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Phytochemical Investigation of Flavanoid of Calotropis Procera in Iraq, Isolation and Identification of Rutin, Quercitin and Kampferol

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Abstract:

The aim of our study was to investigate chemical constituents of *Calotropis Procera* since no phytochemical investigation had been done previously in Iraq, the aerial parts leaves and stems of *C. Procera* were macerated in 95% aqueous methanol for three days and fractionated with petroleum ether, chloroform, ethyl acetate, and n-butanol. The ethyl acetate fraction was analyzed by high-performance liquid chromatography (HPLC) and high-performance thin-layer chromatography (HPTLC) for its flavonoid contents. flavonoids were isolated from this fraction and identified by mass spectrometry, infrared, HPLC, and HPTLC.

INTRODUCTION

CalotropisProcera (Arka) is an important medication source in the monograph of Ayurveda, and it is in India well known from the earliest time. It was revealed by writers of the Hindu and the earliest sacrificial rites many years ago. There are two common species of Calotropis reported, *C. Procera*. and *CalotropisGigantea* known by the earliest writers. The two species of Calotropis consists of comparable types of phytoconstituents revealed till now despite of differences in percent from area to area and environmental conditions[1].

It is commonly used in the Indian traditional medicinal system as well as in the other available treatments such as Arabic, Unani, and Sudanese for various diseases.

C. Procera is also used by many tribes of the world as a medicinal agent for diseases such as some skin disease, elephantiasis, toothache, respiratory diseases , leprosy, and rheumatoid diseases [1]. Traditionally*CalotropisProcera* has been used as an antifungal [2]antipyretic [3]and analgesic agent [4]. The leaves of the plant are used to treat joint pain in osteoarthritis and reduce knee swelling [5]. Different parts such as leaves, roots and bark, flower, fruits, stem, and latex of the plant have been reported to have various phytochemicals which might possess many pharmacological activities. The coarse shrub possesses acaricidal, schizonticidal , antimicrobial, anthelmintic, insecticidal, anti-inflammatory, antidiarrheal, anticancer , and larvicidal activities with other beneficial properties [1, 6] .*CalotropisProcera*has a potential of others uses in the traditional medicine [7] .The roots, stems, leaves, flowers, fruits or seeds of the plant contain different percentage of the phytochemicals some are rich in it other with low concentration or even not contain it at all. Many phytochemicals, particularly the pigment molecules, are often concentrated in the outer layers of the various plant tissues [8]

A vast number of articles and researches are published on the phytochemical investigation and chemical properties of *C. Procera*. Besides the cardenolides, which took the highest importance, other phytochemicals are also reported from the plant such as sterols, flavonoids, coumarins, alkaloids, triterpenes, saponins, tannins, and hydrocarbons were reported and isolated from *CalotropisProcera*different parts. *CalotropisProcera* are considered toxic in all of its parts, due to the existence of cardenolides (cardiac glycosides). The milky secretion (latex) considered the more toxic one due to the higher concentration of cardiac glycoside[9][10]

Scientific Classification

Taxonomy
Kingdom: Plantae
Division: Magnoliophyte
Class: Magnoliopsida
Order: Gentianales
Family: Asclepiadaceae
Genus: Calotropis
Species : *Calotropisprocera*(Aiton). [11]

Flavonoids

Flavonoids are important group of polyphenolic compounds that are widely distributed among the plant. Structurally, they are made of more than one benzene ring in its structure (a range of C15 Aromatic compounds) and numerous studies support their use as antioxidants or scavengers of free Radical[12]. Flavonoids are derived from parent compounds known as flavones.

In vitro many studies also showed that flavonoids have anti-inflammatory, anti-allergic, antiviral, and anti-carcinogenic properties [13]. Flavonoids also protect the plants from UV radiation and atmospheric exposure. In addition, flavonoids contribute to nutraceutical qualities of fruits and vegetables and have long been recognized to possess anti-oxidant, anti-inflammatory, anti-allergic, hepato-protective, anti-thrombotic, antiviral, and anti-carcinogenic activities [2, 5]. The healthbeneficial effects of flavonoids may relate to interactions with key enzymes of the body, signaling cascades involving cytokines and transcription factors, or antioxidant systems [7].

METHODS

Collection of plant materials

Calotropisprocera aerial parts were obtained from ALYarmouk area in Baghdad city where it was cultivated in house garden of Iraqi citizen. *Calotropisprocera* was identified and authenticated by Prof. Dr. SukaenaAbbas /Department of Biology /College of Sciences/ University of Baghdad.

Equipment and chemicals

The instruments used were rotary evaporator (BÜCHI Rotavapor R-205, Swiss), high-performance liquid chromatography (HPLC) (model (SYKAM) Germany, and highperformance thin-layer chromatography (HPTLC) (Eike Reich/CAMAGLaboratory, Switzerland). All chemicals and solvents used were of analytical grade and obtained from Riedel-de Haen, Germany, except methanol for HPLC grade purchased from Sigma-Aldrich,

Germany. The standard Rutin,Quercetin and Kaempferol were purchased from Chengdu Bio purify Phytochemicals, China (purity >97). Thin-layer chromatography (TLC) aluminum plates pre-coated with silica gel 60 F 254 (100 mm×100 mm, 0.2 mm thick) used were obtained from E. Merck Ltd., India.

Extraction

Leaves of *C. procera* were thoroughly washed and dried in the shade. The dried plant was powdered in anelectrical grinder. 400 g powders of *C. procera*were macerated in 95% methanol for 3 days and filtered, and the filtrate was evaporated to dryness under vacuum using rotary evaporator. the residue was suspended in water and subsequently fractionated by partitioning with petroleum ether, chloroform, ethyl acetate, and n-butanol (500 ml ×3) for each fraction. The first three organic layers were dried over anhydrous sodium sulfate, filtered, and evaporated to dryness.

Preparations of standards and samples for analysis

Standard solutions for HPLC of rutin, kaempferol andQuercetin were prepared by dissolving 0.04 mg in 1 ml of methanol HPLC grade. Dried samples were prepared for HPLC analysis by dissolving them in methanol and subjecting them to ultrasonication for 30 minutes at 25°C followed by centrifugation at 7500 rpm for 15 minutes. The clear supernatant of each sample was evaporated under vacuum. The residues were resuspended individually, in 1 ml of methanol HPLC grade, homogenizing using vortex mixer, and passing them through 2.5 µm disposable filter, and stored at 4°C for further analysis. 20 µl of the sample was injected into HPLC system for analysis. Standards used for HPTLC analysis were prepared by dissolving few milligrams from each sample in 1 ml methanol.

Phytochemical investigation

Test for flavonoids

Few milligrams of the ethyl acetate fraction were suspended in ethanol and few drops of 5% ethanolic KOH were added, and then, few drops of 5% HCl were added. The changes in colors were recorded.

Isolation of flavonoid

Rutin, Quercetin and Kaempferol was isolated by preparative TLC, utilizing ethyl acetate fraction of the aerial parts

Preparation of stationary phase

Readymade silica gel GF254 plates with a layer thickness of 0.5 mm dimension 20×20 cm. The plates were reactivated by heating in the oven at 120°C for 15-20 minutes, left to cool, and used for application after allocation of the baseline and the solvent front

Preparation of mobile phase (solvent system)

The constituents of the mobile phase for flavonoidswas composed from Chloroform: ethyl acetate: methanol: formic acid (70: 14: 14: 10)were mixed in a conical flask and introduced in the jar. The jar was lined with a filter paper, closed tightly, and left for saturation

Application of sample

About 1 g of the sample was dissolved in absolute methanol and applied on the baseline of TLC plates using a capillary tube.

Detection of separated spots

Detection was done by examination under UV light with wave length of 254nm. The purity of each band was checked by analytical TLC till single spot on TLC plate is obtained for identification with reference standard

HPLC analysis

HPLC technique ((SYKAM)) was applied for the detection of different constituents found in the ethyl acetate fraction using a mobile phase composed of A: methanol: B: 0.05 Trifluoroaceticusing gradient flow A= 70 % (0 – 5 min), A= 40 % (5 – 8 min), A= 90 % (8 – 15), the Column of HPLC was C18 – ODS (25cm * 4.6 mm) the detection done at –280 nmwith Flow rate 1.0 ml / min

HPTLC analysis

Ethyl acetate fraction was analyzed also for its flavonoids contents utilizing HPTLC (Eike Reich/CAMAGLaboratory, Switzerland), using silica gel GF254 plates developed in a mobile phase composed ofChloroform: ethyl acetate: methanol: formic acid (70: 14: 14: 10) examined at 280 and 366 nm wavelength.

Identification of isolated flavonoid

The isolated flavonoids were identified by different spectroscopic and chromatographic techniques listed below:

- Mass spectrometry (MS): Shimadzu GCMS - QP2010 Ultra
- Infrared (IR): IR spectra was recorded in KBr disk, the range of scanning 4000-400 cm−1
- HPLC: As listed before
- HPTLC: As listed before

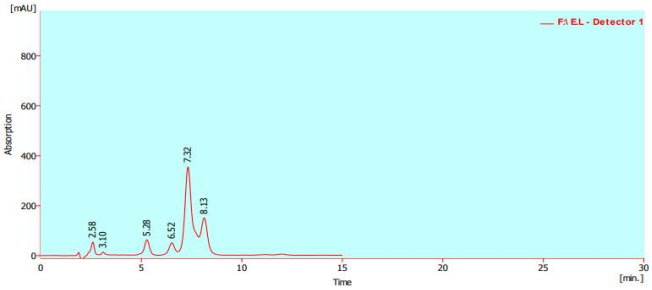


FIG.1: High performancechromatographychromatogram of ethyl acetate fraction

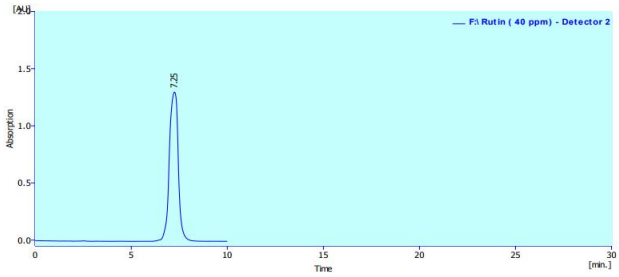


FIG. 2: High performance chromatography chromatogram of Rutin standard

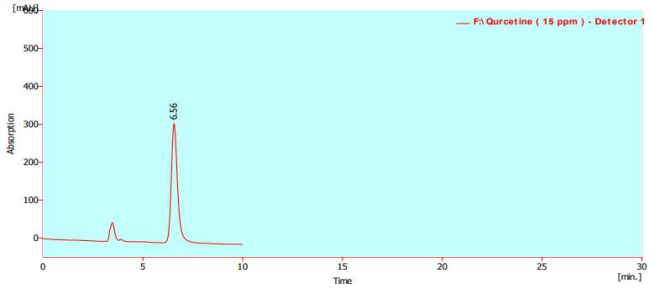


FIG. 3: High performance chromatography chromatogram of Quercetin standard

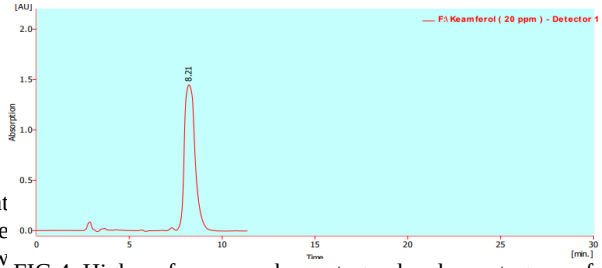
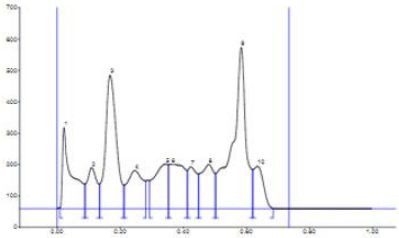


FIG.4: High performance chromatography chromatogram of kaempferol standard

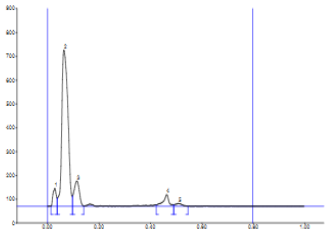
Table 1: Retention Time of Ethyl acetate fraction and Standards

Retention time	Ethyl acetate fraction	RUTIN	Quercetin	Kaempferol
	2.85			
	3.1			
	5.28			
	6.52		6.56	
	7.32	7.25		
	8.13			8.21

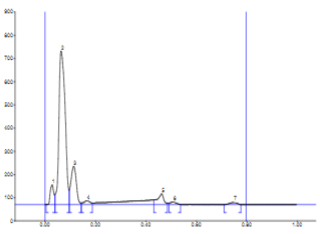


Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.01	3.0	0.02	258.4	12.07	0.09	77.9	6063.5	9.52	unknown *
2	0.09	78.3	0.11	130.8	6.11	0.13	77.4	3154.2	4.95	unknown *
3	0.14	78.0	0.17	425.7	19.87	0.21	74.7	11495.3	18.06	unknown *
4	0.21	74.7	0.25	121.7	5.68	0.28	88.2	4688.1	7.36	unknown *
5	0.29	88.8	0.35	142.3	6.64	0.35	140.2	4909.7	7.71	unknown *
6	0.35	140.2	0.36	140.9	6.58	0.41	122.0	5441.2	8.55	unknown *
7	0.41	122.5	0.42	132.9	6.21	0.45	110.9	2952.3	4.64	unknown *
8	0.45	111.0	0.48	140.6	6.57	0.50	111.7	4485.7	7.05	unknown *
9	0.50	111.7	0.58	513.5	23.97	0.62	124.9	17134.6	26.92	unknown *
10	0.62	125.2	0.64	135.1	6.31	0.69	0.1	3334.7	5.24	unknown *

FIG.5: High performance Thin Layer chromatography chromatogram of Ethyl acetate fraction

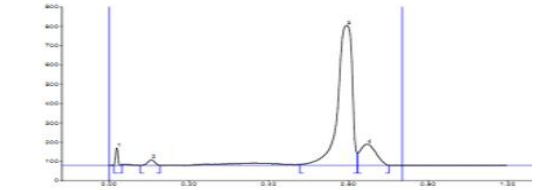


Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.01	0.5	0.03	75.9	8.41	0.04	34.8	772.5	4.87	unknown *
2	0.04	34.9	0.06	656.7	72.79	0.10	42.9	12239.6	77.18	unknown *
3	0.10	45.6	0.11	106.7	11.82	0.14	1.7	1697.8	10.71	unknown *
4	0.42	6.5	0.46	50.0	5.54	0.49	6.6	877.9	5.54	unknown *
5	0.49	6.9	0.51	13.0	1.44	0.55	0.7	270.6	1.71	unknown *

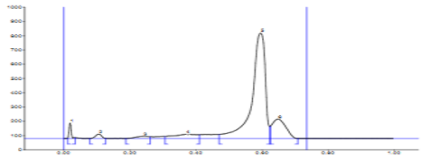


Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.00	0.3	0.03	86.0	8.60	0.04	43.0	955.7	5.23	unknown *
2	0.04	44.7	0.06	662.9	66.24	0.10	62.4	12761.7	69.84	unknown *
3	0.10	64.6	0.11	166.0	16.58	0.14	7.3	2789.5	15.27	unknown *
4	0.14	7.5	0.17	16.6	1.66	0.19	7.3	354.2	1.94	unknown *
5	0.43	20.6	0.46	47.3	4.72	0.49	6.8	930.3	5.09	unknown *
6	0.49	6.5	0.51	11.8	1.18	0.54	0.9	238.8	1.31	unknown *
7	0.71	0.1	0.75	10.1	1.01	0.78	0.1	241.9	1.32	unknown *

FIG.6: High performance Thin Layer chromatography chromatogram of Rutin standard and Isolated B band



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.01	2.7	0.02	91.8	9.59	0.03	4.7	445.4	1.79	unknown *
2	0.08	1.2	0.11	29.4	3.07	0.13	1.4	422.6	1.70	unknown *
3	0.48	5.6	0.60	725.3	75.79	0.62	64.0	20656.0	82.88	unknown *
4	0.62	64.6	0.65	110.5	11.54	0.70	0.9	3399.6	13.64	unknown *



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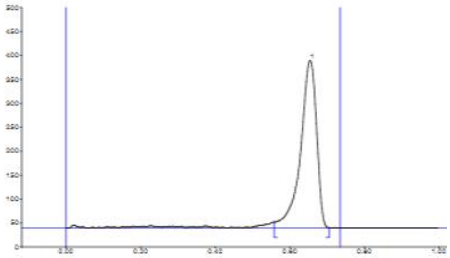
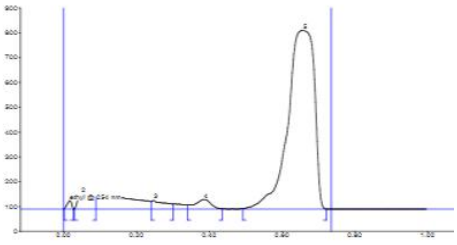
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.01	2.7	0.02	108.4	10.24	0.03	5.3	554.5	1.82	unknown *
2	0.08	0.4	0.11	30.8	2.91	0.13	1.5	436.8	1.43	unknown *
3	0.19	0.2	0.24	15.7	1.49	0.26	11.7	461.2	1.51	unknown *
4	0.31	14.6	0.37	29.6	2.80	0.41	26.5	1679.0	5.50	unknown *
5	0.47	28.6	0.60	738.5	69.72	0.62	83.5	23048.6	75.48	unknown *
6	0.63	85.5	0.65	136.2	12.86	0.71	0.1	4356.1	14.27	unknown *

FIG.7: High performance Thin Layer chromatography chromatogram of Quercetin standard and Isolated E band



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.56	12.6	0.66	349.9	100.00	0.71	0.6	11747.9	100.00	unknown *



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.00	1.3	0.02	34.7	3.89	0.03	0.4	330.1	0.72	ethyl
2	0.03	0.7	0.05	62.8	7.04	0.09	50.5	2025.3	4.43	unknown *
3	0.24	31.0	0.25	35.6	3.99	0.30	20.5	1076.1	2.35	unknown *
4	0.34	16.7	0.39	39.4	4.41	0.44	0.8	1339.4	2.93	unknown *
5	0.49	2.0	0.66	720.1	80.67	0.72	0.4	40984.7	89.57	unknown *

FIG.8: High performance Thin Layer chromatography chromatogram of Kaempferol standard and Isolated F band

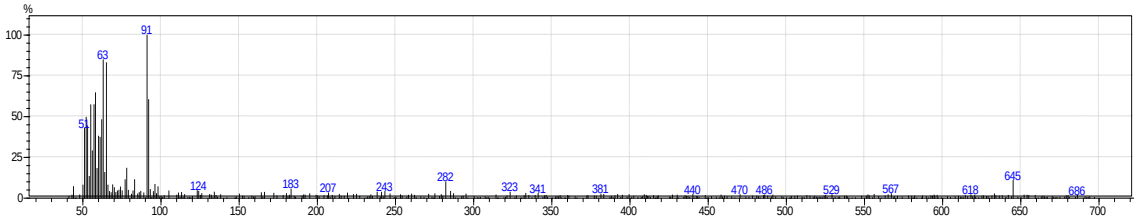


Figure .9: Mass fragmentation pattern Of Isolated B Band (Rutin)

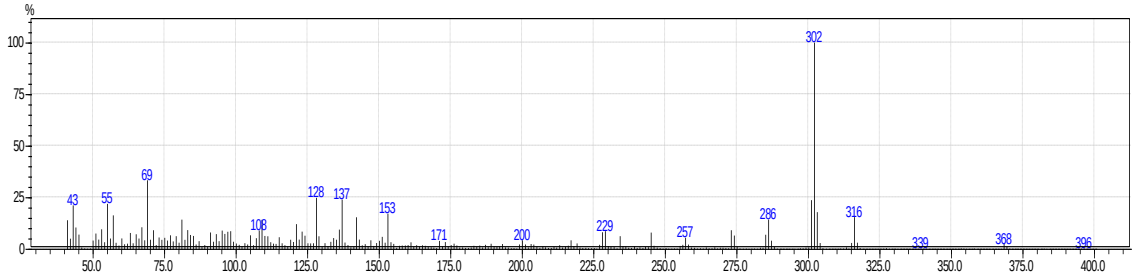


Figure .10: Mass fragmentation pattern Of Isolated E Band (Quercetin)

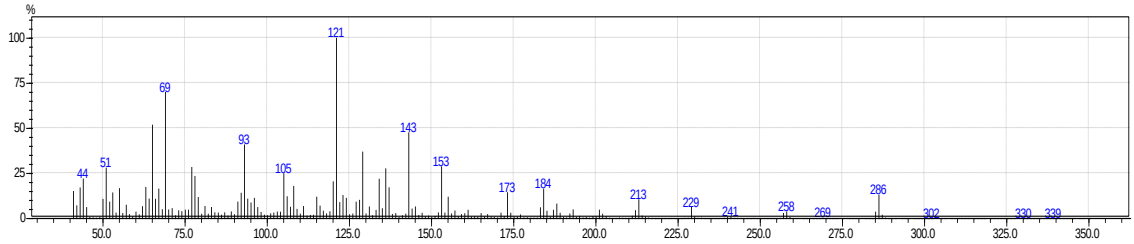


Figure .11: Mass fragmentation pattern Of Isolated F Band (kaempferol)

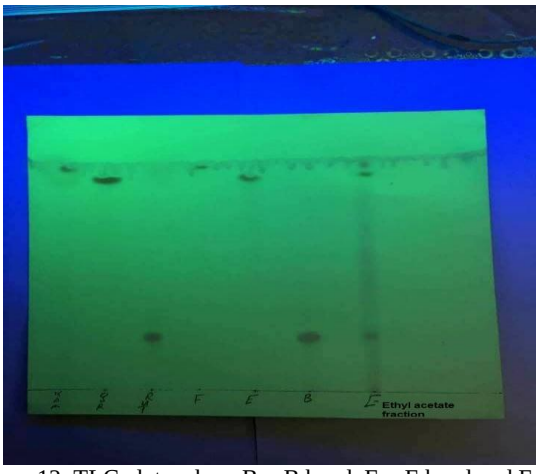


Figure 12: TLC plate where B = B band, E = E band and F = F band comparing with Quercetin, Rutin and Kaempferol Standers

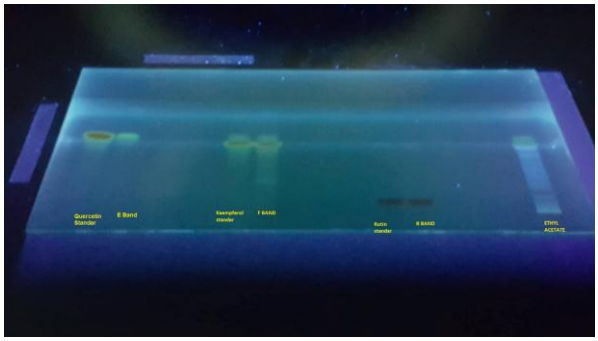


Figure 13: HPTLC Images compare the Ethyl acetate fraction and the isolated bands with standers.

DISCUSSION:

Regarding isolated B compound the data obtained from IR, HPLC, MASS, and HPTLC of the isolated compound **B** were identical with those reported for RUTIN , which indicates that B could be RUTIN

For E isolated compound the data obtained from IR, HPLC, MASS, and HPTLC of the isolated compound **E** were identical with those reported for QUERCITIN, which indicates that E could be QUERCITIN

And finally, for F compound the data obtained from IR, HPLC, MASS, and HPTLC of the isolated compound **F** were identical with those reported for kaempfrol, which indicates that F could be kamferol

Conclusion: The different chromatographic and spectroscopic results revealed the presence of rutin, quercetin and kaempferol. The results of the current study showed the presence of rutin, quercetin and kaempferol flavonoids in *Calotropis Procera* aerial parts.

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