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Flavonoids and isoenzymes as chemotaxonomic markers in *Cleome* L. (Cleomaceae Bercht and J. Presl)

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ABSTRACT

These studies were conducted on the two flavonoid compounds of quercetin and kaempferol in addition electrophoretic studies of esterase (Est) and peroxidase (Prx) isoenzymes by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for five *Cleome* L. species. Extraction of these flavonoids was carried out using 90% ethanol and 85% methanol. Identification and quantification of quercetin and kaempferol were done using thin layer chromatography (TLC) and further augmented using high-performance liquid chromatography and then isolated, purified and identified using melting point (MP) and NMR analysis. Seven quercetin and seven kaempferol compounds are isolated and identified with different percentages. Electrophoretic patterns of two isoenzymes of Est and Prx considered a good taxonomic marker techniques. A total of 50 Est and Prx bands with different migration distances obtained scanning the gels. The highest combined Est and Prx bands (13 bands) are found in *Cleome scaposa* DC, whereas the lowest seven bands are recorded in *Cleome paradoxa* B. BR.34 obtained data of two flavonoid compounds and two isoenzymes were subjected to numerical analysis which separated the studied species into two groups, the first has *Cleome gynandra* and *Cleome viscosa* L., the second group included three species of *C. scaposa*, *Cleome brachycarpa*, and *C. paradoxa*. From the dendrogram of statistical program, the highest correlation appeared between *C. paradoxa* and *C, brachycarpa*

KEW WORDS: *Cleome* L., flavonoids, HPLC, isoenzymes, SDS-PAGE

INTRODUCTION

Cleome L. is the largest genus from Cleomaceae Bercht and J. Presl family, with over 200 species distributed in drier areas of the tropics and subtropics, used in traditional medicine and many of them have been the subject of pharmacological and phytochemical studies (Aparadh *et al*., 2012). About 38 species of Capparidaceae including *Cleome* had been analyzed based on biochemical studies for their content of isothiocyanate-producing glucosides for the taxonomic significance of species and possible biogenesis of the glucosides (Kjaer and Thomsen, 1963). Mnzava (1990) studied biochemical properties (amino acid analysis, iodine and saponification numbers, crude protein, lipid, oleic and linoleic acids of the total fatty acids) of seeds of *Cleome gynandra* L. Different workers have isolated different compounds from different *Cleome* L. species for example, kaempferide 3-glucuronide from the roots (Chauhan *et al.,* 1979). Ahmad and Alvi (1987) have isolated a cabralealactone, ursolic acid and new trinortriterpenoid dilactone, deacetoxybrachycarpone from *Cleome brachycarpa* Vahl ex DC and determined its structure. Sharaf *et al*. (1992) examined aerial parts of four *Cleome* L. species for their surface flavonoids content. About 10 methylated flavonoids were isolated and identified. Sharaf *et al*. (1997) isolated and identified 13 flavonoid glycosides from four *Cleome* L. and three *Capparis* L. species. Qin *et al*. (2000) isolated and elucidated new trinortriterpenoid, 1-epibrachyacarpone from *Cleome chrysantha* L. Songsak and Lockwood (2002) have isolated 2 volatile glucosinolate hydrolysis compounds in *Cleome chelidonii*. Seif *et al*. (1984) have reported some flavonoids from *Cleome droserifolia* (Forssk.) Del. which were identified as kaempferol-3-O-glucoside, rutin, kaempferol, and luteolin-7-o-glucoside. Furthermore, Quercetin 3-*o*-(2-acetyl)-glucoside reported in *Cleome viscosa* L. by Senthamilselvi *et al*., 2012. Molecular systematics for 22 taxa belonging to 9 tribes of Brassicaceae

in Egypt using isoenzymes electrophoretic techniques were studied by Hasan (2009). In *Cleome paradoxa* B. BR. quercetin-3-O-β-D-glucopyranoside (isoquercetrin), β-amyrin, sitgmasterol, and sitgmasterol-3-O-β-D-glucopyranoside are reported by Essam *et al*. (2009). Leaf seed protein and isoenzymes not influenced by environmental factors also use for taxonomic studies in some genera and (Forde and Gardiner and, 1986). Evaluate the affinities between the *Cleome*. The objective of this study is analysis of identification and quantification two flavonoid compounds of quercetin and kaempferol in addition molecular systematics of peroxidase (Prx) and esterase (Est) which in turn these parameter used as chemotaxonomic support of the position of species in the genus *Cleome* L.

MATERIALS AND METHODS

Fresh samples of aerial parts (stem, leaves and flowers) of the five species of the *Cleome* named *Cleome gynandra* L., *C. viscosa* L, *Cleome scaposa* DC, *C. paradoxa* B. BR, and *C. brachycarpa* Vahl ex DC related to genus *Cleome* L. obtained different localities of southwest of Saudi Arabia. Specimens are identified according to Alfarhan *et al*., 2005 and Masrahi, 2012.

Estimation of Flavonoid

40 gm of powdered plants were packed in a thimble of soxhlet extractors with. 200 ml of petroleum ether was used in a soxhlet extractor for three hours to get rid of lipids and fat (Neil, et al., 2006). 20 defatted gm were ground in a blender containing ETOH, another the 20 gm of defatted extract were ground at high speed in a blender and extracted with 85% MEOH at room temperature overnight. Determination of flavonoid by ethanolic extract (F 1) according to (Boham and Kocipai, 1974), for estimation by the methanolic extract (F 2) according to Harbone and King (1976) and Singab (1995). The preliminary analysis of the flavonoid extracts employed two-dimensional TLC (Thin layer chromatography). Cellulose TLC plate The extracts of each species were spotted on (Merck, 20 cm × 20 cm). Two two different detection methods, F1 sprayed by using aluminum chloride (AlCl3), F2 sprayed by using iodine vapor. After locating of quercetin and kaempferol of the extract in comparison with standards, preparative thin layer chromatography was done to isolate and purify them. The portion of ethanolic and methanolic extract (F1 and F2) was used to obtain the final product by applying it as a concentrated solution in arrow of spots using the capillary tube and the standard sample was applied in one side of the plate. The used developing solvent systems according to Wagner and Bladt (1996) and Nicola (2006). Toluene: chloroform: aceton (40: 25: 35) For ethanolic extract. For methanolic one chloroform:aceton:formic acid (75:17:8). Then, the filtrate was evaporated to dryness under reduced pressure to give yellow precipitate. The precipitate then recrystallized using hot 50% ethanol. To separation and identification of two flavonoid compounds, the band corresponding to the standard was scrapped out and collected in a beaker and eluted with gentle heating and filtered. Then, the filtrate was evaporated to dryness under reduced pressure to give yellow precipitate. The precipitate then recrystallized using hot ethanol and maintained for TLC and measuring melting point (MP) and infra-red their sharp MP. Qualitative and quantitative estimations of quercetin and kaempferol were done by using high-performance liquid chromatography (HPLC), in which identifications were made by comparison of retention time obtained at identical chromatographic conditions of analyzed samples and authentic standards (Pouchert, 1978). Flavonoids were detected by their absorbance under UV light, standard methods of identification were followed (Harborne, 1967; Mabry et al., 1970; Markham, 1982). H-NMR spectra of the flavonoid compounds were recorded as DMSO-d8 on a Bruker AMX-400 spectrometer (400 MHz for 1H-NMR and 100 MHz for 13C-NMR). Mass spectra were recorded using a Finnigan SSQ-7000 (El and CI modes) and Kratos MS 80 (FAB mode).

Estimation of Isoenzyme Electrophoresis

Seeds of the samples were collected, washed in distilled water, dried and ground to fine powder. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted as the method outlined by Stegemann *et al.* (1988). The gels were stained after electrophoresis; the specific staining solution used for each studied isoenzyme was prepared according to Graham *et al*., 1964 and Jonathan and Wendel, 1990. For Esterase (EST) isoenzyme staining, the gels were stained by adding 1 ml of 1% alpha naphthyl acetate in 60% acetone was added to 25 ml 0.11 l phosphate buffer (pH 6.5). 20 mg of fast blue RR were added to 25 ml of the same buffer. The gel washed and filtered. For Peroxidases (prx) isoenzyme staining, gel soaked ino-dianisidine dissolved in 150 ml of 95% ethanol, 20 ml of acetate buffer (0.88 M sodium acetate, 0.62 glacial acetic acid, pH 4.7), 20 ml of distilled water, and 5 ml of 3% hydrogen peroxide which added just before using. The gel was incubated at room temperature and washed and filtered (Jonathan and Wendel, 1990). All gels resulted from isoenzyme electrophoresis were scanned using gel documentation system GS 900. A statistical analysis of

Table 1: Rf values of flavonoid compounds in different developing solvent systems in TLC in the studied species.

Rf (%)	Species				
	C. gynandra	*C. viscosa*	*C. pardoxa*	*C. scopasa*	*C. brachycarpa*
Ethanolic extract					
Rf1	0.26	0.34	0.22	0.34	0.72
Rf2	-	-	0.34	-	0.45
Rf3	0.19	0.54	0.17	-	-
Rf4	0.34	0.41	0.31	0.22	0.35
Rf5	-	-	0.34	-	0.67
Methanolic extract					
Rf1	-	0.18	-	-	0.47
Rf2	-	0.37	0.22	0.84	0.27
Rf3	0.22	0.34	0.12	0.34	0.29
Rf4	-	0.42	0.26	0.39	0.82
Rf5	0.32	0.43	0.67	0.89	0.34
Rf6	0.52	-	-	0.42	-
Rf7	0.39	-	0.39	-	-
Rf8	-	0.39	-	-	0.39

TLC: Thin layer chromatography

Table 2: Quercetin and Kaempferol compounds in the studied species by ethanolic extracts by HPLC

Compounds	Species				
	C. gynandra	*C. viscosa*	*C. pardoxa*	*C. scopasa*	*C. brachycarpa*
Quercetin 7-rhamnoside	0.24	0.13	0.024	0.044	0.027
Quercetin 3-rutinoside	0.099	0.011	-	0.022	0.049
Quercetin 7-rutinoside	0.33	0.22	0.023	-	0.022
Quercetin 3,7-dirhamnoside	0.02	0.025	-	-	0.21
Quercetin 3-glucoside-7-rhamnoside	-	0.034	-	-	0.52
Quercetin-3-glucoside	0.025	0.044	0.24	0.019	0.021
Quercetin 7-rhamnoside	0.023	0.05	0.41	-	-
Kaempferol-3-o-glucoside	0.31	0.22	0.074	0.024	0.021
Kaempferol 7-rhamnoside	0.064	0.021	0.029	0.025	-
Kaempferol 3-rutinoside	0.14	0.2	0.43	0.19	0.28
Kaempferol 3,7-dirhamnoside	-	0.24	-	-	-
Kaempferol 3-glucoside-7-rhamnoside	0.11	0.15	-	0.025	0.2
Kaempferol 3-rhamnoside-7-glucoside	-	0.012	0.015	0.02	0.32
Kaempferol-7-o-glucoside	-	0.022	-	0.055	-

HPLC: High performance liquid chromatograph

the identified data was carried out by multivariate cluster analysis using Minitab (version 13.0) statistical software.

RESULTS AND DISCUSSIONS

Two extraction portions were obtained the experimental work in which ethanolic extract (F1) and methanolic extract (F2). Our results showed that the methanol extract portion was the best because the amount of both extract and flavonoid were higher than the two other extract portions. As shown in Table 2, Rf of flavonoid compounds extracted and assigned by TLC of the extracts (F1 and F2) obtained dried aerial parts of the studied species confirms the presence of quercetin and kaempferol in all extraction portions of *Cleome* species. Isolated compounds in both F1 and F2 have the same color and varied only in spot numbers, also Rf value as that of reference standards differed where the highest quercetin spots found in *C. viscosa,* extracted by methanol than ethanol extract as represented in Table 1 and Figure 1.

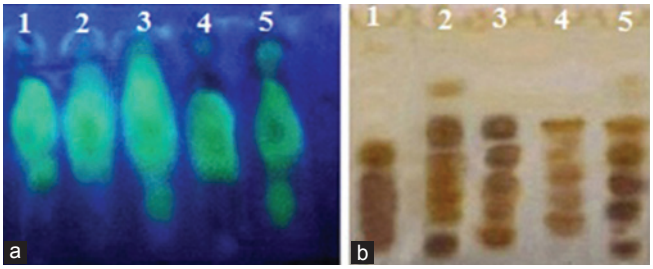

Figure 1: Thin layer chromatography of two flavonoid compounds in five *Cleome* species. (a) Ethanolic extract sprayed by aluminum chloride ($AlCl_3$) vapor. (b) Methanolic extract sprayed by iodine vapor. (1) *Cleome gynandra,* (2) *Cleome viscosa,* (3) *Cleome paradoxa,* (4) *Cleome scaposa* and (5) *Cleome brachycarpa*

From the investigation of the aerial extracts (fractions) in the studied species on TLC plates, flavonoid compounds present in all studied species with different percentages calculated by Rf as shown in Table 1. The highest Rf of flavonoid compounds with ethanolic extract found in *C. paradoxa (five spots),* whereas the lowest one found in *C. scaposa*. On the other hand, the highest flavonoid

compounds with methanolic extract found in *C. viscosa* and *C. brachycarpa (six spots),* whereas the lowest one found in *C. gynandra.* In Table 2, seven quercetin and seven kaempferol compounds are identified with different percentages. It is clear that, quercetin 7-rhamnoside and Quercetin-3-glucoside are found in all the studied species. Also, Quercetin-3-rutinoside and quercetin 7-rutinoside recorded. Analysis of the ethanolic portion by HPLC appeared seven quercetin and seven kaempferol compounds are identified with different percentages. In addition, quercetin 3-glucoside-7-rhamnoside found in the two species of *C. viscosa* and *C. brachycarpa*. Quercetin 3,7-dirhamnoside absent only in *C.paradoxa* and Cleome scaposa. In the same sense, Kaempferol-3-o-glucoside and kaempferol 3-rutinoside found with high percentages in all studied species. Kaempferol 7-rhamnoside evaluated in all species except *C.brachycarpa*, also kaempferol 3,7-dirhamnoside found only in *C. viscosa*. In the same sense, kaempferol 3-rhamnoside-7-glucoside found in all species except *C. gynandra*. Moreover, kaempferol -7-o-glucoside found only in two species of *C. viscosa* and *C. scaposa*.

Isoenzyme Analysis

Esterases (Est)

Nine Est groups obtained scanning the gel with migration distances ranged from 0.59 mm to 1.60 mm. These patterns indicate that Est 3 and Est 5 groups found in all the studied species. The highest number of six Est bands was found in *C. scaposa* and *C. brachycarpa* followed by five bands found in *C. viscosa,* whereas the lowest number of three bands was noticed in *C. gynandra* and *C.paradoxa*. From Table 3, all studied species are shared in Est 3 and Est 5. In addition, Est 1 found only in C. *scaposa* and Est 2 found only in *C. brachycarpa*. It is that Est 8 found in all studied species except *C. gynandra* and *C. paradoxa.* In addition, Est 4 found in the two species of *C. scaposa* and *C. brachycarpa*. From the Est gel, *C. paradoxa, C. scaposa* and *C. bracycarpa* are shared in three Est 3, Est 5 and Est 6 groups, respectively. Est 6 found in all species absent only in *C. viscosa* (Figure 2a).

Peroxidases (Prx)

About 11 Prx groups of isoenzyme were electrophoretically obtained scanning the gel in the studied species (Table 4 and Figure 2b). The highest number of seven Prx bands are found in *C. scaposa,* followed by five bands in *C. gynandra* and *C. viscosa*, while the lowest three bands found in *C. pardoxa*. It is obvious that Prx 3 and Prx 8 are found in all studied species, Prx 1 found in all species except *C. gynandra* and *paradoxa,* and also Prx 4 found in all species except *C. paradoxa* and *C. brachycarpa.* In addition, Prx 9 found

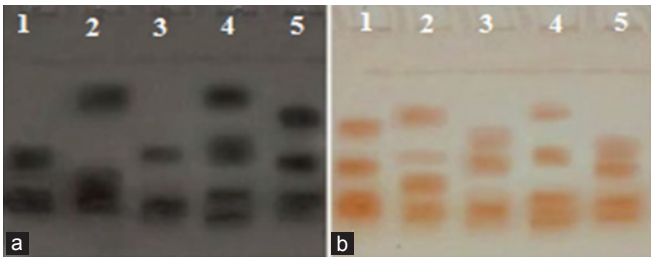

Figure 2: Electrophoretic patterns of esterase isoenzyme (a) and peroxidase isoenzymes (b) of the studied species. (1) *Cleome gynandra,* (2) *Cleome viscosa,* (3) *Cleome paradoxa,* (4) *Cleome scaposa* and (5) *Cleome brachycarpa*

Table 3: Migration distances of Est isoenzymes in *Cleome*

Migration distance (mm)	Species				
	C. gynandra	*C. viscosa*	*C. pardoxa*	*C. scopasa*	*C. brachycarpa*
0.59	-	-	-	22.1	-
0.79	-	-	-	-	32.6
0.92	21.8	2.11	0.27	11.8	19.3
0.97	-	-	-	0.67	0.28
1.0	0.25	1.25	0.27	0.27	3.21
1.20	22.4	-	22,8	6.81	2.43
1.34	2.76	3.88	-	-	-
1.48	-	0.18	-	0.27	0.27
1.60	-	-	0.54	-	-

Est: Esterase

Table 4: Migration distances of Prx isoenzymes in *Cleome*

Migration distance (mm)	Species				
	C. gynandra	*C. viscosa*	*C. pardoxa*	*C. scopasa*	*C. brachycarpa*
1.71	-	0.16	-	2.67	0.33
1.83	0.15	-	-	-	-
1.90	0.67	0.56	5.55	1.27	6.89
2.01	0.80	7.27	-	0.34	-
2.10	-	-	-	0.57	9.54
2.18	33.4	22.5	-	-	-
2.22	0.33	-	-	-	-
2.35	0.19	0.69	0.32	3.62	3.65
2.54	-	4.44	-	-	-
2.61	-	-	3.98	0.30	-
2.69	-	-	-	2.45	-

Prx: Peroxidase

only in *C. viscosa*. Both of Prx 2 and Prx 7 are characterized with the migration distance of 1.83 and 2.22, respectively are characterized the *Cleome gynandra* also Prx 10 found only *C. scaposa* and *C. paradoxa*. The last Prx group (Prx 11) with migration distance of 2.69 found in *C. scaposa*. From Table 5, the highest combined Est and Prx bands (13 bands) are found in *Cleome scaposa* fellowed by 10 bands in Both *C. gynandra* and *C. Brachycarpa*, whereas the lowest seven bands are recorded in *Cleome paradoxa*.

Using the 34 data of two flavonoid compounds of quercetin and kaempferol and electrophoretic data of Prx and Est isoenzymes in the numerical analysis Table 5 result in

Table 5: Using 34 of flavonoids and isoenzymes data used in statiscal program between the five species

Obtained data	Species				
	C. gynandra	*C. viscosa*	*C. pardoxa*	*C. scopasa*	*C. brachycarpa*
Quercetin 7-rhamnoside	1	1	1	1	1
Quercetin 3-rutinoside	1	1	0	1	1
Quercetin 7-rutinoside	1	1	1	0	1
Quercetin 3,7-dirhamnoside	1	1	0	0	1
Quercetin 3-glucoside-7-hamnoside	0	1	0	0	1
Quercetin-3-glucoside	1	1	1	1	1
Quercetin 7-rhamnoside	1	1	1	0	0
Kaempferol-3-o-glucoside	1	1	1	1	1
Kaempferol 7-rhamnoside	1	0	1	1	0
Kaempferol 3-rutinoside	1	1	1	1	1
Kaempferol 3,7-dirhamnoside	0	1	0	0	0
Kaempferol 3-glucoside-7-rhamnoside	1	1	0	1	1
Kaempferol 3-rhamnoside-7-glucoside	0	1	1	1	1
Kaempferol -7-o-glucoside	0	1	0	1	0
Est 1	0	0	0	1	0
Est 2	0	0	0	0	1
Est 3	1	1	1	1	1
Est 4	0	0	0	1	1
Est 5	1	1	1	1	1
Est 6	1	0	1	1	1
Est 7	1	1	0	0	0
Est 8	0	1	0	1	1
Est 9	0	0	1	0	0
Prx 1	0	1	0	1	1
Prx 2	1	0	0	0	0
Prx 3	1	1	1	1	1
Prx 4	1	1	0	1	0
Prx 5	0	0	0	1	1
Prx 6	1	1	0	0	0
Prx 7	1	0	0	0	0
Prx 8	1	1	1	1	1
Prx 9	0	1	0	0	0
Pr×10	0	0	1	1	0
Pr×11	0	0	0	1	0

1: Present, 0: Absent; Est: Esterase, Prx: Peroxidase

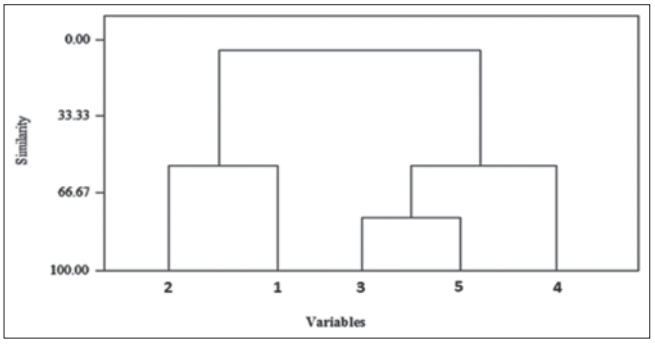

Figure 3: The relationship between the *Cleome* species based on two flavonoid compounds and two isoenzymes. (1) *Cleome gynandra,* (2) *Cleome viscosa,* (3) *Cleome paradoxa,* (4) *Cleome scaposa* and (5) *Cleome brachycarpa*

two groups. The first group composed of two species of *C. gynandra* and *C. viscosa*, the second group included three species: *C. scaposa, C. brachycarpa,* and *C. paradoxa*. In the last group, *C. scaposa* is separated in a single level (Figure 3). Results of the molecular data and flavonoid compounds support the between the *C. brachycarpa* and *C. paradoxa*. Furthermore, *C. viscosa* has a distinctive quercetin and kaempferol compounds, so it appears in a separate group with *C. gynandra*. Biochemical assay for the studied species reveals similar functional groups observed as per their peaks. It is known that the flavonol contents may be affected by a wide range of environmental conditions including temperature, soil, light levels, nutritional conditions. It is obvious that, kaempferol 3,7-dirhamnoside found in high percentages in *C. viscosa* which is agreement with the result of Senthamilselvi *et al.*, 2012. Furthermore, quercetin 3,7-dirhamnoside and kaempferol 3-rhamnoside-7-glucoside in *C. brachycarpa* supported by results of Sharaf *et al*. 1997. Molecular techniques in the field of biology have helped researchers to establish genetic relationship between the different members of different taxonomic also can provide genomic information and genetic variability (Bernatzky and Tanksley, 1986 a,b). Est isozymes are one of the frequently used biochemical markers in genetic analysis of higher plants and the staining is an important process in electrophoresis analysis. A maximum gene expression of Est isoenzyme was found in *C. scaposa* and *C. brachycarpa* while the lowest one present in *C. gynandra*. Furthermore,

Est 3 and Est 5groups are monomorphic (found in all species) which may be considered positive markers. Moreover, Prx 3 and Prx 8 groups are monomorphic bands. The highest gene expression was found in *C. scaposa*. On the other hand, all the species studied showed similar nature of absorption peaks; however, at different transmission percentages, genes and environments are responsible for the various expressions of characters in plants. This determines the genetic relationships between the five morphotypes species. Our results of two flavonoids compounds and two isoenzymes markers are support previous studies by Kasem (2016) which support the separation of *Cleome gynandra* and *Cleome viscosa* in addition *Cleome scaposa* in distinctive levels.

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