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ORIGINAL ARTICLE

High-Performance Liquid Chromatography-Fingerprint Analyses, *In vitro* Cytotoxicity, Antimicrobial and Antioxidant Activities of the Extracts of Two *Cestrum* Species Growing in Egypt

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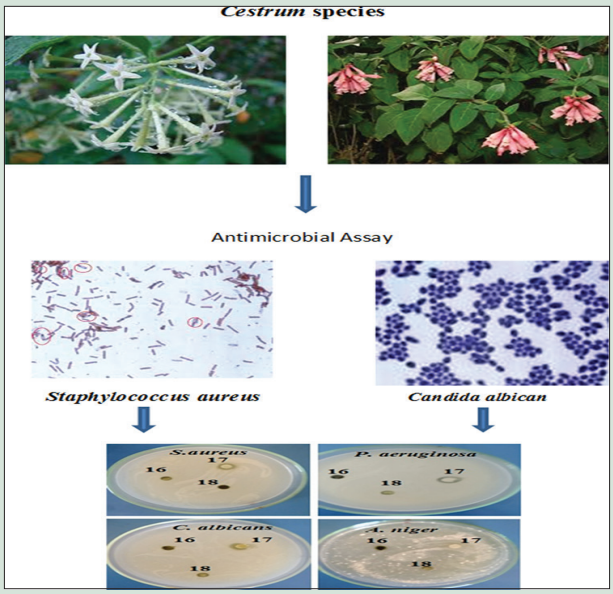
ABSTRACT

Background: Cancer diseases and microbial resistance are serious health disorders associated with oxidative stress and infectious diseases. Their risks can be reducing via using polyphenols-rich plants. **Methodology:** Different solvent extracts from two *Cestrum* species (*Cestrum nocturnum* and *Cestrum elegans*) were evaluated for their biological and chemical activities. Also, the chemical profiles of the most promising extracts were investigated via high-performance liquid chromatography (HPLC)-fingerprint analyses. **Results:** The tested extracts showed weak to moderate cytotoxicity against Vero cell line with IC_{50} values ranged from 133.67 µg/ml to 57.634 µg/ml. The only noncytotoxic extractive fraction was the dichloromethane extract of *C. elegans* leaves with an IC_{50} value of 204.732 µg/ml, while the most toxic extract was the ethyl acetate extract of *C. elegans* flowers with an IC_{50} value of 19.22 µg/ml. The antimicrobial activity results revealed that the *n*-BuOH extract of *C. nocturnum* was the most active against four tested microbial strains with inhibition zones (10–13 mm). Also, the water and *n*-BuOH extracts of *C. elegans* leave exhibited moderate activities with inhibition zones (7–9 mm), while for *C. elegans* flowers both of water and methanol extracts showed strong activities (9–14 mm). In the 2,2′-diphenyl-1-picrylhydrazyl assay, the most active fraction was EtOAc with IC_{50} values of 100.52 µg/ml and 64.40 µg/ml for *C. elegans* leaves and flowers respectively, while for *C. nocturnum* the most active fraction was methanol with an IC_{50} value of 161.16 µg/ml, all relative to 7.60 µg/ml of ascorbic acid. HPLC-fingerprint analyses revealed that the major identified compounds in the ethyl acetate extract of *C. elegans* flowers are caffeic acid, coumaric acid, vanillin, and rutin, while for the *n*-butanol extract of *C. nocturnum* leaves are coumaric acid and vanillin. **Conclusion:** The obtained results revealed that the two species can be used as natural sources of antioxidant compounds with low cytotoxic effect on the mammalian cell line.

Key words: 2,2′-Diphenyl-1-picrylhydrazyl, antimicrobial, *Cestrum* species, cytotoxicity, high-performance liquid chromatography-fingerprint analyses, Solanaceae

SUMMARY

- The current research work tested the antimicrobial, antioxidant and cytotoxic activities of different solvent extracts from two Egyptian *Cestrum* species
- Among the tested extracts, the ethyl acetate extract of *C. elegans* flowers and the *n*-butanol extract of *C. nocturnum* leaves are the most promising extracts
- HPLC-fingerprint analyses of the most promising extracts demonstrate the presence of four major compounds including; rutin and vanillin as well as caffeic and coumaric acids.



Abbreviations Used: HPLC: High-performance liquid chromatography; IC_{50}: Median inhibitory concentration; DPPH: 2,2′-Diphenyl-1-picrylhydrazyl; ATCC: American Type Culture Collection; ECACC: European Collection of Animal Cell Cultures; HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; GM: Growth medium; EDTA: Ethylene diamine tetra acetic acid; MM: Maintenance media; ELISA: Enzyme-linked immuno-sorbent assay; G^{+ve}: Gram-positive; G^{-ve}: Gram-negative; IP: Inhibition percentage; RP-HPLC: Reversed phase-high performance liquid chromatography; DAD: Diode array detection; NCI: National Cancer Institute; DCM: Dichloro methane.

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INTRODUCTION

Genus *Cestrum* (Family Solanaceae) comprises more than 300 species and is widely distributed in tropical and subtropical areas around the world like Bangladesh, India, United States, Australia, South America and southern China.[1,2] *Cestrum* species have a long history in folk medicine for the treatment of several diseases and health disorders.[1,3] Different species belonging to *Cestrum* genus were previously investigated for their chemical constituents, e.g., parquine, carboxyparquine and steroid. Glycosides were also identified in *C. diurnum*[4] whereas, saponins were identified in *C. parqui*.[5,6]

Cestrum nocturnum is one of the most important species in the genus *Cestrum*; it is commonly known as "Queen of Night."[7] *C. nocturnum* is an evergreen shrub native to South America and the West Indies, the leaves of *C. nocturnum* have been used in Chinese folk medicine for the treatment of some health problems.[8] The plant showed noticeable biological activities including; insecticidal,[9] pesticidal,[10] cytotoxic,[11] antimicrobial,[12] hepatoprotective,[7] anticonvulsant,[13] antidiabetic,[14] larvicidal,[15] anti-inflammatory & analgesic,[16] antitumour,[17] antioxidant[2,18] and wound healing.[19] Different parts of the plant were investigated for their chemical components such as essential oils,[3,9,20] alkaloids,[21] steroidal saponins,[11,22] spirostanol, steroidal glycosides,[23,24] trisesquiterpenoid[25] and flavonol glycosides.[11] To the best of our knowledge, there is no enough available reported data about *Cestrum elegans*. *C. elegans* leaves contain cinnamic acid derivatives and aromatic acids.[26]

Free radicals are highly energetic unstable reactive species containing odd electrons that able to penetrate cells and tissues of our human bodies led to abnormal cell growth which known by mutation.[27] Moreover, the high accumulation rate of such harmful species in the human body is known by oxidative stress which is the starting point of cancer disease. The plant containing antioxidant compounds can be used to overcome such phenomena.[28-30] Cancer can be defined as uncontrolled cells growth and the second causing of death after heart diseases. The plant-derived naturally occurring compounds are considered as good chemotherapeutic anticancer agents.[31] Plants are considered a vital source of the bioactive chemical ingredients used for the treatment of many diseases especially cancer.[30] Most of the developed anticancer drugs and chemotherapeutic agents were derived from medicinal plants as natural sources.[32,33]

Recently, the resistance of the pathogenic microbial strains against antibiotics develops much faster than ever. Infectious diseases caused by bacterial and fungal infections are still a major threat to public health, despite the tremendous progress in human medicine. The past three decades have seen a dramatic increase in microbial resistance to antimicrobial agents. Such situation stimulates the development of new antimicrobial agents to treat the infectious disease in an effective manner. So this matter continued to an era to identify the potential antimicrobial agent from the natural resources.[34] Therefore, our research has been undertaken to evaluate the *in vitro* antimicrobial, cytotoxic and antioxidant activities of two *Cestrum* species growing in Egypt as well as high-performance liquid chromatography (HPLC)-finger print analyses.

MATERIALS AND METHODS

Chemicals and reagents

2,2′-Diphenyl-1-picrylhydrazyl (DPPH) radical, ascorbic acid, and standard phenolic compounds were purchased from Sigma-Aldrich (Steinheim, Germany).

Plants materials

The leaves of *C. nocturnum* were collected from the Zoo Garden, Giza, Egypt in February, 2014. While, the leaves of *C. elegans* were collected from Paraghel, Giza, Egypt in February, 2014. The two plants were kindly identified via Dr. Threase Labib, Consultant in Orman Botanical Garden and National Gene Bank. Voucher specimens (No. C14/3/21) and (No. C14/3/10) were kept at the herbarium of the garden (respectively) for *C. nocturnum* and *C. elegans*.

Extraction and fractionation

Leaves dry powder of *C. nocturnum* (300 g) and *C. elegans* (250 g) were separately macerated in Methanol at room temperature for 4 days. The resulting extracts were concentrated via rotatory evaporator (Buchi, Switzerland) at 45°C ± 2°C. The crude methanolic extracts were successively fractionated using petroleum ether (60–80°C), CH_2Cl_2, EtOAc and *n*-BuOH.

Cytotoxicity evaluation

Vero cells obtained from adult African green monkey kidney epithelial cells according to American Type Culture Collection and the European Collection of Animal Cell Cultures repositories No. CCL-81, passage number: 136, kindly obtained from VACSERA, Egypt. Vero cell line was grown in liquid growth medium (GM) of Dulbecco's modification of Eagle medium (MEM).[35,36] Vero cells were stored in liquid nitrogen vapor phase and cells usually take 2–3 passages to reach their regular growth rate after recovery from the frozen state.[37] Dulbecco's MEM with fetal bovine serum (FBS), antibiotic (penicillin/streptomycin) fungi zone solution (LONZA), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer 1M pH values ranging from 6.8 to 8.2 (LONZA), Earle's salt and L-glutamine, sterile polystyrene 75 cm^2 tissue culture flasks with vented caps, sterile serological pipettes and 70% ethanol solution, all to be used in cell propagation. Cell culture incubations were in a 37°C humidified CO_2 incubator and solutions were wormed to 37°C just before headway.[38-40] In a laminar flow hood, Vero cell suspension from the cryovial was transferred into a 75 cm^2 tissue culture flasks containing 25 mL GM of MEM supplemented with heat inactivated 10% FBS, 1% HEPES and 1% antibiotic, A cryovial of Vero cells containing 1 × 106 cells/1 ml was thawed by gently swirling in a 37°C water bath for 2 min and added to incubated flask in 37°C, Vero cells grown to a 90%–95% confluency, GM from confluent monolayer of Vero cells was removed and cells washed with 1x PBS twice, 5 mL of 1X trypsin-EDTA added and cells were incubated at 37°C for 2–3 min, until cells detached from the flask, 50 ml GM was added. Cells checked for confluency using inverted microscope. When cells reached a >90% confluent monolayer, it was passaged at suitable concentration of cells needed (30.000 cells/100 ul/well). Samples were diluted in 100 µl Maintenance media as 2% FBS, 1% HEPES, 1% antibiotic, and 96% MEM Earle's and the used dilutions were 100 µg, 50 µg and 10 µg per well in triples in 96 well plate. Cell then added in each well and mixed well to be sure of its homogeneity. Plate was covered with sealing and incubated at 37°C overnight in CO_2 incubator, wells were washed with PBS twice to be ready for staining with crystal violet stain (0.5% crystal violet, 5% formalin, 50% ethanol, 0.85% NaCl, H_2O) 10 µl for 10 min followed by washing with distal H_2O three times and let it to dry and measure the plate readings in enzyme-linked immunosorbent assay plate reader at wavelength 630 nm.[39-41] Half maximal inhibitory concentration (IC_{50}) in pharmacological research is the factor used to evaluate antagonist drug potency and the dose-response curve of drug different concentrations effect on mammalian cell line growth.[42] The average cell viability obtained from triplicate determinations at each concentration was plotted as a dose-response curve. The IC_{50} of the active substances was determined as the lowest concentration which reduced cell growth by 50% in treated compared to untreated culture. The IC_{50} values were compared for their activities.[41] According to the National Cancer Institute (NCI), the criteria and the conditions of cytotoxic activity for

the crude extract IC_{50} against mammalian cell line growth based on U.S. NCI and Geran protocol, as follows: $IC_{50} \leq 20$ μg/ml = Highly cytotoxic, IC_{50} 21–100 μg/ml = Moderately cytotoxic, IC_{50} 101–200 μg/ml = Weakly cytotoxic and IC_{50} >501 μg/ml = Not cytotoxic.[40,43] The isolated fractions from number 1–18 were evaluated for their *in vitro* cytotoxic potentiality against mammalian cell line. Six concentrations 100 μg/ml, 80 μg/ml, 60 μg/ml, 50 μg/ml, 30 μg/ml and 10 μg/ml of each were used to determine the cytotoxic effect on Vero cell line in compare with the control. Cell viability was determined according the following formula:

$$\text{Cell viability} = \frac{\text{Average absorbances of triplicate treated cells}}{\text{Average absorbances of control cells}} \times 100$$

Antimicrobial activity evaluation (disc agar plate assay)

Disc agar plate method was used to estimate the antimicrobial activities of different solvent extracts.[44-47] Four different test microbes; *Staphylococcus aureus*, *Escherichia coli*, *Candida albicans* and *Aspergillus niger* were selected to evaluate the antimicrobial activities as representatives of G^{+ve} bacteria, G^{-ve} bacteria, yeast and fungal groups. The micro-organisms were obtained from Northern Utilization Research and Development Division, United State Department of Agriculture, Peoria, Illinois, USA.

2,2′-Diphenyl-1-picrylhydrazyl radical scavenging activity

The scavenging activity of the stable DPPH free radical was determined according to the method described by Marwah *et al.* (2007) with some modifications.[48] Briefly, the reaction medium contained 2 mL of 100 μM DPPH purple solution in methanol and 2 mL of plant extract, ascorbic acid was used as standard. The reaction mixture was incubated in the dark for 20 min and the absorbance was recorded at 517 nm. The assay was carried out in triplicate. The decrease in absorbance on addition of test samples was used to calculate the antiradical activity, as expressed by the inhibition percentage (%IP) of DPPH radical, following the equation: %IP = (Ac − As)/Ac × 100; where Ac and As are the absorbances of the control and of the test sample after 20 min, respectively.

Separation and quantification of phenolic compounds

Separation and determination of phenolic compounds were performed by reversed phase HPLC/diode array detection (Agilent Technologies 1260 infinity) using; a column Eclipse plus C18, 5 μm (250 mm × 4.6 mm id), detector (MWD, G1365D, 1260 MWD VL), pump (Quat pump, G1311C, 1260 Quat pump VL) and sampler (G1329B, 1260 ALS). The solvent system used was a gradient of A (CH_3COOH 2%) and B (Acetonitrile). The best separation was obtained with the following gradient: at 0 min, 90% A and 10% B; at 15 min, 45% A and 55% B; at 17 min, 20% A and 80% B; at 18 min, 90% A and 10% B; at 20 min, 90% A and 10% B. The solvent flow rate was 0.8 ml/min, and separation was performed at 25°C. The volume injected was 10 μl. Phenolic compounds were assayed by external standard calibration at 280 nm. All values were the mean of two injections.[49]

RESULTS AND DISCUSSION

Cytotoxicity

The results are presented as mean ± standard deviation for three measurements. Microsoft Excel was used to calculate $P < 0.05$ (A small P value [typically ≤ 0.05]) indicates strong evidence against the null hypothesis, so we reject the null hypothesis. A large P value (>0.05) indicates weak evidence against the null hypothesis, so we fail to reject the null hypothesis) for each fraction against the control (+ve). The dose-response curves were plotted to enable the calculation of IC_{50} for each sample. Statistical analysis of the count of viable Vero cells grown in serial dilutions of the effect of the extracted fractions compared to control. Referring to Table 1, it was found that fractions 1, 2, 3, 5, 6, 7, 8, 10, 11, 14, 15, 16, 17 and 18 showed moderately cytotoxic effect while fractions 4, 9 and 18 showed weak cytotoxic activity and fraction 13 is highly cytotoxic effect and fraction 12 has not cytotoxic effect.

Antimicrobial activity

The results in Table 2, revealed that the methanolic extract of *C. nocturnum* leaves showed a moderate antimicrobial activity against four pathogenic microbial strains with inhibition zones; *S. aureus* (9 mm), *Pseudomonas aeruginosa* (10 mm), *C. albicans* (10 mm) and *A. niger* (12 mm). On the other hand, the *n*-butanol extract showed strong activity with inhibition zones; *S. aureus* (12 mm), *P. aeruginosa* (13 mm), *C. albicans* (13 mm) and *A. niger* (10 mm) [Figure 1]. The activities of the two above mentioned extracts were compared with two standard antibiotics the first one, Penicillin G was used at 50 μg per disk with inhibition zones *S. aureus* (19 mm), *P. aeruginosa* (20 mm), *C. albicans* (23 mm), while the second one, Griseofulvin was used as specific antifungal antibiotic at 50 μg per disk with inhibition zone *A. niger* (29 mm).

On the other side, both of the water and *n*-butanol extracts *C. elegans* leaves showed a moderate antimicrobial activity with inhibition zones in the manner (H_2O/*n*-BuOH); *S. aureus* (8/7 mm), *P. aeruginosa* (8/7 mm), *C. albicans* (9/8 mm) and *A. niger* (8/9 mm) as represented in Table 3. Furthermore, the methanol, water, and *n*-butanol extracts *C. elegans* flowers showed strong to moderate antimicrobial activities with inhibition zones in the manner (MeOH/H_2O/*n*-BuOH); *S. aureus* (11/10/7 mm), *P. aeruginosa* (13/9/7 mm), *C. albicans* (14/11/8 mm) and *A. niger* (0/12/9 mm). While the ethyl acetate extract showed only a moderate activity against *S. aureus* (8 mm) and there is no any activities were recorded against the other strains [Table 4 and Figure 1]. Khan *et al.*[12] reported on the antimicrobial activity of the methanolic extract of *C. nocturnum* growing in Pakistan and its derived fractions against certain bacterial and fungal strains namely; *P. aeruginosa, Staphylococcus aureus, Bacillus subtilis, E. coli* and *Shigella flexenari* with inhibition zones ranged from 8 to 17 mm.[12] Also, the different solvent extracts (e.g., ethanol, methanol, butanol, propanol, and acetone) of *C. nocturnum* growing in India were evaluated for their antimicrobial activity against *S. typhi, P. aeruginosa*, *K. pneumoniae*, *Aspergillus* and *Trichoderma*; the *n*-butanol extract showed a potent activity against *Aspergillus* with inhibition zone of 16 mm.[2]

Free radical antioxidant activity (2,2′-diphenyl-1-picrylhydrazyl assay)

The DPPH$^{\cdot}$ radical is a stable chromogen widely used to assess the antioxidant potentials of extracts, fractions or pure isolates derived from medicinal plants.[50] Moreover, the *in vitro* DPPH model is based on the characteristic absorption at 517 nm (purple in color), which decreases significantly when exposed to radical-scavengers (due to hydrogen atoms transfer from antioxidant sample to the DPPH radical to become DPPH-H with yellow colour.[51] For *C. elegans* leaf part, the IC_{50} values were varied from 100.52 μg/ml to 181.72 μg/ml, and the results are in the order, EtOAc (100.52) > MeOH (102.47) > H_2O (139.75) > *n*-BuOH (143.41) > CH_2Cl_2 (147.10) > Pet. ether (181.72) μg/ml, in comparison to ascorbic acid as a positive control with IC_{50} equal to 7.60 μg/ml [Table 5].

On the other hand, the IC_{50} values for flower part were varied from 64.40 μg/ml to 107.62 μg/ml, and the results are in the order, EtOAc (64.40) > H_2O (107.62) > MeOH (134.38) > *n*-BuOH (149.53) > CH_2Cl_2 (185.84)

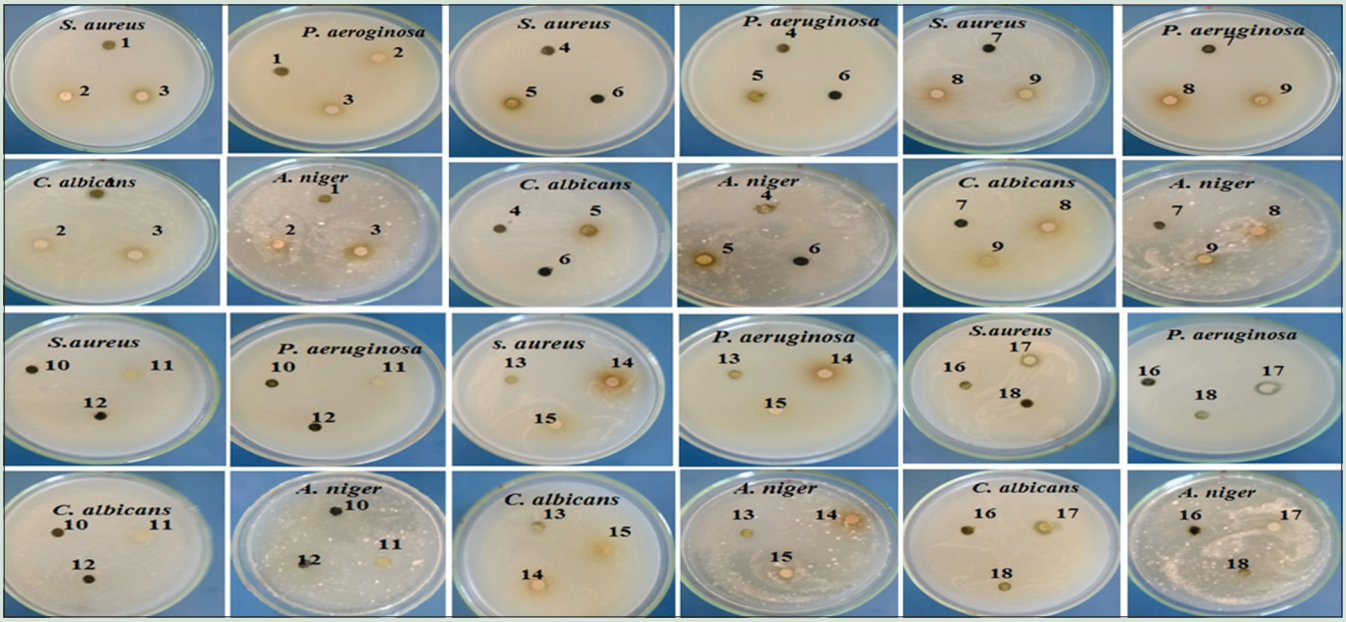

Figure 1: The antimicrobial inhibition zones (mm) of the different solvent extracts from *C. nocturnum* and *C. elegans* against four pathogenic microbial strains; *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Candida albicans* and *Aspergillus niger*. 1-*Cn*-L (EtOAc), 2-*Cn*-L (H_2O), 3-*Cn*-L (*n*-BuOH), 4-*Cn*-L (Petroleum ether), 5-*Cn*-L (MeOH), 6-*Cn*-L (CH_2Cl_2), 7-*Ce*-L (EtOAc), 8-*Ce*-L (H_2O), 9-*Ce*-L (*n*-BuOH), 10-*Ce*-L (Petroleum ether), 11-*Ce*-L (MeOH), 12-*Ce*-L (CH_2Cl_2), 13-*Ce*-F (EtOAc), 14-*Ce*-F (H_2O), 15-*Ce*-F (*n*-BuOH), 16-*Ce*-F (Petroleum ether), 17-*Ce*-F (MeOH) and 18-*Ce*-F (CH_2Cl_2). *Cn*-L: refer to *C. nocturnum* leaves; *Ce*-L: refer to *C. elegans* leaves and *Ce*-F: refer to *C. elegans* flowers

Table 1: Cytotoxicity evaluation using concentrations 100, 80, 60, 50, 30, 10 µg/ml, respectively of different solvent extracts of *Cestrum nocturnum* and *Cestrum elegans* flowers and Leaves against Vero cell line and the equivalent concentration to half maximal inhibitory concentration each

	Sample	Toxicity	Mean concentration						Equation	Concentration equivalent to IC_{50} µg
			100 µg	80 µg	60 µg	50 µg	30 µg	10 µg		
1	CEF/MeOH	Moderate	0.119	0.144	0.173	0.199	0.211	0.133	y=−353.06x+112.61	77.304
2	CEF/H_2O	Moderate	0.136	0.146	0.141	0.142	0.118	0.204	y=−554.02x+136.9	81.498
3	CEF/n-BuOH	Moderate	0.139	0.161	0.168	0.184	0.409	0.17	y=−150.66x+85.91	70.844
4	CEF/EtOAc	Weak	0.149	0.181	0.138	0.165	0.22	0.249	y=−607.71x+166.62	105.849
5	CEF/petroleum ether	Moderate	0.094	0.078	0.103	0.287	0.146	0.296	y=−239.82x+95.131	71.149
6	CEF/DCM	Moderate	0.086	0.108	0.339	0.384	0.37	0.307	y=−187.63x+104.85	86.087
7	CEL/MeOH	Moderate	0.064	0.165	0.125	0.113	0.182	0.271	y=−383.92x+113.87	75.478
8	CEL/H_2O	Moderate	0.07	0.069	0.177	0.138	0.233	0.381	y=−258x+100.92	75.12
9	CEL/n-BuOH	Weak	0.127	0.134	0.166	0.183	0.196	0.195	y=−1027.2x+226.37	123.65
10	CEL/EtOAc	Moderate	0.095	0.081	0.116	0.259	0.264	0.232	y=−310.08x+109.11	78.102
11	CEL/petroleum ether	Moderate	0.088	0.148	0.172	0.2	0.264	0.255	y=−474.07x+144.05	96.643
12	CEL/DCM	Not toxic	0.229	0.229	0.248	0.23	0.29	0.277	y=−994.88x+304.22	204.732
13	CN/MeOH	Highly	0.079	0.07	0.083	0.091	0.096	0.099	y=−2618x+281.02	19.22
14	CN/H_2O	Moderate	0.055	0.094	0.102	0.12	0.123	0.123	y=−1115.2x+169.68	58.16
15	CN/n-BuOH	Moderate	0.066	0.207	0.243	0.156	0.3	0.141	y=−154.81x+83.717	68.236
16	CN/EtOAc	Moderate	0.065	0.076	0.109	0.101	0.18	0.1	y=−509.06x+108.54	57.634
17	CN/petroleum ether	Moderate	0.145	0.145	0.187	0.173	0.3	0.198	y=−375.12x+126.77	89.258
18	CN/DCM	Weak	0.151	0.256	0.279	0.288	0.293	0.3	y=−488.14x+182.49	133.676
Control	Vero cells without extracts		0.2	0.2	0.2	0.2	0.2	0.2		
Control IC_{50}			0.1	0.1	0.1	0.1	0.1	0.1		

y: It is the concentration equivalent to IC_{50} per µg; x: It is the control IC_{50} absorbance at wavelength 630 nm; CEF: *Cestrum elegans* flowers; CEL: *Cestrum elegans* leaves; IC_{50}: Half maximal inhibitory concentration

> Pet. ether (382.55) µg/ml [Table 5]. The results also revealed that the most extracts of *C. nocturnum* leaf part showed a weak activity with IC_{50} values were varied from 161.16 µg/ml to 274.41 µg/ml [Table 5].

Prasad *et al*.[2] reported on the antioxidant activity of the different solvent extracts (e.g., ethanol, methanol, butanol, propanol and acetone) of *C. nocturnum* growing in India via DPPH assay. The different solvent extracts of *C. nocturnum* flowers growing in Bangladesh exhibited free radical scavenging potential with an IC_{50} values of 25.58, 36.70, 41.86 µg/ml, respectively for methanol, ethyl actate and chloroform.[18] Moreover, the DPPH free radical scavenging activities of different solvent extracts of the Egyptian *C. nocturnum* stem bark were evaluated, the DPPH free radical scavenging percent values were 86.6%, 74.9%, 66.7% and 43.8%, respectively for 80% MeOH, EtOAc, *n*-BuOH and aqueous extracts (Rashed, 2013).

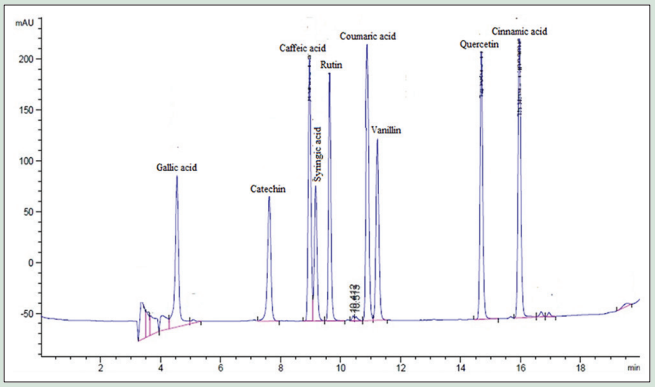

Figure 2: High performance liquid chromatography chromatogram of nine standard phenolic compounds; gallic acid (1), catechin (2), caffeic acid (3), syringic acid (4), rutin (5), coumaric acid (6), vanillin (7), quercetin (8), and cinnamic acid (9)

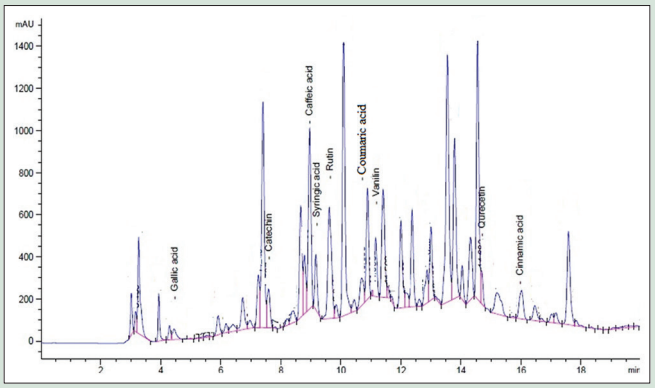

Figure 3: High-performance liquid chromatography-fingerprint chromatogram of the ethyl acetate extract of *C. elegans* flowers

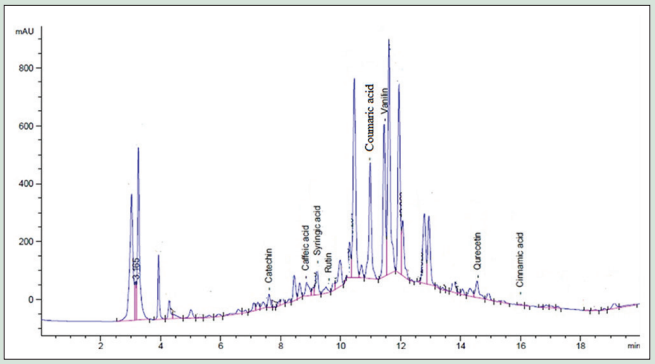

Figure 4: High-performance liquid chromatography-fingerprint chromatogram of the *n*-butanol extract of *C. nocturnum* leaves

High performance liquid chromatography -fingerprint analyses

Fingerprint analyses approach are widely used to identify the chemical composition and relative proportions of phenolic compounds in different medicinal plant extracts due to their simplicity and reliability.[52] Among them, HPLC has been the most widely used technology for identifying differences in chemical compositions among medicinal herbal samples.[53] Owing to the high *in vitro* antimicrobial activity of the ethyl acetate extract of *C. elegans* flowers and also the high *in vitro* antioxidant activity of the *n*-butanol extract of *C. nocturnum* leaves compared to the rest of the tested extracts, the two mentioned extracts were subjected to further phytochemical investigations via HPLC-fingerprint analyses aiming to identify their chemical constituents and to correlate the obtaining activities with these chemical ingredients.

Nine standard phenolic compounds namely; gallic acid (1), catechin (2), caffeic acid (3), syringic acid (4), rutin (5), coumaric acid (6), vanillin (7), quercetin (8), and cinnamic acid (9) were used in this study [Table 6 and Figure 2]. The results revealed that the above-mentioned standards were present in the tested extracts in different proportions. Caffeic acid, coumaric acid, vanillin, and rutin were recognized as major constituents, while syringic acid, catechin, quercetin, cinnamic acid and gallic acid were detected as minor components in the ethyl acetate extract of *C. elegans* flowers [Table 6 and Figure 3]. On the other hand, coumaric

Table 2: *In vitro* antimicrobial activity of the methanolic extract of *Cestrum nocturnum* leaves as well its derived sub-fractions

Sample	Clear zone (Θmm)			
	S. aureus	*P. aeruginosa*	*C. albicans*	*A. niger*
MeOH	9	10	10	12
Petroleum ether	0	0	0	0
DCM	0	0	0	0
EtOAc	0	0	0	0
H^2O	0	0	0	0
n-BuOH	12	13	13	10
Penicillin G[a]	19	20	23	0
Griseofulvin[b]	0	0	0	29

[a]Penicillin G was used at 50 µg per disk; [b]Griseofulvin was used at 100 µg per disk. *S. aureus: Staphylococcus aureus; P. aeruginosa: Pseudomonas aeruginosa; C. albicans: Candida albicans; A. niger: Aspergillus niger;* DCM: Dichloromethane

Table 3: *In vitro* antimicrobial activity of the methanolic extract of *Cestrum elegans* leaves as well its derived sub-fractions

Sample	Clear zone (Θmm)			
	S. aureus	*P. aeruginosa*	*C. albicans*	*A. niger*
MeOH	0	0	0	0
Petroleum ether	0	0	0	0
DCM	0	0	0	0
EtOAc	0	0	0	0
H_2O	8	8	9	8
n-BuOH	7	7	8	9
Penicillin G	19	20	23	0
Griseofulvin	0	0	0	29

S. aureus: Staphylococcus aureus; *P. aeruginosa: Pseudomonas aeruginosa*; *C. albicans: Candida albicans*; *A. niger: Aspergillus niger*; DCM: Dichloro methane

Table 4: *In vitro* antimicrobial activity of the methanolic extract of *Cestrum elegans* flowers as well its derived sub-fractions

Sample	Clear zone (Θmm)			
	S. aureus	*P. aeruginosa*	*C. albicans*	*A. niger*
MeOH	11	13	14	0
Petroleum ether	0	0	0	0
DCM	0	0	0	0
EtOAc	8	0	0	0
H_2O	10	9	11	12
n-BuOH	7	7	8	9
Penicillin G	19	20	23	0
Griseofulvin	0	0	0	29

S. aureus: Staphylococcus aureus; *P. aeruginosa: Pseudomonas aeruginosa*; *C. albicans: Candida albicans*; *A. niger: Aspergillus niger*; DCM: Dichloro methane

Table 5: Free radical scavenging antioxidant activities (2,2'-diphenyl-1-picrylhydrazyl) of the different solvent extracts of *Cestrum elegans* leaves, *Cestrum elegans* flowers and *Cestrum nocturnum* leaves

Sample	DPPH (IC_{50})[a] (µg/ml)		
	***C. elegans* leaves**	***C. elegans* flowers**	***C. nocturnum* leaves**
MeOH	102.47±1.92	107.62±8.06	275.41±13.84
Petroleum ether	181.72±5.82	134.38±11.40	161.16±20.40
CH_2Cl_2	147.10±4.94	382.55±4.83	262.39±7.40
EtOAc	100.52±6.98	185.84±7.22	228.24±24.01
H_2O	139.75±1.68	64.40±3.42	202.34±9.26
n-BuOH	143.41±1.08	149.53±4.47	174.69±7.98
Ascorbic acid	7.60±0.85	7.60±0.85	7.60±0.85

Results are expressed as mean values±SD (n=3). [a]IC_{50} - Concentration of sample required to scavenge 50% of free radicals. IC_{50} values are expressed as µg dry extract/ml (µg/ml). SD: Standard deviation; DPPH: 2,2'-diphenyl-1-picrylhydrazyl; IC_{50}: Half maximal inhibitory concentration; *C. elegans: Cestrum elegans*; *C. nocturnum: Cestrum nocturnum*

Table 6: Areas under peaks and concentrations of the ethyl actate extract of *Cestrum elegans* flowers and *n*-butanol extract of *Cestrum nocturnum* leaves against nine standard phenolic compounds

	Standards			Ethyl actate extract of *C. elegans* flowers			*n*-butanol extract of *C. nocturnum* leaves		
	R_t	Concentration (µg/ml)	Area	Area	Concentration (µg/ml)	Concentration (µg/g extract)[a]	Area	Concentration (µg/ml)	Concentration (µg/g extract)[a]
Gallic acid	4.53	60	1243.57	462.10	22.30	445.91	0.00	0.00	0.00
Catechin	7.62	200	951.20	1328.19	279.27	5585.34	981.56	49.08	233.42
Caffeic acid	8.96	60	1557.86	5282.77	203.46	4069.26	302.08	15.10	392.16
Syringic acid	9.16	40	837.79	1483.58	70.83	1416.67	547.54	27.38	573.40
Rutin	9.63	200	1310.44	4500.76	686.91	13,738.14	632.27	31.61	207.14
Coumaric acid	10.88	40	1709.93	2980.49	69.72	1394.44	1160.29	58.01	2480.02
Vanillin	11.22	40	1205.39	1702.99	56.51	1130.25	1917.73	95.89	2889.51
Quercetin	14.68	160	1644.99	685.18	66.64	1332.89	881.81	44.09	453.30
Cinnamic acid	15.95	20	1848.68	1280.96	13.86	277.16	6.44	0.32	29.76

[a]Sample concentration=50 mg/ml. *C. elegans: Cestrum elegans; C. nocturnum: Cestrum nocturnum*

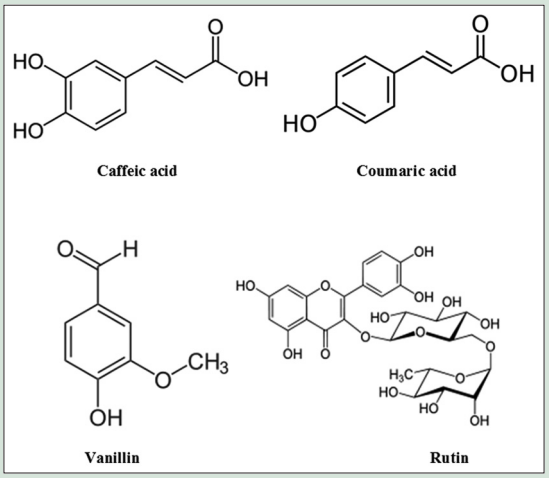

Figure 5: Chemical structures of the four major identified compounds

acid and vanillin were recognized as major constituents, while caffeic acid, rutin, syringic acid, catechin, quercetin and cinnamic acid were detected as minor components in the *n*-butanol extract of *C. nocturnum* leaves, and the absence of gallic acid [Table 6 and Figure 4]. Accordingly, the presence of such identified phenolic compounds in the two extracts may be responsible for their own activities. The chemical structures of the four major identified compounds are presented in Figure 5.

Many authors have been reported in the correlation between the existence of phenolic compounds in medicinal plant extracts and their biological activities. The phenolic compounds exhibited potent antioxidant potential due to the presence of the characteristic structural criteria for effective free radical scavenging activity like; heavy hydroxylation pattern, extended conjugation system and ketonic groups.[54-57] While for the antimicrobial action these compounds have specific modes of actions like; cell walls damage.[58-62]

CONCLUSION

Leaves from two *Cestrum* species in addition to flowers of one of them, collected from zoo garden in Egypt, showed noticeable cytotoxic and antimicrobial activities as well as a moderate antioxidant activity. This finding provides an insight into the usage of the tested species as a source of naturally occurring cytotoxic and antimicrobial agents. Accordingly, we recommended the chromatographic isolation of the most promising extracts from the two plants to identify their bioactive secondary metabolites.

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Conflicts of interest

There are no conflicts of interest.

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