Austin Journal of Plant Biology

Open Access

Austin Publishing Group

Research Article

Chemotaxonomic Significance of Flavonoids in Some Species of *Galium* (Rubiaceae) from Libya

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Received: July 22, 2016; **Accepted:** September 06, 2016; **Published:** September 12, 2016

Abstract

The flavonoid profiles of five Libyan *Galium* L. (Rubiaceae) species collected from different localities and habitats were investigated. Fourteen flavonoid compounds were isolated and identified using the direct comparison of chromatographic and UV spectral analyses with standard samples. The flavone compounds were identified as apigenin (1) and its 7-glycoside (2); luteolin 7-diglycoside (3), diosmetin (4) and its 7-monoglycoside (5), as well as its 7-diglycoside (6). In addition, the detected flavonol compounds were kaempferol 3-glycoside (7), 3-diglycoside (8) and 3,7-diglycoside (9); quercetin (10) and its 3-glycoside (11), 3-rutinoside (12), 3,7-diglycoside (13), and 3-diglycoside-7-glycoside (14). The chemotaxonomic relationships of the studied species of *Galium* and their significance were also discussed.

Keywords: Chemosystematics; *Galium*; Rubiaceae; Flavonoid glycosides; biochemistry; Multivariate analysis

Introduction

Galium L. is one of the largest genera of Rubieae (Rubiaceae) with more than 400 species included into 16 sections containing annual and perennial herb that are distributed in temperate and tropical regions of the world [1,2]. Certain species of *Galium* are found even in the Arctic zone or high elevations on tropical mountain ranges. *Galium* itself is problematic taxonomically, because taxa from different sections exhibit similar habit, many species are widely distributed and polymorphic, and species groups often are poorly differentiated both morphologically and geographically [3].

The flavonoid chemistry of *Galium* have been studied, and reported to contain predominantly flavones and flavonol aglycones and their glycosides. Earlier study by [4] isolated rutin and a mixture of flavonoids and diosmetin from *Galium palustre* L. Also, the glycosides of quercetin, luteolin, apigenin and kaempferol were isolated from *Galium aparine* [5,6]. Reported that *Galium* has long been known to contain substantial amounts of anthraquinones, with the roots being especially rich sources of these secondary metabolites. Bedstraw species, including *G. mollugo*, contain mollugin [7], flavonoids [8], coumarins, phenolic acids, and iridoid glucosides [9]. Recently, two new flavonoids; diosmetin glycoside and biflavone, were isolated and identified in *G. verum* L., in addition to isorhamnetin and its glycosides, kaempferol, quercetin, diosmetin and its glycosides [10,11].

Bedstraw species, including *G. mollugo*, also contain mollugin [7], flavonoids [8], coumarins, phenolic acids, and iridoid glucosides [12,13]. Some of these compounds have allelopathic, fungistatic, or repellent effects, and may also be used to flavour food or wine [14]. Recently, extracts from this plant were evaluated for their anti-cancer and anti-malarial activities and for their ability to inhibit HIV–1 reverse transcriptase, but initial results showed no activity [15].

The flora of Libya is not rich in the number of species; however, the Al-Jabal Al-Akhdar Mountain landscape comprises the richest vegetation and the highest number of species known from Libya [16]. The geographical affinity of the flora is mainly East Mediterranean rather than neighboring regions of North Africa [17,18].

In Libya, Rubiaceae is one of the eight species-rich families which represented by 50 genera and 90 species [19]. The genus *Galium* is represented by 10 species; *G. mollugo L., G. spurium L., G. aparine L., G. tricornutum Dandy, G. verrucosum Huds., G. parisiense L., G. setaceum Lam., G. recurvum* Req. ex DC., *G. cossonianum* Jafri, and *G. murale* (L.) All.

As from the beginning of 1990s, the increasing interest of molecular approach in taxonomic studies, especially those based on nucleic acids sequences, a remarkable decrease in number and importance of investigations dealing with chemotaxonomic evidence. Therefore, some authors urged this trend, and recommended research work on non-molecular evidences [20].

There is often confusion between different samples of this genus in herbariums and so the aim of this study is to develop a method to find characters providing data of both taxonomical and pharmaceutical use. No phytochemical studies were reported so far from *Galium* species in North African countries. The present work aims to establish foliar flavonoid patterns of five *Galium* species from Libya, in an attempt to determine chemical affinities among species and compare the results obtained with affinities indicated by evidences from morphology.

Materials and Methods

Plant material

Table 1 shows locations, types of habitats, dates of collection, GPS coordinates of localities, elevation and number of populations that were examined for the selected 5 species of *Galium*. Aerial parts of all plants were collected from natural populations. For each species at least two populations from distant habitats were studied. Altogether,

Citation: Moubasher H, Abd El-Ghani M, Al-Wakeel S and Bahoor A. Chemotaxonomic Significance of Flavonoids in Some Species of *Galium* (Rubiaceae) from Libya. Austin J Plant Biol. 2016; 2(1): 1014.

Table 1: List of studied species with binomials, habitats, localities, collection dates and GPS coordinates of localities. Figures between parentheses refer to the population numbers. NA=Not Available.

Species	Localities	Habitats	Elevation (m ASL)	GPS coordinates
Galium aparine L.	Gasr Libya (S4)	Mountainous areas	280.7	32° 37'N, 21° 23'E
	Shahat Ruins (S1)	Mountainous areas	560.2	32° 50'N, 21° 55'E
	Wadi Qaam (S12)	Coastal wadis	20.7	32° 28'N, 14° 25'E
G. murale L.	Wadi Elkouf (S5)	Inland desert wadis	264.6	32° 41'N, 21° 33'E
	Sharshara (S8)	Canal banks	327.4	32° 27'N, 13° 37'E
G. setaceum Lam.	Wadi Derna (S6)	Inland desert wadis	85.3	32° 42'N, 22° 36' E
G.tricornutum Dandy	Lamluda (S2)	Farmlands barley fields	668.1	32° 44'N, 22° 06'E
	Almansoura (S3)	Farmlands	NA	32° 50'N, 21° 55'E
	Stwa (S7)	Farmlands	566.9	32° 49'N, 22° 09'E
G. verrucosum Huds.	Wadi Qaam (S11)	Coastal wadis	20.7	32° 28'N, 14° 25'E
	Almansoura (S9)	Farmlands	NA	32° 50'N, 21° 55'E
	Wadi Derna (S10)	Inland desert wadis	85.3	32° 42'N, 22° 36' E

80 specimens were collected from their natural habitats in different locations of Libya. Voucher specimens of the studied species were deposited at the herbarium of Cairo University (CAI).

Biochemical procedures

The whole plant of *Galium* was dried in the shade and ground. The powder was extracted with 70% MeOH three times at room temperature and evaporated under reduced pressure. The aqueous layer was stored in a refrigerator until needed to test the presence of flavonoids in different samples.

The separation of flavonoid mixture was detected by spotting all the flavonoid extracts using one-dimensional paper chromatography on Whatman No. 3mm along with the standard samples, water, 15% acetic acid (acetic acid: water; 15:85) and BAW (n-butanol: acetic acid: water; 4: 1: 5) upper phase as solvent systems. After drying the paper chromatograms, the developed spots were examined under UV light at a wave length of 365nm using an Ultraviolet Lamp (Model, UV GL-25) then in the presence of ammonia fumes.

The qualitative analysis of the crude plant extracts containing complex mixture of flavonoid compounds was carried out on Whatman No. 3mm chromatographic paper, using the solvent system BAW in the first run and 15% acetic acid in the second. The chromatograms should be dried in the hood between the first and the second chromatographic runs. All the flavonoid spots on the dried chromatograms were detected under UV light with and without the presence of ammonia.

The separation of a flavonoid mixture was achieved by elution techniques using one-dimensional paper chromatography on Whatman No. 3mm. The used solvent system in purification of flavonoid mixture was selected after preliminary one-dimensional runs in different systems to observe which has effectively separated the mixture. The used solvent systems were BAW (n-butanol: acetic acid: water; 4: 1: 5) upper phase; 15% acetic acid (acetic acid: water; 15:85) and water. The crude plant extract was applied as a band at 10cm from the top of the chromatograms and developed in the selected solvent. UV–detectable bands observed on dried chromatograms with and without exposure to ammonia fumes were cut out and eluted with 95% methanol. Each methanolic extract was concentrated by evaporation on rotary evaporator. Additional purification of the isolated flavonoids was carried out until a pure flavonoid compound was obtained.

Ultra Violet (UV) spectral analyses were performed according to standard procedures performed by Mabry et al. [21] and Mabry and Markham [21].

The diagnostic reagents used for the UV spectral measurements of the isolated flavonoid compounds were: Sodium Methoxide (NaOMe), Aluminium Chloride ($AlCl_3$), Hydrochloric Acid (HCl), Sodium Acetate (NaOAc), and Boric Acid (H_3BO_3). The absorption spectra were measured in methanolic solution against methanolic blank using automatic recording Spectrophotometer (UV–3101 PC, UV-VIS-NIR) scanning Spectrophotometer using standard Quartz cuvettes of 1cm path length. A fresh stock solution of 0.1mg pure flavonoid in about 10ml spectral methanol was prepared and adjusted on the spectrophotometer so that the major absorption peaks between 240 and 460nm. Then, the following steps were carried out:

1. The MeOH spectrum was recorded at normal scan speed, using 2-3ml of the fresh stock solution.

2. The NaOMe spectrum was recorded immediately after addition of 3 drops of NaOMe stock reagent to the solution used for step (1). After 5 minutes, the spectrum was rerun to check for any decomposition in the compound, this solution was discarded,

3. The $AlCl_3$ spectrum was recorded immediately after the addition of 6 drops of $AlCl_3$ to 2-3ml of the fresh stock methanol solution,

4. The $AlCl_3$/HCl spectrum was recorded immediately after addition of 3 drops of stock HCl reagent to the cuvette containing $AlCl_3$ used for step (3), the solution was then discarded,

5. The NaOAc spectrum of the flavonoid was determined by adding enough anhydrous NaOAc to the cuvette containing 2-3ml fresh stock solution of the flavonoid. After shaking, recording out within two minutes and then after 5-10 minutes to check for any decomposition of the compound,

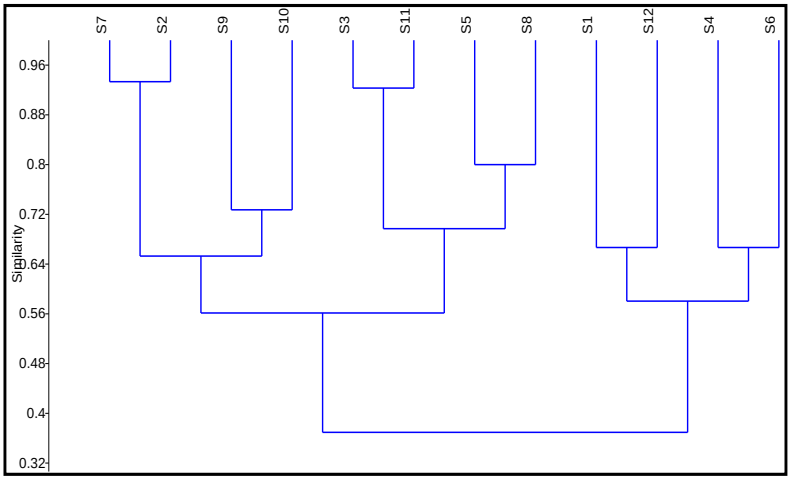
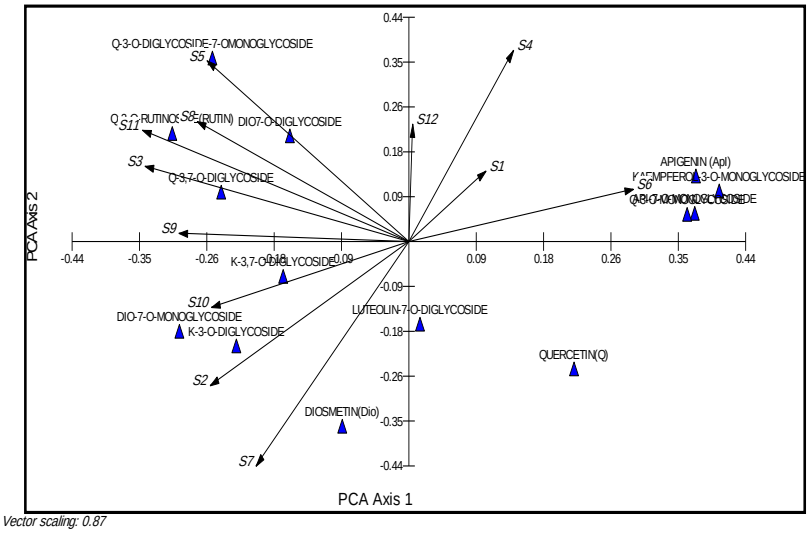


Figure 1: Dendrogram of *Galium* sp. based on flavonoid distributions and UPGMA clustering method. A, B and C are the three separated groups. For species abbreviations, see (Table 1).



Vector scaling: 0.87

Figure 2: PCA scatter biplot showing the relations between the separated compounds and the studied species (S1-S10). A, B and C are the separated groups in (Figure 1).

6. The NaOAc/H_3BO_3 spectrum was recorded after addition of anhydrous H_3BO_3 to the cuvette which contained NaOAc used for step (5).

Multivariate analysis, using the species analyzed as Operational Taxonomic Units (OUT) and the flavonoids as characters, was carried out with PAST ver. 2.11 [22]. The resulting UPGMA dendrogram is shown in (Figure 1). Principal Components Analysis (PCA) was performed sing MVSP ver. 3.1 [23] and the resulting biplot is shown in (Figure 2).

Results and Discussion

According to recent modifications to the classification of *Galium* [23], the 5 studied species are included in two sections: Sect. Kolgyda (*Galium* murale L., G. tricornutum Dandy, G. aparine L. and G. verrucosum Huds.) and Sect. Jubo*galium* (G. setaceum Lam.).

Fourteen flavonoid compounds were detected in the five *Galium* species collected from different locations of Libya. Identification of the isolated flavonoids was mainly based on the direct comparison of chromatographic and UV spectral studies with standard samples. The present data clearly established that the investigated species have a simple and highly substituted flavone and flavonol compounds including flavonoid, aglycones, glycosides and methylated compounds. The isolated flavonoids based on six flavones and eight flavonols. The flavone compounds identified were apigenin (1) and its 7-glycoside (2), luteolin 7-diglycoside (3), diosmetin (4) and its 7-monoglycoside (5), as well as its 7-diglycoside (6). In addition, the detected flavonol compounds were kaempferol 3-glycoside (7), 3-diglycoside (8) and 3,7-diglycoside, (9) quercetin, (10) and its 3-glycoside (11), 3-rutinoside (12), 3,7-diglycoside (13), and 3-diglycoside-7-glycoside (14).

Identification of the isolated flavonoids

Paper chromatography: The colour reaction of the aglycone

Table 2: Paper chromatographic data of the isolated flavonoid compounds. Y=yellow; P=purple; dp=dull purple; YG=yellow green; britY=bright yellow.

No.	Compounds	R_f value ×100		Colour reaction	
		BAW	15% acetic acid	UV	UV/NH_3
1	Apigenin	92	2	P	P
2	Apigenin-7-O-glycoside	47	27	P	YG
3	Luteolin-7-O-diglycoside	33	29	P	Y
4	Luteolin-4`-O-Me (Diosmetin)	76	4	P	dP
5	Diosmetin-7-O-glycoside	34	40	P	dP
6	Diosmetin-7-O-diglycoside	20	63	P	dP
7	Kaempferol-3-O-glycoside	57	35	P	Y
8	Kaempferol-3-O-diglycoside	35	60	P	Y
9	Kaempferol-3,7-O-diglycoside	25	70	P	Y
10	Quercetin	69	3	Y	brit Y
11	Quercetin-3-O-glycoside	56	35	P	Y
12	Quercetin-3-O-rutinoside (Rutin)	33	46	P	Y
13	Quercetin-3,7-O-diglycoside	24	72	P	Y
14	Quercetin-3-O-diglycoside-7-O-glycoside	16	81	P	YG

Table 3: UV spectral data of isolated flavonoid compounds (λ_{max}, nm); sh=shoulder.

Compound	MeOH	NaOMe	$AlCl_3$	$AlCl_3$/HCl	NaOAc	NaOAc/H_3BO_3
(1) Apigenin	336	392	382	381	340	337
	268	325	345	342	302 sh	269
	. . .	275	301	300	269	. . .
	. . .	. . .	277	278	. . .	. . .
(2) Apigenin-7-O-glycoside	331	380	381	380	385	337
	288 sh	348 sh	347	340	352 sh	265
	265	302	296	295	265	. . .
	. . .	270	272	274	. . .	. . .
(3) Luteolin-7-O-diglycoside	347	392	428	386	407	369
	265 sh	263	328	357	365 sh	260
	250	. . .	296 sh	295 sh	265	253 sh
	. . .	. . .	275	272	257 sh	. . .
(4) Luteolin-4`-O-methyl (Diosmetin)	344	382	384	355	343	342
	289	270	360	295	271	270
	270	. . .	295	276	. . .	. . .
	. . .	. . .	273	. . .	. . .	. . .
(5) Diosmetin-7-O-glycoside	343	372	382	383	340	342
	265	300 sh	360	355	264 sh	264 sh
	250	266	292 sh	293 sh	255	254 sh
	. . .	. . .	272	269	. . .	. . .
(6) Diosmetin-7-O-diglycoside	343	370	381	383	340	342
	267	300 sh	360 sh	360 sh	282 sh	264 sh
	251	266	293 sh	293 sh	264 sh	253
	. . .	. . .	270	269	253	. . .
(7) Kaempferol-3-O-glycoside	352	403	397	395	372	352
	295 sh	326	350	346	302	295
	265	274	304	303	275	265
	. . .	. . .	274	274	. . .	. . .

(8) Kaempferol-3-O-diglycoside	352	406	403	400	393	352
	320 sh	325	358	346	312	320 sh
	267	278	304	302	277	295 sh
	. . .	. . .	276	275	. . .	266
(9) Kaempferol-3,7-O-diglycoside	352	392	394	394	400	352
	315 sh	350 sh	353	348	358 sh	300 sh
	263	300 sh	300 sh	300 sh	265	268
	. . .	268	270	270	. . .	. . .
(10) Quercetin	372	419	452	430	373	386
	306 sh	331	337	362	256	260
	256	. . .	271	267	. . .	. . .
(11) Quercetin-3-O-glycoside	357	405	428	400	390	377
	295	326	301 sh	358	271	288 sh
	266 sh	267	273	29 sh	. . .	260
	255	. . .	. . .	269	. . .	. . .
(12) Quercetin-3-O-rutinoside (Rutin)	358	417	429	400	363	380
	306 sh	328	275	364	267	263
	259	273	. . .	299	. . .	. . .
	. . .	. . .	. . .	270	. . .	. . .
(13) Quercetin-3,7-O-diglycoside	354	430	433	400	437	380
	266 sh	277	300 sh	356	385 sh	282 sh
	255	. . .	273	298	262	265
	. . .	. . .	. . .	268	. . .	. . .
(14) Quercetin-3-O-diglycoside-7-O-glycoside	356	410	438	400	418	375
	295 sh	269	330 sh	364 sh	375 sh	262
	268 sh	. . .	298 sh	298 sh	297 sh	. . .
	258	. . .	273	268	263	. . .

(apigenin) appeared as purple spot on 1D and 2D paper chromatogram under UV light and with ammonia fumes, whereas the aglycone: quercetin showed yellow spot with UV light, that changed to bright yellow when fumed with ammonia (Table 2). The methylation of C-4` position with and without glycosidation at C-7 appeared as purple spots on paper chromatogram upon exposure to UV light and after fumed with ammonia. Additionally, most of the other glycosides appeared as purple spots with UV light and changed to yellow with ammonia fumes.

The characteristic Rf values of each compound on 1D paper chromatograms in different solvent systems (Table 2) indicated the position of glycosides attachment. The mobility of a glycoside is closely correlated with the number and position of sugar substitution. Increasing glycosylation at C-3 position decreased Rf value in BAW and increased the mobility in 15% acetic acid relative to its aglycone. The 3,7-diglycosidation behave like the 3-glycosidation, but moved very slower in BAW and faster in 15% acetic acid. In addition, increasing glycosidation at C-7 position with methylated C-4` of luteolin showed faster movement in 15% acetic acid and lower Rf value in BAW relative to its aglycone (diosmetin).

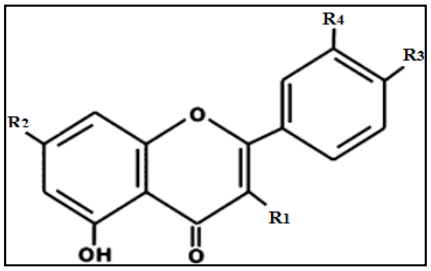
Ultra Violet spectra (UV): The UV spectral data of isolated flavonoid compounds are shown in (Table 3). In addition, the chemical structures of the isolated flavone and flavonol compounds are illustrated in (Table 4).

Distribution patterns of the isolated flavonoids

The qualitative and quantitative analyses of the 14 flavonoids among the five *Galium* species that collected from different locations of Libya are represented in (Table 5). The flavonoid patterns between the *Galium* species showed a predominant flavonol glycoside being quercetin-3-rutinoside (12). However, the other compounds were variable in quality, quantity, and in their distribution among the investigated samples.

In addition to the main flavonol compound (12), there are three other major flavonoids in *Galium* setaceum collected from Wadi Derna identified as apigenin-7-glycoside (2), kaempferol-3-glycoside (7) and quercetin-3-glycoside (11), accompanied by less major amount of apigenin (1) and quercetin (10), whereas kaempferol-3,7-diglycoside (9) present in trace amount. *Galium* aparine was represented by three samples collected from various locations of Libya showed resemble flavonoids pattern, but qualitatively different. These samples have a major quercetin-3-rutinoside (12), along with trace amount of quercetin-3-diglycoside-7-glycoside (14). Additionally, sample collected from Gasr Libya contained major amount of kaempferol-3-glycoside (7) with less major amount of apigenin (1)

Table 4: Chemical structure of isolated flavonoids from the five investigated *Galium* species.



No.	Compound	R_1	R_2	R_3	R_4
1	Apigenin	H	OH	OH	H
2	Apigenin-7-O-glycoside	H	O-monoglycosyl	OH	H
3	Luteolin-7-O-diglycoside	H	O-diglycosyl	OH	OH
4	Luteolin-4`-O-Me (Diosmetin)	H	OH	O-CH_3	OH
5	Diosmetin-7-O-glycoside	H	O-monoglycosyl	O-CH_3	OH
6	Diosmetin-7-O-diglycoside	H	O-diglycosyl	O-CH_3	OH
7	Kaempferol-3-O-glycoside	O-monoglycosyl	OH	OH	H
8	Kaempferol-3-O-diglycoside	O-diglycosyl	OH	OH	H
9	Kaempferol-3,7-O-diglycoside	O-monoglycosyl	O-monoglycosyl	OH	H
10	Quercetin	OH	OH	OH	OH
11	Quercetin-3-O-glycoside	O-monoglycosyl	OH	OH	OH
12	Quercetin-3-O-rutinoside (Rutin)	O-rhamnoglucosyl	OH	OH	OH
13	Quercetin-3,7-O-diglycoside	O-monoglycosyl	O-monoglycosyl	OH	OH
14	Quercetin-3-O-diglycoside-7-O-glycoside	O-diglycosyl	O-monoglycosyl	OH	OH

and its 7-glycoside (2). However, G. aparine was collected from Shahat Ruins showed less major amounts of luteolin-7-diglycoside (3), kaempferol-3-glycoside (7) and quercetin-3-glycoside (11), along with trace amount of quercetin (10). On the other hand, apigenin (1), luteolin-7-diglycoside (3) and quercetin-3-glycoside (11) were isolated from G. aparine that collected from Wadi qaam (khoms) in less major amount, but kaempferol-3,7-diglycoside (9) is present in trace value.

With respect to *Galium* tricornutum, qualitative and quantitative variations of flavonoid profiles characterized by the presence of luteolin-7-diglycoside (3), diosmetin (4) and its 7-glycoside (5) and 7-diglycoside (6) with complete absence of apigenin (1) and its 7-glycoside (2), as well as the 3-glycoside of both kaempferol (7) and quercetin (11). Sample collected from Stwa afforded seven flavonoids including major amount of compounds (3), (4) and (12), along with lesser content of glycosides (8) and (9), while the flavonoids (5) and (10) detected in trace amount. There are two major flavonoids: (3) and (12) in G. tricornutum collected from Lamluda, in addition to less major compounds of (4), (5), (9) and (14) along with trace level of compounds (8) and (10). On the other hand, the major flavonoids isolated from G. tricornutum collected from Almansoura are diosmetin-7-glycoside (5) and quercetin-3-rutinoside (12), along with less major amount of flavone derivatives (3) and (6), as well as flavonol glycosides (8), (13) and (14). *Galium* verrucosum and G. murale showed a nearly similar pattern of flavonoid compounds, particularly the methylated luteolin (diosmetin) (4), its 7-glycoside (5) and 7-diglycoside (6); with complete absence of the aglycones and simple substituted compounds including apigenin (1) and its 7-glycoside (2); luteolin-7-diglycoside (3); kaempferol-3-glycoside (7); quercetin (10) and its 3-glycoside (11). The flavonoid patterns of G. verrucosum collected from Almansoura characterized by the presence of higher amount of compounds (4), (9) and (12), accompanied by lesser content of compounds (8) and (14), as well as trace amount of flavonoid (13). In addition, sample collected from Wadi Derna contained higher levels of compound (4) and (12), with less major content of compounds (5) and (9) and trace level of flavonoid (13). On the other hand, G. verrucosum collected from Wadi qaam (khoms) has major amounts of flavonoid (5), (12) and (14), along with less major amount of compounds (6) and (13), while compound (8) present in trace level.

The two samples of *Galium* murale collected from Wadi Elkouf and Sharshara (Tarhuna) contained nearly similar flavonoid patterns with respect to the predominant of diosmetin-7-diglycoside (6), quercetin-3-rutinoside (12) and quercetin-3-diglycoside-7-glycoside (14), along with less major amount of compound (9). However, the quercetin-3,7-diglycoside (13) was isolated only in the first sample that replaced by diosmetin-7-glycoside (5) in the second sample only.

Multivariate analyses: Morphologically similar and taxonomically closer species have similar even same flavonoid profiles. So, they clustered together in the dendrogram. Three groups of species can be separated in the dendrogram (Figure 1). The PCA biplot in (Figure 2) showed that each group is correlated to more than one compound.

Chemotaxonomic significance: This study confirmed that flavonoid content is a useful marker in the taxonomy of *Galium*. Kaempferol-3,7-O-diglycoside and Quercetin-3-O-rutinoside (Rutin) are found in all species. The isolated and structure elucidation of flavonoid compounds afford the presence of simple and relatively complex flavonoids identified among the five *Galium* species. The flavonoid complexity appeared in the substitution patterns of flavone and flavonol aglycones with O-glycosylated and O-methylated attachments. Generally, the flavonoid chemistry in all studied *Galium* species appears to be relatively homogeneous based on the predominance of flavonol glycoside, particularly quercetin-3-rutinoside (12).

The first group (group C) included *Galium* setaceum and G. aparine, which are characterized by simple O-glycoside of apigenin, kaempferol and quercetin. However, the detection of luteolin-7-diglycoside (3) along with trace amount of complex O-glycosylated quercetin (14) only in *Galium* aparine and their lacking from G. setaceum indicates the more specialized of the former species relative to the second one. *Galium* verrucosum and G. murale represent the second group (group B), which was encountered to produce methylated flavone: diosmetin (4) and its glycosidic attachments (5) and (6), along with the presence of complex O-glycosylated kaempferol and quercetin, particularly a C-3 and C-7 positions (9 and 14). In addition, the frequent lacking of simple aglycones of apigenin

Table 5: The distribution of flavonoids in *Galium* species collected from different localities of libya. For localities of *Galium* species.

Compound	*G. setaceum*	*G. aparine*			*G. tricornutum*			*G. verrucosum*			*G. murale*	
	S6	S4	S1	S12	S7	S2	S3	S9	S10	S11	S5	S8
1 - Apigenin	+	+	-	+	-	-	-	-	-	-	-	-
2 - Apigenin-7-O-glycoside	++	+	-	-	-	-	-	-	-	-	-	-
3 - Luteolin-7-O-diglycoside	-	-	+	+	++	++	+	-	-	-	-	-
4 - Luteolin-4`-O-Me (Diosmetin)	-	-	-	-	++	+	-	++	++	-	-	-
5 - Diosmetin-7-O-glycoside	-	-	-	-	t	+	++	-	+	++	-	+
6 - Diosmetin-7-O-diglycoside	-	-	-	-	-	-	+	-	-	+	++	++
7 - Kaempferol-3-O-glycoside	++	++	+	-	-	-	-	-	-	-	-	-
8 - Kaempferol-3-O-diglycoside	-	-	-	-	+	t	+	+	-	t	-	-
9 - Kaempferol-3,7-O-diglycoside	T	-	-	t	+	+	-	++	+	-	+	+
10 - Quercetin (Q)	+	-	t	-	t	t	-	-	-	-	-	-
11 - Q-3-O-glycoside	++	-	+	+	-	-	-	-	-	-	-	-
12 - Q-3-O-rutinoside (Rutin)	++	++	++	++	++	++	++	++	++	++	++	++
13 - Q-3,7-O-diglycoside	-	-	-	-	-	-	+	T	t	+	+	-
14 - Q-3-O-diglycoside-7-O--glycoside	-	t	t	t	-	+	+	+	-	++	++	++

(1) and quercetin (10), as well as their simple glycosidic forms (2), (3), (7) and (11) indicates that both species are of evolutionary advanced than the two species of first group.

With respect to their classification, Group (A) comprised of *Galium* verrucosum (S9, S10) and G. tricornutum (S2, S7). Morphologically, these two species are linked together as they share some characters such as mericarp size, petal length, flower diameter size, and petal width. They are clustered together as a result of the same flavonoid profiles (Figure 2). Group (B) included G. murale (S5, S8), G. verrucosum (S11) and G. tricornutum (S3) as well. The former species is characterized by its seed and mericarp shapes. Group (C) included two different species: G. setaceum (S6) and G. aparine (S1, S4, S12). Both species are correlated with petal colour, style length, leaf width, leaf shape, and pedicel length. Remarkably, G. aparine (S1, S4, S12) that collected from distant mountainous and isolated areas do not affected by any flavonoid except for the two which are common to all species. The phytochemical results are relatively congruent with morphological data.

However, qualitative and quantitative variations in the flavonoid profiles among the five investigated *Galium* species collected from Libya could respond to the environment-dependent variability [24]. Such change may be related to genetic variation amongst individuals that affected by environmental factors. On the other hand, Puff [25] described that variation change in the quality and quantity in the flavonoid profiles in the most taxa of *Galium* sect. aparinoides is related to change of the environment. Separate publications on the effect of some environmental variables, and genetic variations among the studied *Galium* are in preparation.

In Conclusion, the flavonoid patterns in the *Galium* species showed a predominant flavonol glycoside being quercetin-3-rutinoside [26]. However, the other compounds were variable in quality, quantity and in their distribution among the investigated samples. The distribution patterns of the isolated fourteen flavonoid compounds showed the presence of biosynthetic divergence in the glycosidic and methylated attachments that possible to distinguish between two main groups with an intermediate group.

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Austin J Plant Biol - Volume 2 Issue 1 - 2016
ISSN: 2472-3738 | www.austinpublishinggroup.com

Citation: Moubasher H, Abd El-Ghani M, Al-Wakeel S and Bahoor A. Chemotaxonomic Significance of Flavonoids in Some Species of *Galium* (Rubiaceae) from Libya. Austin J Plant Biol. 2016; 2(1): 1014.