-Ghazaly, S. A. Kenawy, M. Seif-el-Nasr, L. G. Mahran, Y. A. Kafafi, and S. N. Okpanyi, *Arzneimittelforschung*, **51(7)**, 545(2001), https://doi.org/10.1055/s-0031-1300078
5. P. A. Belinky, M. Aviram, B. Fuhrman, M. Rosenblat, and J. Vaya, *Atherosclerosis*, **137 (1)**, 49(1998), https://doi.org/10.1016/S0021-9150(97)00251-7
6. T. Fukai, K. Satoh, T. Nomura, and H. Sakagami, *Fitoterapia*, **74(7)**, 624(2003), https://doi.org/10.1016/S0367-326X(03)00164-3
7. T. Fukai, K. Satoh, T. Nomura, and H. Sakagami, *Fitoterapia*, **74(7)**, 720(2003), https://doi.org/10.1016/j.fitote.2003.07.004
8. H. Haraguchi, N. Yoshida, H. Ishikawa, Y. Tamura, K. Mizutani, and T. Kinoshita, *Journal of Pharmacy Pharmacology*, **52(2)**, 219(2000), https://doi.org/10.1211/0022357001773724
9. K. Okada, Y. Tamura, M. Yamamoto, Y. Inoue, R. Takagaki, K. Takahashi, S. Demizu, K. Kajiyama, Y. Hiraga, and T. Kinoshita, *Chemical and Pharmaceutical Bulletin*, **37(9)**, 2528(1989), https://doi.org/10.1248/cpb.37.2528
10. J. Vaya, P. A. Belinky, and M. Aviram, *Free Radical Biology & Medicine*, **23(2)**, 302(1997), https://doi.org/10.1016/S0891-5849(97)00089-0
11. P. Montoro, M. Maldini, M. Russo, S. Postorino, S. Piacente, and C. Pizza, *Journal of Pharmaceutical and Biomedical Analysis*, **54(3)**, 535(2011), https://doi.org/10.1016/j.jpba.2010.10.004
12. V. Viswanathan, and A. P. Mukne, *Journal of AOAC International*, **99(2)**, 374(2016), https://doi.org/10.5740/jaoacint.15-0239
13. C. J. Ma, G. S. Li, D. L. Zhang, K. Liu, and X. Fan, *Journal of Chromatography A*, **1078(1)**, 188(2005), https://doi.org/10.1016/j.chroma.2005.01.053
14. S. S. Wei, M. Yang, X. Chen, Q. R. Wang, and Y. J. Cui, *Chinese Journal of Natural Medicines*, **13(3)**, 232(2015), https://doi.org/10.1016/S1875-5364(15)30009-1
15. M. T. Ren, J. Chen, Y. Song, L. S. Sheng, P. Li, and L. W. Qi. *Journal of Pharmaceutical and Biomedical Analysis*, **48(5)**, 1351(2008), https://doi.org/10.1016/j.jpba.2008.09.037
16. O. V. Astafeva, and L. T. Sukhenko, *Bulletin of Experimental Biology and Medicine*, **156**, 829(2014), https://doi.org/10.1007/s10517-014-2462-8
17. R. Tilton, A. A. Paiva, J. Q. Guan, R. Marathe, Z. Jiang, W. V. Eyndhoven, J. Bjoraker, Z. Prusoff, H. Wang, S. H. Liu, and Y. C. Cheng, *Chinese Medicine*, **5**, 30(2010), https://doi.org/10.1186/1749-8546-5-30
18. R. B. V. Breemen, *Journal of Medicinal and Pharmaceutical Chemistry*, **58(21)**, 8360(2015), https://doi.org/10.1021/acs.jmedchem.5b00417

19. D. Prashanth, A. Amit, S. Yogisha, and R. Padmaja, *Journal of Natural Remedies*, **3(2)**, 166(2003), https://doi.org/10.18311/jnr/2003/158
20. R. Tanemoto, T. Okuyama, H. Matsuo, T. Okumura, Y. Ikeya, and M. Nishizawa, *Biochemistry and Biophysics Reports*, **2**, 153(2015), https://doi.org/10.1016/j.bbrep.2015.06.004
21. T. Saitoh, T. Kinoshita, and S. Shibata, *Chemical and Pharmaceutical Bulletin*, **24(4)**, 752(1976), https://doi.org/10.1248/cpb.24.752
22. T. Kinoshita, K. Kajiyama, Y. Hiraga, K. Takahashi, Y. Tamura, and K. Mizutani, *Heterocycles*, **43(3)**, 653(1996), https://doi.org/10.3987/COM-95-7296
23. C. V. Chandrasekaran, H. B. Deepak, P. Thiyagarajan, S. Kathiresan, G. K. Sangli, M. Deepak, and A. Agarwal, *Phytomedicine*, **18(4)**, 278(2011), https://doi.org/10.1016/j.phymed.2010.08.001
24. B. Auddy, M. Ferreira, F. Blasina, L. Lafon, F. Arredondo, F. Dajas, P. C. Tripathi, T. Seal, and B. Mukherjee, *Journal of Ethnopharmacology*, **84(2)**, 131(2003), https://doi.org/10.1016/S0378-8741(02)00322-7
25. C. V. Chandrasekaran, A. Gupta, and A. Agarwal, *Journal of Ethnopharmacology*, **129(2)**, 203(2010), https://doi.org/10.1016/j.jep.2010.03.007
26. Y. Wang, L. Yang, Y. Q. He, C. H. Wang, E. W. Welbeck, S. W. A. Bligh, and Z. T. Wang, *Rapid Communications in Mass Spectrometry*, **22**, 1767(2008), https://doi.org/10.1002/rcm.3536
27. T. Xu, M. Yang, Y. Li, X. Chen, Q. Wang, W. Deng, X. Pang, K. Yu, B. Jiang, S. Guan, and D. A. Guo, *Rapid Communications in Mass Spectrometry*, **27**, 2297(2013), https://doi.org/10.1002/rcm.6696
28. I. Kitagawa, W. Z. Chen, K. Hori, M. Kobayashi, and J. Ren, *Chemical and Pharmaceutical Bulletin*, **46(10)**, 1511(1998), https://doi.org/10.1248/cpb.46.1511
29. M. A. Farag, A. Porzel, and L. A. Wessjohann, *Phytochemistry*, **76**, 60(2012), https://doi.org/10.1016/j.phytochem.2011.12.010
30. K. Kajiyama, Y. Hiraga, K. Takahashi, S. Hirata, S. Kobayashi, U. Sankawa, and T. Kinoshita, *Biochemical Systematics and Ecology*, **21(8)**, 785(1993), https://doi.org/10.1016/0305-1978(93)90090-E
31. Lipidomics gateway structure Database: LIPID MAPS, 2016. Available at http://www.lipidmaps.org/data/structure/LMSDSearch.php?Mode=ProcessClassSearch&LMID=LMPK1205, Accessed 15 November 2016.
32. R. Kupfer, L. Swanson, S. Chow, R. E. Staub, Y. L. Zhang, I. Cohen, and U. Christians, *Drug Metabolism and Disposition*, **36(11)**, 2261(2008), https://doi.org/10.1124/dmd.108.021402
33. N. Fabre, I. Rustan, E. D. Hoffmann, and J. Q. Leclercq, *Journal of the American Society for Mass Spectrometry*, **12(6)**, 707(2001), https://doi.org/10.1016/S1044-0305(01)00226-4
34. M. Kuroda, Y. Mimaki, S. Honda, H. Tanaka, S. Yokota, and T. Mae, *Bioorganic & Medicinal Chemistry*, **18(2)**, 962(2010), https://doi.org/10.1016/j.bmc.2009.11.027
35. S. Fang, Q. Qu, Y. Zheng, H. Zhong, C. Shan, F. Wang, C. Li, and G. Peng, *Journal of Separation Science*, **39(11)**, 2068(2016), https://doi.org/10.1002/jssc.201600073
36. Y. J Li, J. Chen, Y. Li, Q. Li, Y. F. Zheng, Y. Fu, and P. Li, *Journal of Chromatography A*, **1218(45)**, 8181(2011), https://doi.org/10.1016/j.chroma.2011.09.030
37. T. Fukai, C. B. Sheng, T. Horikoshi, and T. Nomura, *Phytochemistry*, **43(5)**, 1119(1996), https://doi.org/10.1016/S0031-9422(96)00391-3
38. T. Fukai, C. B. Sheng, K. Maruno, Y. Miyakawa, M. Konishi, and T. Nomura, *Phytochemistry*, **49(7)**, 2005(1998), https://doi.org/10.1016/S0031-9422(98)00389-6

[RJC-6831/2021]