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Characterization of polyphenols by HPLC, their antioxidant and GC-MS analysis of wild Calotropis procera leaves and fruit extracts

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Abstract : Medicinal plants are considered as important source of promising bioactive compounds. *Calotropis procera* is a traditional medicinal plant which is known to have biochemical constituents with potential medicinal properties. The present study was aimed to evaluate the phytochemicals and antioxidant properties of crude methanolic extracts of wild *C.procera*. The total phenolic, flavonoid and DPPH antioxidant activity were measured in methanol extract of (leaves and fruits) of *C.procera*. Additionally, HPLC analysis of both extracts showed that Ellagic acid (18.03%), and Tannic (6.30%) were the major phenolic compounds in *C.procera*. Various phenolic compounds such as rutin, chlorogenic, caffeic, ferulic, coumaric acids were also identified. The chemical composition of hexane extract derived from leaves and fruits were analyzed using Gas chromatography-mass spectrometry (GC–MS) and have an interesting contribution to the total antioxidant activity. Results of the present study show that *C. procera* plant is rich source of polyphenolic agents that might be playing an important role in inhibition the progress of several diseasess.
Keywords : Antioxidant activity, *Calotropis procera*, DPPH free radical scavenging, flavonoid, phenolic.

Introduction

Bioactive compounds in medicinal plants are compounds produced by plants that having pharmacological or therapeutic properties such as, antimalarial, antidiabetic, antioxidant, antimicrobial, anticarcinogenic, and, anticholinergic activities. According to the previous studies, antioxidants are reported to relief the function of immune cells against free radicals[1]. A number of clinical studies suggested that the antioxidants in medicinal plants are contributed in reducing the chronic disorder including heart disease and protect cell constituents against oxidative damage[2]**.** The use of medicinal plants has an ancient origin in different cultures around the world and their preparation is basically due to producing a spectrum of secondary metabolites. To promote the use of medicinal plants as potential sources of antioxidant, it is important to thoroughly find out their composition and activity and thus confirm their use.

Calotropis procera is known as Alarka, Surya, Tabana, Vasuka and Ashar. It is widely grown in many places all over the world especially in Bangladesh, India and Indonesia, it is belongs to Asclepiadaceae family, which includes more than 280 genera and approximately 2,000 species [3]. In many countries *C.procera* leaves are used in folk medicine to reduce blood glucose in patients suffering from diabetes mellitus [4]. Different parts of Calotropis are reported to have abundant phytochemical constituent as flavonoids, tannins, sterols, alkaloids,

cardiac glycosides, sterols and tri-terpenes[5]**.** Indeed**,** two new flavonoid constituents were identified from this plant, which are known as quercetin 3-O-galactoside and rutin[6].

Calotropis procera is considered as a source of digitalis-like therapeutic agents and is highly toxic to the land snail [7]**.** The latex of Calotropis is used in the treatment of eczema, inflammations, malarial and low hectic fevers [8], it exhibits also antidiarrheal properties duo to its desensitizing effect on the smooth muscles of the gastrointestinal tract [9]. While the leaves, fruits and roots are used in rheumatism, as anti-inflammatory, antimicrobial, antioxidant and hepatoprotective agents [10]. The remarkable anti-diarrheal activity of *Calotropis procera* leaves extract against castor-oil induced diarrhea model proved its utility in a good rang of diarrheal cases [11].

However, *C. procera* are used traditionally in Nigeria, it has been used to treat diseases like fever, eczema, leprosy, ringworm, cough, asthma and convulsion [12]. GC-MS is considered as best technique to identify the chemical composition of long chain hydrocarbons, alcohols, acids, esters, alkaloids, steroids, amino and nitro compounds etc.[13]. The leaf of Calotropis contains ascorbic acid, o-pyrocatechic acid and also contains β-amyrin, taxasterol, tarasterol and β-sitosterol. Therefore, the present study was undertaken to evaluate the total phenolic, flavonoid and DPPH antioxidant activity of *C. procera* leaves and fruits extract. Furthermore, GC-MS analysis has carried out to identify the bioactive constituent present in this plant.

Experimental

Chemical reagents and solvents

Folin-Ciocalteu reagent, sodium carbonate, aluminum chloride and gallic acid were purchased from Merck Company (Darmstadt, Germany). Butylatedhydroxyltoluene (BHT) and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma Chemical Co., Ltd (St.Louis, MO, USA). All other reagents and solvents were of analytical grade.

Sample collection and preparation:

Calotropis procera wild plant was collected from the desert around Makkah Provence, Saudia Arabia, during May 2016 during the flowering stage. Authentication of the plant was performed by Dr. Mona M. Marzouk (Ph.D.) Department of Phytochemistry and Plant Chemo-systematics, National Research Center (NRC), Cairo, Egypt. The healthy plant parts (leaves and fruits) were used for samples extraction. After washing by distilled water, plant parts were cut into small pieces using a kitchen knife and the leaves were shade dried for 7 days while the fruits were shade dried for 10 days. The dried plant parts were separately ground in a mill in which the ground samples were passed through a mini test sieve to obtain pure processed sample used for the analysis.

Methanol extraction

The ground plant materials (10.0 g) were extracted by stirring in 100 ml of 80% (v/v) methanol for 24 h at room temperature (on magnetic stirrer). The extract was then filtered under reduced pressure to separate the insoluble plant material and the solvent evaporated at 50 °C under reduced pressure in a rotary evaporator (BuchiRotavapor B-480, Buchi Australia). The resulting concentrate was then mixed with 10 ml of methanol to obtain a crude methanol extract. Then all the sample constituents stored at 4 °C until use

Determination of total phenolic content (TP):

Phenolic compounds were determined based on a method described previously [14]**.**One ml of methanolic extract was mixed with 1 ml of FolinCiocalteu reagent. After 3 min, 1 ml of saturated sodium carbonate solution (30%) was added to the mixture and adjusted to 10 ml with distilled H_2O. The reaction mixture was kept in the dark for 1 h with intermittent shaking. The absorbance was measured at 725 nm using a spectrophotometer (UNICAM UV300). Phenolic contents were calculated on the basis of the standard curve for gallic acid (GAE). The results were expressed as mg of gallic acid equivalent per g of dry extract.

Determination of total flavonoid contents (TF):

The TF was determined using a modified aluminum chloride assay method[15]. Briefly, extracts of plant material (1 ml) were diluted with water (4 ml) in a 10 ml volumetric flask. Initially, 5% $NaNO_2$ solution (0.3 ml) was added to each volumetric flask; at 5 min, 10% $AlCl_3$ (0.3 ml) was added; and at 6 min, 1.0 M NaOH (2 ml) was added. Water (2.4 ml) was then added to the reaction tube and mixed well. Absorbance of the reaction mixture was read at 510 nm. TF were determined as Quercetin equivalents (mg QE /g of dry weight).

Determination of DPPH free radical scavenging activity

Quantitative measurement of radical scavenging properties of different samples leaves and fruits extract was carried out according to the pervious described method [16].Solution of DPPH (0.1 mM) in methanol was prepared and 1 ml of this solution was added to 3 ml of each extract at different dilutions (100, 200 and 300 µg/ml). Butylatedhydroxyltoluene (BHT) was used as a positive control. Discoloration was measured at 517 nm after incubation for 30 min. The activity to scavenge the DPPH radical was calculated using the following equation:

DPPH scavenging activity (%) = [ADPPH-AS / ADPPH] x100

Where, ADPPH is the absorbance of the DPPH solution and AS is the absorbance of the solution when the sample extract was added.

Analysis of polyphenolic compounds by HPLC

Identification of individual polyphenolic compounds in methanolic extract was performed using JASCO HPLC (Agilent technologies 1260 infinity), with a hypersil C18 reversed-phase column Eclipse plus (250x4.6 mm) and 5 µm particle size. HPLC analysis of methanolic extract was performed by re-dissolving 100 mg of extract in 1 ml of methanol (80%) and filtered through a 0.2 µm filter sterilized membrane prior to HPLC analysis. Injection by means of a Rheodyne injection value (Model 7125) with 50 pJ fixed loop was used. A constant flow rate of 1 ml/min was used with two mobile phases: (A) 0.5% acetic acid in distilled water at pH 2.65; and solvent (B) 0.5% acetic acid in 99.5% acetonitrile. The elution gradient was linear starting with (A) and ending with (B) over 50 min, using an UV detector set at wavelength 254 nm. The concentration of individual polyphenolic compounds was calculated on the basis of peak area measurements[17].

Gas chromatography/mass spectrometry (GC/MS) analysis

The **GC/MS** analysis of leaves and fruits *C.procera* hexane extract were performed using a Thermo Scientific capillary gas chromatography (model Trace GC ULTRA) directly coupled to ISQ Single Quadruple MS and equipped with TG-5MS non polar 5% phenyl methylpolysiloxane capillary column (30 m × 0.25 mm ID × 0.25 µm). The operating condition of GC oven temperature was maintained as: initial temperature 40^{o}C for 3 min, programmed rate 5^{o}C/min up to final temperature 280^{o}C with isotherm for 5 min. For GC/MS detection, an electron ionization system with ionization energy of 70 eV was used. Helium was used as a carrier gas at a constant flow rate of 1.0 ml/min. 1 µl of each sub-fraction was injected automatically in the splitless mode. Detection was performed in the full scan mode from 40 to 500 m/z. The quantification of the components was based on the total number of fragments (total ion count) of the metabolites as detected by the mass spectrometer. The identification of the chemical components was carried out based on the retention time of each component (R_t) compared with those of the Wiley 9 and NIST 08 mass spectra libraries [18].

Statistical Analysis

All data are presented as means ± SD; the mean values were calculated based on the data taken from at least three independent experiments conducted on separate days using freshly prepared reagents.

Results and Discussion

Polyphenolic content

The total polyphenolic contents of *C.procera* methmolic extracts (leaves and fruits) are given in Table 1. The *C.procera* extracts showed the presence of high value of phenolic compounds. Highest phenolic content (56.3 ±0.77mg GAE /g) was observed in leaves extract followed by fruits extract (31.4±1.02 mg GAE /g).Plant phenolics present in the medicinal plants have received considerable attention in recent years due to their diverse pharmacological properties including antioxidant activity[19]**.** Phenolic compounds comprises from non-polar to very polar, thus choosing the most appropriate solvent is crucial in maximizing the extraction process. Methanol is usually preferred for the extraction of antioxidant compounds from plant matrices mainly due to its good extraction efficacy. Moreover, total phenolic and flavonoid in methanol extract of leaves of *C. procera* were 3.8 and 2.1 mg/g, respectively[20]**.** These amounts are very low compared to our result. Such variations of TP and TF among different studies might be due to the varied agro-climatic factors of the regions from where the plant materials were collected. Normally, the concentrations of flavonoid in plant extract range from 3.53 to 149.97 mg QU/g. The highest value of flavonoid is detected in *C. procera* leaves (41.4±0.30 mg of QE/g) ,while fruits extract is contain low value of flavonoid (19.5±0.22 mg of QE/g).Leaves and fruits show an average value of flavonoids/phenolic ratio 0.74, and 0.62 respectively. The differences in the values of TP and TF in the tested two parts (leaves and fruits)may be explained by the fact that presence of phenolics is affected by growing conditions or plant tissues. Additionally, sometimes there is a vague correlation between TPC and antioxidant activity of several medicinal plants[21]. It is known that flavonoid with a certain structure and particularly hydroxyl position in the molecule can associated with health beneficial effects, such as lowering the incidence of aging, inflammation, cardiovascular diseases and certain cancers nevertheless these flavonoids compounds are also act as proton donating and show radical scavenging activity [22].

Table (1): Total phenolic (TP) and total flavonoid (TF) contents and their ratio of *C.procera* methmolic extract (leaves and fruits)

Polyphenolic compounds	Plants parts	
	Leaves	Fruits
Total phenolic (mg GAE /g)	56.3 ±0.77	31.4±1.02
Total flavonoid (mg QE /g)	41.4±0.30	19.5±0.22
Total flavonoid/ Total phenolic	0.74	0.62

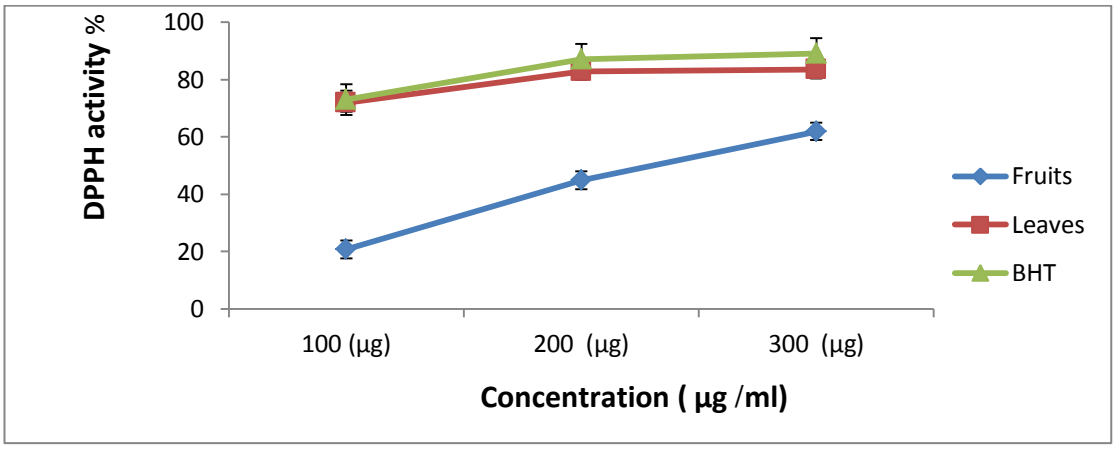

Figure 1: DPPH free radical scavenging activity of *C. procera* methanol extracts (Each value is expressed as mean ± SD, n = 3).

Antioxidant activity

The antioxidant activity of plant extracts from *C. procera* is detected using methanol solution of DPPH reagent. It is accepted that, as the concentration of polyphenolic compounds of the phenolic compounds increases, DPPH radical scavenging activity and hence antioxidant activity of a plant extract also increased.

The inhibition percent of free radicals by leaves and fruits extracts was investigated against DPPH. The DPPH radical scavenging activity results are presented in Fig. (1) as comparable with known positive control (BHT). From the data of Fig. (1), we can conclude that the scavenging effects of leaves extract at all concentrations (100, 200 and 300 µg/ml) on DPPH radicals were excellent, compared to BHT. The scavenging effects of methanolic extracts from both parts on the DPPH· radical decreased in the order of BHT >leaves >fruits which were 89.1%, 83.5%, and 61.9% at the concentration of 300 µg/ml, respectively. These results indicated that the methanolic extracts of *C. procera* leaves have a noticeable effect on scavenging free radicals. However, the scavenging effect of BHT is higher than our methanolic extracts of *C. procera* leaves. This observation may be of applied value in utilization of leaves, as this plant grows wildly in the Saudia desert. Although many studies support that total phenols and flavonoids contribute significantly to the total antioxidant[23]**.** Similarly antioxidant activity of leaf extract of *C. procera* through DPPH was also evaluated in other study [24] and it was reported that *C. procera* possess high antioxidant properties due to more phenols and flavonoids. It has been reported that methanol extract of *C. procera* latex exhibited positive activity to scavenge free radicals [25]**.** Also, the present results showed that the methanolic extract of *C. procera* fruits exhibited the medium radical scavenging activity. The effectiveness of the leaves could be due to the hydroxyl groups existing in the phenolic compounds that can provide the necessary component as a radical scavenger [26]**.**

HPLC of polyphenolic composition of methanolic extract of *C. procera*

It is noticed that the total phenolic content detected by the Folin-Ciocalteu reagent does not exhibit e full picture of the quantity of the phenolic composition in the extracts as reported in previous literature [27]. Therefore, HPLC of polyphenolic composition were detected. The phenolic compounds which identified by HPLC were 17 compounds as Ellagic acid, Tannic acid, Gallic acid, Chlorogenic acid, Coumaric acid, Myrecitin, Ferulic acid, Quercetin, Coumarin, Cinnamic acid, Kaempferol, Benzoic acid, Catechin, Luteolin, Rutin, Acacetin and Caffeic acid as shown in (Table 2). The major polyphenolic compounds presented in the extract were Ellagic acid (18.03%), in leaves and Tannic acid (6.30%) in fruit. These natural polyphenolic compounds could thus be a good source of antioxidants for applications in food industry. Among phenol phytochemicals, ellagic acid has been target of previous studies and has shown important biological activity. One of the most studied properties of ellagic acid is their antioxidant capacity [28]. Tannic acid has known applications in the food, cosmetic and pharmaceutical industries and has antimicrobial properties [29]. The other phenols identified in leaves extract also have important biological effects. It is reported that chlorogenic acid has antioxidant activity.

Table2. Concentration of phenolic compounds (%) identified in methanol extract of leaves and fruits of *C. procera* by HPLC.

No	Compounds	Leaves	Fruits
1	Ellagic acid	18.03	ND
2	Tannic acid	ND	6.30
3	Gallic acid	2.03	2.69
4	Chlorogenic acid	7.08	ND
5	Coumaric acid	6.77	ND
6	Myrecitin	1.52	0.02
7	Ferulic acid	1.65	1.82
8	Quercetin	0.19	0.44
9	Coumarin	0.58	2.35
10	Cinnamic acid	1.13	0.54
11	Kaempferol	ND	0.42
12	Benzoic acid	0.84	0.93
13	Catechin	0.26	0.50

14	Luteolin	0.16	1.05
15	Rutin	0.14	0.46
16	Acacetin	0.18	1.83
17	Caffeic acid	4.27	1.29

Table 3.Chemical composition profile (retention time (RT) & relative area %) of hexane extract of *C.procera* (leaves)

Peak no	[a]R_t	Compounds name	Area (%)[b]	Molecular formula
1	15.76	Diphenyl 2methoxycarbonyl2, c5diphenylpyrrolidinec3,t4dicarboxylate	0.64	$C_{32}H_{27}NO_6$
2	20.88	(+)guattaminone	0.68	$C_{27}H_{25}C_{l4}NO_7$
3	24.25	1[2,4,6tris(trimethylsiloxy)phenyl]3[3,4di(tri methylsiloxy)phenyl]2propen1one	0.76	$C_{30}H_{52}O_6Si_5$
4	25.77	{(Iridiumchloride) bis[(methoxycarbonyl)ethy nyl]bis(triisopropylphosphanyl)}	0.72	$C_{26}H_{48}ClIrO_4P_2$
5	26.63	11,23Ditertbutyl5,17 diethoxycarbonyl25,26 ,27,28tetrahydroxycali x[4]arene	0.78	$C_{42}H_{48}O_8$
6	27.59	Nonane	3.21	C_9H_{20}
7	30.72	Docosane	2.41	$C_{22}H_{46}$
	33.72	9[4,5Diacetoxy6(azidomethyl)tetrahydrop yran2yl]11hydroxy1,2,3,4tetrahydronaphth o[c]quinoline1,7(H),1 2(H)trione	1.55	$C_{27}H_{24}N_4O_9$
9	34.65	4nButylbenzoic acid,2ethylcyclohexylester	0.90	$C_{19}H_{28}O_2$
10	34.73	2[3,4Bis(tetradecyloxy)phenyl]4,4,5,5tetram ethyl1,3,2dioxaborolane	1.19	$C_{40}H_{73}BO_4$
11	42.64	Antipain	0.67	$C_{27}H_{44}N_{10}O_6$
12	52.74	9-Octadecenamide	23.64	$C_{18}H_{35}NO$
13	59.58	Dodecachloro3,4benzophenanthrene	0.63	$C_{18}Cl_{12}$
14	60.65	13-Docosenamide	9.90	$C_{22}H_{43}NO$
15	62.35	Tetratetracontane	5.25	$C_{44}H_{90}$
16	66.10	1chloro1,1,2trifluoro2tridecene	2.30	$C_{13}H_{22}ClF_3$

[a]Rt: retention time (min).
[b]The percentage composition was computed from the gas chromatography peak areas.

GC/MS analysis

The GC/MS spectral results and comparison of results with library search successfully enabled the identification of the total 36 and 37 compounds in *C. procera* leaves and fruits hexane extracts respectively. However few of them are presented in (Tables 3 and 4). The GC-MS study of *C. procera* leaves has shown many phytochemical compounds which contribute to the medicinal activity of the plant (Table 3). The major components present are Diphenyl 2methoxycarbonyl2,c5diphenylpyrrolidinec3,t4dicarboxylate(RT:15.76), guattaminone (RT: 20.88), Nonane (RT: 27.59) and Antipain (RT: 42.64). In this concern, Tetratetracontane ,Docosane and Tetracosane, these compounds have been already proposed to have a certain antimicrobial activity [30]. Also, Antipain and its related compounds are potential therapeutic compounds as anti-microbial properties [31].

Moreover, the results in Table (4) reveal that the fruit hexane extracts have a quite number of chemical constituents, which responsible for some pharmacological activities. For instance, Ginkgetin has antitumor properties [32]. Tetratetracontane was detected in benzene extract of GC-MS analysis of fruits of *C. procera*[33]. In addition, Nonacosane is a squalane wax and can be incorporated in anti-aging creams, lipsticks, hair or skin care products and other beauty products industry. Further studies are needed on these extracts in order to isolate, identify, characterize and elucidate the structure of these compounds.

Table 4.Chemical composition profile (retention time (RT) & relative area %) of hexane extract of *C.procera*(fruits)

No.	[a]R_t	Compounds name	Area (%)[b]	Molecular formula
1	9.18	Methylsulfinato[2,3,7,8,12,13, 17,18octaethylporphyrinato]indium	1.45	$C_{37}H_{47}InN_4O_2S$
2	9. 84	(4Bromophenyl) bis(2,4 dibromophenyl) amine	1.83	$C_{18}H10Br_5N$
3	10.57	Acetic acid,1,1',4'triacetoxy5,5'd iisopropyl6,7,6',7'tetra methoxy3,3' dimethyl[2,2']binaphthalenyl4ylester	1.49	$C_{40}H_{46}O_{12}$
4	12 .39	(4Bromophenyl) bis(2,4 dibromophenyl) amine	1.40	$C_{18}H_{10}Br_5N$
5	18.79	2,6Bis[5cyano6(4bromophenyl)1,2,4triazin 3yl]pyridine	1.39	$C_{25}H_{11}Br_2N_9$
6	34.79	5,15Bis(3methoxyphenyl)10phenyl20prop ylporphyrin	1.35	$C_{43}H_{36}N_4O_2$
7	34.84	Methylsulfinato[2,3,7,8,12,13,17,18octaeth ylporphyrinato]indium	1.52	$C_{37}H_{47}N_4O_2S$
8	39.44	3,4,5,6Tetrakis(pchlorophenoxy)1,2dicyano benzene	1.43	$C_{32}H_{16}C_{l4}N_2O_4$
9	52.48	5Mercapto2,4dimethy l1,2,4triazoline3thione	1.31	$C_4H_7N_3S_2$
10	52.82	3-Hydroxy1(4{13[4(3hydroxy3phenylac ryloyl)phenyl]tridecyl}phenyl)3phenylprop 2en1one	1.71	$C_{43}H_{48}O_4$
11	54.64	1,8-Octandial	1.67	$C_{16}H_{14}O_2$
12	56.45	Tetratetracontane	3.84	$C_{44}H_{90}$
13	61.05	4,4'dibromotriphenyla mine	1.37	$C_{18}H_{13}Br_2N$
14	61.68	Nonacosane	20.21	$C_{29}H_{60}$
15	62.71	Ginkgetin	1.89	$C_{32}H_{22}O_{10}$
16	63.34	Nonacosane	11.74	$C_{29}H_{60}$
17	64.14	8-O-MethylFalconerine	2.15	$C_{35}H_{51}NO_{10}$
18	66.60	Dipyridamole	1.73	$C_{24}H_{40}N_8O_4$

[a]Rt: retention time (min).
[b]The percentage composition was computed from the gas chromatography peak areas.

Conclusion

This study provides a comprehensive profile of polyphenolic compounds, profiles of phenolic compounds, and DPPH antioxidant activity of *C. procera* leaves and fruits methanol extracts. This plant, a wild growing plant, exhibits potent DPPH antioxidant properties. The phenols and scavenging potential of leaves and fruits extracts of plants growing in Saudia Arabia may higher compared to the same plant grown in different area.The antioxidant potency confirms that the leaves of *C. procera* are worth for further chemical isolation and pharmacological investigations. In our present study a new phytochemicals have identified from the *C. procera* leaves and fruits hexane extracts by GC-MS. One important point might be taking into the consideration, *C. procera*plant is toxic in nature and in folk medicine the leaves are used in fresh. But we have used the dried leaves and fruits. There is a pressing need to study the differences in therapeutic compounds nature and pharmacological efficacy of *C. procera* plant parts and when used fresh and dried.

Conflict of interest statement

We declare that we have no conflict of interest.

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