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ANTIBACTERIAL ACTIVITY AND PHYTOCHEMICAL INVESTIGATION OF LEAVES OF *CALOTROPIS PROCERA* PLANT IN IRAQ BY GC-MS

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ABSTRACT: *Calotropis procera* plant belongs to the Asclepiadaceae family. It is prescribed to treat bronchitis, asthma, cough, infections, cancer, ascites, intestinal worms, cutaneous diseases, eczema, leprosy and also aid to stimulate the immune system. The leaves of *C. procera* were investigated for their phytochemical and anti-microbial activity. Chemical screening of leaves ethanolic extract exhibited to contain active compounds like alkaloids, terpenoids, flavonoids, saponins, and reducing sugar. The antibacterial activity of *C. procera* leaves against four different bacteria revealed that the ethanolic extracts could kill only *Staphylococcus aureus* and *Basillus subtilis*, but more resisted by *Pseudomonas sp.*, and *Escherichia coli* compare to the standard antibiotic streptomycin. GC-MS analysis of the ethanolic extracts indicated that the highest percentage in 100% ethanolic leaves extract belongs to camphene 6.22%, thebaine 7.59% dodecanoic acid 19.15%, and linolenic acid ethyl ester 14.87%, while the highest percentage in 70% ethanolic leaves extract belongs to hexa-hydro-farnesol 9.87%, gamolenic acid 12.71%, and linolenic acid ethyl ester 6.83%. This is the first phytochemical, and antibacterial study on *C. procera* leaves in Iraq.

INTRODUCTION: Medicinal plants have no doubt remained the major sources of traditional medicine worldwide[1]. Today, many of the available drugs were derived from the medicinal plants which still till now the major source of drug [2]. The plant is rich in many types of secondary metabolites, that found to have *in-vitro* anti-microbial properties [3, 4, 5]. Because most plants have medicinal potency [6], recently their extracts have been developed and proposed for use in food as natural antimicrobials [7, 8].

Some of the plant's secondary metabolites have been used successfully for the prevention and treatment of many disease infections, cancer or aid to stimulate the immune system [9]. These bioactivities are attributed to the presence of chemical components such as alkaloids, phenolic compounds, flavonoids, tannins and others [10, 11].

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FIG. 1: *CALOTROPIS PROCERA* LEAVES, FLOWERS, AND FRUITS

Calotropis procera was a xerophytic, evergreen, erect, perennial shrub, **Fig. 1**. It is a flowering plant known as Sodom apple or Arka. It is grown widely in the tropical and sub-tropical areas of Africa and Asia. This plant is popularly known because it produces a large quantity of latex [12, 13]. Many parts of *C. procera* plant have been reported to possess anticancer [14], antimicrobial [15], anti-oxidant [16], anti-viral [17], anti-fungal [18], anti-inflammatory [19, 20], and wound healing [21, 22] and antiemetic properties [23, 24].

In Northern Nigeria, the leaves of *C. procera* are used as a remedy for cough, asthma, *etc*. The powder of dried root used for asthma, bronchitis, eczema, leprosy and elephantiasis, splenic and hepatic enlargement [25]. *C. procera* used to treat diarrhea, sinus fistula, rheumatoid/joints, stomatic, expectorant, jaundice, anthelmintic and skin disease [26].

C. procera produces many bioactive constituents such as alkaloids. The alkaloids, calotropin, uskerin and calotaxein have been reported from *C. procera*, act as heart stimulant. It is also used to treat ringworms [27, 28]. Based on the medicinal treatments and pharmacotherapeutic uses of *C. procera*, more phytochemical studies in different methods are needed to investigate the chemical components of this plant, therefore, this study was aimed to extract the leaves of *C. procera* growing in Iraq, using two different polarities of solvent extractor, and to identify the chemical components using GC-MS Technique. Also to study the antibacterial activity of leaves extract. This is the first study on the phytochemical and antimicrobial activity of the leaves of *C. procera* in Iraq.

MATERIALS AND METHODS:

Plant Sample Preparation: *Calotropis procera* plant obtained from Kerbala City, Iraq. The sample was identified and authenticated by Pharmacognosy and the Medicinal Plant Department at College of Pharmacy-Al-Mustansiriyah University, Iraq. The plant leaves are collected, washed, and dried in Pharmacognosy Department at College of Pharmacy Kerbala University, Iraq.

Solvents and Chemicals: Analytical grade, ethanol 99%, ethanol 70%, *n*-hexane, methanol, potassium mercuric iodide solution, bismuth subnitrate, glacial acetic acid, potassium iodide, ethyl acetate, chloroform, ferric chloride, sulphuric acid, ammonia, and hydrochloric acid were bought from Sigma-Aldrich.

Extraction Methods: The dried plant parts were powdered in a mechanical grinder, and then extracted by two different methods:

Extraction Method No. 1: One hundred gram of powdered *C. procera* leaves were macerated in 200 ml of *n*-hexane for 3 days then filtered. The leaves residue were re-extracted by Soxhlet with 600 ml of ethanol 95% for 10 h, then alcoholic extract cooled at room temperature and filtered, the clear filtrate is evaporated to dryness under reduced pressure by rotatory evaporator at temperature 40ºC to get 10.03 g (10.03%) ethanolic crude extract of *C. procera* leaves ETCP100 (10.03%) [29].

Extraction Method No. 2: One hundred gram of powdered *C. procera* leaves were extracted under reflux for 10 h with 600 ml of 70% ethanol. The ethanolic extract was filtered, and the clear filtrate evaporated to dryness under reduced pressure by using a rotatory evaporator at temperature 40 ºC to yield hydro-ethanolic extract ETCP70 (7.4%) [30]. A preliminary phytochemical investigation was done by alkaline reagent test for flavonoids, foam test for saponins, terpenoids test for terpenoids, Fehling's reagent for reducing sugar and Dragendroff's reagent for alkaloids as described literature [31, 32].

Chemical Identification by GC-MS: Ethanolic extracts were analyzed using a GC (Agilent Technologies 7890A) interfaced with a Mass-selective detector (MSD, Agilent 7000) equipped with a polar Agilent HP-5ms (5%-phenyl methyl polysiloxane) capillary column (30 m × 0.25 mm i. d. and 0.25 μm film thickness). The carrier gas was helium with a linear velocity of 1 ml/min. The injector and detector temperatures were 200 ºC and 250 ºC, respectively. The volume injected 1 μl of the sample. The MS operating parameters were as follows: ionization potential 70 eV, interface temperature 250 ºC, and acquisition mass range 50-800.

The identification of components was based on a comparison of their mass spectra and retention time with those of the authentic compounds and by computer matching with NIST and WILEY library as well as by comparison of the fragmentation

pattern of the Mass Spectral data with those reported in the literature

Antibacterial Activity: The antibacterial activity of ethanolic extracts ETCP70 and ETCP100 from *C. procera* leaves were investigated by the disc diffusion method (DD) and minimum inhibitory concentration (MIC). Four types of bacteria were used in this research, gram-positive bacteria which are *Bacillus subtilis* and *Staphylococcus aureus*, gram-negative bacteria which are *Escherichia coli* and *Pseudomonas aeruginosa* [33] were isolated and obtained from Al-Hussein Medical City at Karbala.

Culture Media and Material: Generation of bacteria by culture media was achieved in sterilized nutrient broth (NB) at 37 °C for 16-18 HR. Nutrient broth (NB, 8 g/L), Muller-Hinton (MH, 20 g/L), was dissolved in distilled water. The glasses (pipettes, tubes, Z-rode, and beakers), filter paper discs (6 mm in diameter) and solution (NB, and MH) were sterilized in an autoclave for 2.5 h at 121°C. The concentrations of bacteria, cultures were prepared by comparing with a McFarland solution (9.95 ml of H_2SO_4 solution 1% in broth, and 0.05 ml of $BaCl_2$ solution 1% in broth) equivalent to 150 × 10^6 colony-forming unit (CFU)/ml. Crude extracts (1800 µg/mL) were prepared by dissolving 3.6 mg in 0.5 ml DMSO.

Disc Diffusion Method: The crude extracts were investigated for antibacterial activity by disc diffusion method according to published reports [34] with some modifications. First, the Petri dishes (90×15 mm) were spread with sterilized MH (17 ml) solutions, followed by 200 µl of bacteria stock (150×10^6 CFU/ml); each was spread on the Muller Hinton agar (MH) medium using Z-glass rod, after that, 2 paper discs were individually impregnated with 20 µl of extract (1800 µg/mL), 2 blank discs (with DMSO only), standard disc of streptomycin sulfate (10 µg/disc) for bacteria was placed and arranged on MH petri dish. Finally 37 °C for 24 h.

Determination of Minimum Inhibitory Concentration (MIC): Minimum inhibitory concentration (MIC) for samples was achieved in the 96-well plate. First samples (14.4 mg) were dissolved in 2.0 ml of DMSO. The concentration of stock solution 1800 µg/mL was gradually diluted twofold to get concentration for each sample in the range of 14.07-1800 µg/mL, and then 96-wells were impregnated with 100 µl of an organism and were covered for incubation overnight at 37 °C. All samples were assessed in duplicates [35].

RESULTS: The results showed that the percentage yield of crude extracts from the extraction of *C. procera* leaves by method no.1 was 10.03% higher than that obtained from extraction by method no. 2 which yielded percentage yield 7.4%.

The preliminary phytochemical investigation revealed the presence of alkaloids, saponins, flavonoids, terpenoids, and reducing sugar in the plant leaves, but in different concentrations, as shown in **Table 1**.

TABLE 1: CHEMICAL SCREENING OF THE PHYTO-CHEMICAL COMPONENTS OF *CALOTROPIS PROCERA* LEAVES

Test name	Leaves
Alkaloid test	++
Saponin test	+
Flavonoid test	++
Terpenoid test	+
Reducing sugar test	+

The analysis of both methods that occur by using GC-MS technique, displayed about thirty-five different compounds in the *C. procera* leaves crude extract depending on the extraction method as shown in **Fig. 2-3** and recorded in **Table 2**.

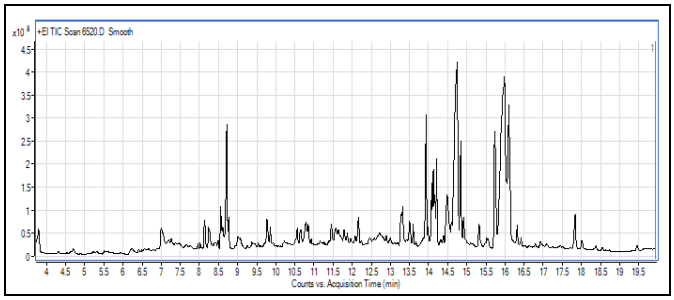


FIG. 2: GC-MS CHROMATOGRAM OF PHYTO-CHEMICALS OF ETHANOLIC EXTRACT ETCP100

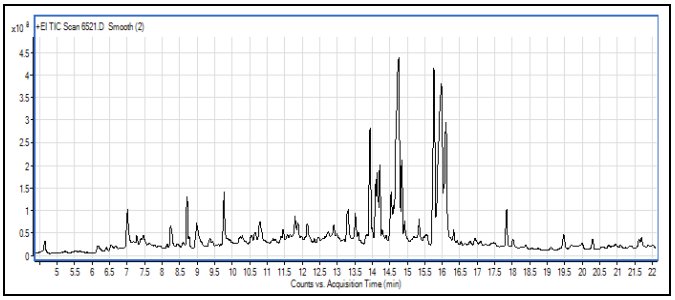


FIG. 3: GC-MS CHROMATOGRAM OF PHYTO-CHEMICALS OF ETHANOLIC EXTRACT ETCP70

TABLE 2: PHYTOCHEMICAL COMPONENTS IN *C. PROCERA* LEAVES IDENTIFIED BY GC MS TECHNIQUE

S. no.	RT (min)	Compounds	Area sum % in 100% ethanolic extract	Area sum % in 70% ethanolic extract
1	7.03	(*R*)-lavandulyl acetate	2	2.45
2	8.12	1-Terpinenol	0.99	0.33
3	8.24	Isopulegol	1.22	1.79
4	8.59	3-Carene	2.33	0.42
5	8.71	Camphene	6.22	3.07
6	9.051	Hydrocoumarin	1.62	2.79
7	9.38	canrenone	0.61	0.79
8	9.76	2-Methoxy-4-vinylphenol	1.56	2.57
9	9.85	O-sec-butyl-phenol	0.86	0.34
10	10.25	trans-calamenene	0.99	1.25
11	10.8	diphenyl(piperidin-2-yl)methanol	1.8	1.67
12	11.4	Geranylisovalerate	0.33	0.36
13	11.59	Melezitose	0.96	0.96
14	11.8	Coniferol	1.07	2.03
15	12.16	1-Eicosene	1.15	1.67
16	12.77	4-Hydroxy-*β*-ionone	0.75	1.03
17	13.29	Tetrahydrospirilloxanthin	1.03	2.37
18	13.5	Minovine	2.77	1.23
19	13.6	cis-Vaccenic acid	0.71	0.44
20	13.93	phytol	0.41	4.38
21	14.13	Nonadecanol	4.32	4.94
22	14.2	*β*-Citronellol	4.89	2.59
23	14.72	n-Hexadecanoic acid	3.23	2.56
24	14.8	Octadecanoic acid	3.08	20.5
25	15.32	Dodecanoic acid	19.15	1.21
26	15.55	Methyl arachidonate	1.04	1.26
27	15.72	Hexa-hydro-farnesol	0.81	9.87
28	15.92	Gamolenic acid	4.11	12.71
29	16.1	Linolenic acid, ethyl ester	14.87	6.83
30	16.33	Thebaine	7.59	0.86
31	17.8	(+)-*α*-Tocopherol	3.61	2.11
32	18.01	Zearalenone	0.83	0.69
33	18.39	Colchicine	0.8	0.34
34	18.56	Z)-9-Tricosene	0.74	0.34
35	19.46	pseudojervine	1.55	1.24

TABLE 3: ANTIBACTERIAL ACTIVITY OF *C. PROCERA* ETHANOLIC EXTRACTS OF LEAVES BY DISC DIFFUSION METHOD ON MULLER-HINTON AGAR

Bacteria species	ETCP70 DD (mm)	ETCP100 DD(mm)	Control (Streptomycin) DD(mm)
Basillus subtilis	17.0 ± 0.0	10.5 ± 0.7	34.5 ± 3.5
Staphylococcus aureus	18.0 ± 1.4	14.0 ± 0.0	19.0 ± 1
Escherichia coli	6.0 ± 0.0	6.0 ± 0.0	22.5 ± 0.7
Pseudomonas aeruginosa	6.0 ± 0.0	6.0 ± 0.0	17.0 ± 0.5

Data represent: mean + standard deviation of duplicated experiments. DD = disc diffusion, mm= millimeter; 6.0 ± 0 = no activity.

TABLE 4: MIC OF ETHANOLIC EXTRACT 70% OF *C. PROCERA* LEAVES

Bacteria species	Ethanolic extract 70% µg/mL
Bacillus subtilis	31.25
Staphylococcus aureus	250

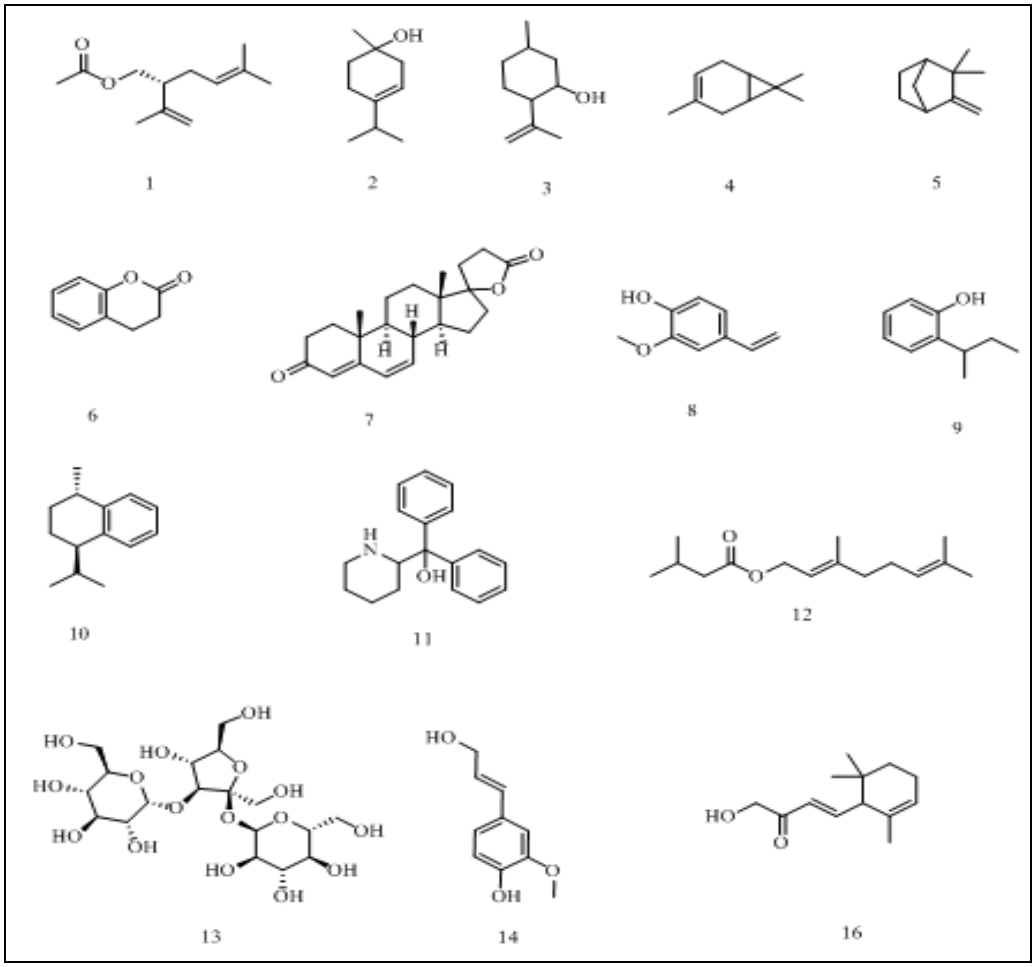
DISCUSSION: The results showed that the best extraction method for *C. procera* leaves was in method no.1 which gives percentage yield 10.03% of ETCP100 extract, while method no. 2 was 7.4% of ETCP70 extract. This difference due to the extraction method, and polarity of the solvent extractors. The phytochemical investigation using chemical reagents exhibited that *C. procera* leaves contain many active compounds like alkaloids, flavonoids, saponins, terpenoids, and reducing sugar.

The chemical components of the ethanolic extract of the leaves of *C. procera* which identified by GC-MS are tabulated in **Table 3**. The 70% and 100% ethanolic extracts consisted mainly from thirty-five compounds ranging from terpenes, fatty acids,

essential oils, an aldosterone antagonist, steroids, alkaloids, phenolic compounds, esters, vitamins, coumarins, and others. The predominant constituents were 1-Eicosene, 4-Hydroxy-β-ionone, phytol, Citronellol, *n*-hexadecanoic acid, octadecanoic acid, dodecanoic acid, methyl arachidonate, hexa-hydro-farnesol, (+)-*α*-Tocopherol, gamolenic acid, linolenic acid ethyl ester, thebaine, colchicine, nonadecanol, camphene, coniferol, tetrahydro-spirilloxanthin, pipradol, pseudojervine, and zearalenone. Most of the compounds are alkaloids according to their structures and that what appear in the phytochemical study as shown in **Fig. 4**. The highest percent in 100% ethanolic leaves extract belongs to camphene 6.22%, thebaine 7.59%, dodecanoic acid 19.15%, and linolenic acid ethyl ester 14.87%, while the highest percent in 70% ethanolic leaves extract belongs to hexa-hydro-farnesol 9.87%, gamolenic acid 12.71%, and linolenic acid ethyl ester 6.83%. The differences in these percent explained by the rule of like dissolve like, the more polar compound appears in 70% ethanol and *vice versa*.

The ethanolic extracts of leaves were examined for antibacterial activity against *S. aureus*, *E. coli*, *B. subtilis* and *Pseudomonas species* which determined by using disc diffusion method [36] and minimum inhibition concentration (MIC). The results of disc diffusion method revealed that ethanol 70% was the best solvent for the antibacterial properties of leaves of *C. procera* because it gave the widest zone of inhibition with gram-positive bacteria 18 ± 1.4 mm against *Staphylococcus aureus* and 17 ± 0.0 mm against *Basillus subtilis* as shown in **Table 3**.

Base on the results of disc diffusion method, only bacteria *S. aureus* and *B. subtilis* were submitted to MIC method, and the results showed strong inhibitory towards *Staphylococcus aureus* (250 µg/mL) and against *Bacillus subtilis* (31.25 µg/mL) as shown in **Table 4**. The antibacterial activity was reported due to the presence of alkaloids, flavonoids, saponins, terpenoids, and reducing sugar and other active compounds.



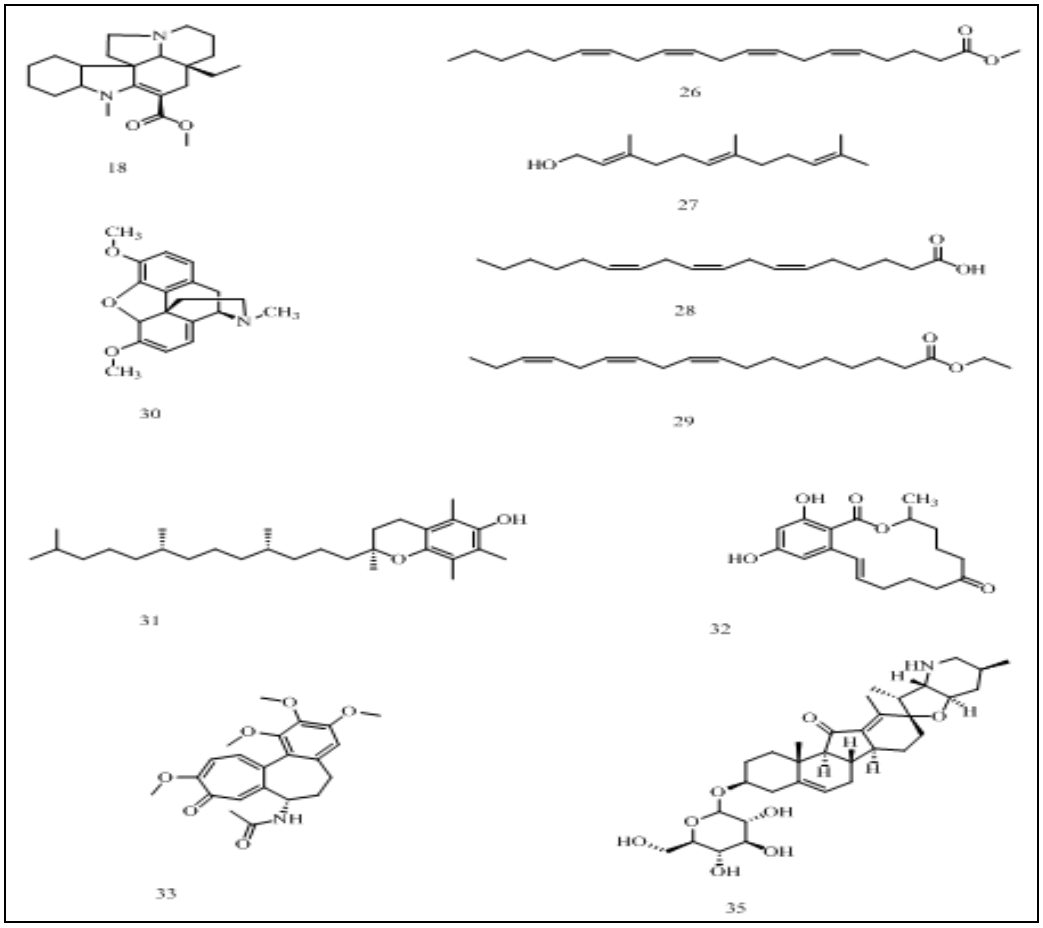


FIG. 4: STRUCTURES OF COMPOUNDS IN THE LEAVES OF *C. PROCERA*

CONCLUSION: The phytochemical screening indicated that alkaloids and phenolic compounds were present in large quantities. The leaves of *C. procera* can be useful for the treatment of cancer due to the antioxidant activity of phenolic compounds. Isolation and identification of active compounds are important to discover new drug from this plant because little articles appear on this side. Further research is required for these parts of the plant in Iraq.

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CONFLICT OF INTEREST: Nil

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