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RESEARCH ARTICLE

Preliminary Phytochemical Investigation and Antibacterial Activity Identification of Stems of Calotropis procera Plant in Iraq by GC. MS

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Abstract

Calotropis procera is a plant from Asclepiadaceae family. The preliminary phytochemical investigation of C. *procera* stems exhibit its high contents of flavonoids, alkaloids, saponins, while terpenoids, and reducing sugar are in lower percent. Its previously used in treatment of many diseases such as cough, bronchitis, ascites, asthma, eczema, leprosy, cutaneous diseases, and intestinal worms. GC. MS analysis of stems ethanolic extract showed that the highest percent in 100% ethanolic stems extract belongs to Octadecanoic acid 20.33%, Hexa-hydro-farnesol 6.16%, and Linolenic acid ethyl ester 39.59 while the highest percent in 70% ethanolic stems extract belongs to (-)-Vincadifformine 17.13%, Dodecanoic acid 13.97%, Methyl arachidonate 9.01%, and (+)-α-Tocopherol 6.66%. The others chemical compounds percent range from about 5%-0.3%. The antibacterial activity of stems of *Calotropis procera* plant compared with streptomycin as standard antibiotic against four different types of bacteria revealed that the ethanolic extracts have no effect on *Escherichia coli, and Pseudomonas sp.* While these extracts have ability to kill *Staphylococcus aureus,* and *Basillus subtilis* bacteria which are more sensitive.

Keywords: *Usher, GC. MS, Calotropis Procera, phytochemical, Sodom apple, Antibacterial.*

Introduction

The medicinal plants that has therapeutic or pharmacological properties such as, antioxidant, anti-malarial, antimicrobial, anti-diabetic, anti-cholinergic, and anti-carcinogenic activity produce bioactive components.

Due to producing many secondary metabolites these medicinal plants used in different cultures around the world [1]. The most important bioactive components are alkaloids, cardiac glycosides, flavonoids, phenolic compounds, tannins and etc [2, 3].

Calotropis procera is a flowering plant that belongs to asclepaiadaceae family [4] which includes approximately 2,000 species and more than 280 genera [5]. C. Procera appears like a shrub that widely distributed in West Africa and other parts of tropics [6].

The plant is perennial, large, tall, erect, and highly branched with milky latex throughout Fig.1. All parts of the plant exude milky latex when it broken or cut, which act as a defense strategy against fungi, insects, and viruses.

The secondary metabolites which are isolated from C. procera include cardiac glycosides, flavonoids, sterols and Triterpenes [7]. C. procera is traditionally used for the treatment of a wide range of infections globally [8, 10].

The antimicrobial activity of C. procera extract against fungi and bacteria was well documented [11, 14]. Pharmacological studies of Calotropis species showed anti-inflammatory, anti-tumor [15], antioxidant [16, 17] antibacterial [18], anti-diarrheal [19], antifungal [20] and Nanoparticles Synthesize activities [21].

Fig.1: *Calotropis procera* stems, leaves, fruits and flowers

Common Names

The habitat of *C. procera* plays important role in its names, such as Usher and Kisher in Arabic, Sodom apple in English, Calotropis, Dead Sea fruit, Calotrope, Mudar fibre, Desert wick, Rubber bush Giant milkweed, Swallow-wort Rubber tree, and Aak or Ak is the local name of this shrub and Akdo, Akada and Madar in Hindi [22,23]. In West of Nigeria, it is called *Bomubomu* by the Yorubas, the Hausas call it *tumfafia*, in Sudan it is called *Oshar*, in Italia it's called *calotrops*, in French it's called *pomme de sodome*, in Brazil it's called cotton silk, silk flower, and "queimadeira" [24].

Medicinal Uses

As folk medicine *C. procera* leaves are used in many countries to decrease blood glucose level in patients whom suffering from diabetes mellitus [25]. Traditionally the stems and secretions from *C. procera* roots are used in India in treatment of intestinal worms, enlargements of abdominal viscera, and skin diseases [26]. In Nigeria, it's used to treat diseases such as leprosy, fever, convulsion, eczema, ringworm, asthma cough, and diarrhea [27]. The latex of *C. procera* is used in treatment of inflammations, low hectic fevers and malarial [28]; it has also antidiarrheal properties because of its desensitizing effect on the smooth muscles of gastrointestinal tract [29].

C. procera fruits are used as anti-inflammatory, anti- rheumatism, antioxidant, and antimicrobial and hepatoprotective agents [30, 31]. The flowers also have anti-inflammatory, hepatoprotective activity, analgesic, antipyretic, antioxidant and antibacterial activities. Also *C. procera* has antidiabetic, cardiovascular, anthelmintic, gastro-protective, anticancer, anticonvulsant, hypolipidemic, wound healing and contraceptive properties in rats [32].

Phytochemistry

GC-MS is the best technique to investigate the chemical compositions of long chain hydrocarbons, alkaloids, alcohols, steroids, acids, amino, nitro compounds and esters, etc [33]. Therefore GC-MS technique used to identify the bioactive compounds present in this plant [34]. Different parts of C. procera are reported to have abundant phytochemical constituent as flavonoids, tannins, sterols, alkaloids, cardiac glycosides, sterols and tri-terpenes [35]. Really, two new flavonoids were identified from *C. procera*, which are rutin and quercetin 3-O-galactoside [36]. *C. procera* is considered as a source of digitalis-like therapeutic agents and is highly toxic to the land snail [37].

Biological Activity

C. procera biological activities include antifungal, anticancer, and insecticidal activity.

Four bacterial strains were used in in our Lab, these bacterial strains are:

- *Bacillus subtilis* is a *gram-* positive bacterium [38] allowing the organism for resistance intensive environmental circumstances.
- *Staphylococcus aureus* is a *gram-* positive bacteria and the major pathogen of increasing importance because of increase in the antibiotic resistance [39]. It can cause impetigo, abscesses, infected wounds, skin infections, and boils carbuncles
- *Escherichia coli* are *gram*-negative bacteria with rod-shape normally harmless, found in lower intestine of the warm- blooded organisms [40].

- *Pseudomonas aeruginosa* is a gram-negative bacterium, found on the surfaces of plants and animals. It causes respiratory tract infection, dermatitis, urinary tract infection, joint and bone infection, soft tissue infection, gastrointestinal infection and a other systemic infections.

Materials and Methods

Plant Sample Preparation

The stems of *Calotropis procera* plant obtained from Kerbala city, Iraq. The sample was authenticated and identified by Pharmacognosy and medicinal plants department at College of Pharmacy/Al-Mustansiriyah University, Iraq. After collection of the plant stems they are washed, and then dried in the Pharmacognosy and medicinal plants department at College of Pharmacy/ University of Kerbala, Iraq.

Extraction Methods

The dried stems of *C. procera* were grind in a mechanical grinder to a coarse powder, then extracted by two different methods:

Extraction Method No.1

one hundred gram of *C. procera* powdered stems were extracted by soxhlet apparatus with 600ml of ethanol 95% for 10 hours, then the ethanolic extract cooled at room temperature and filtered, the clear filtrate evaporate to dryness under reduced pressure by rotatory evaporator at temperature didn't exceed 40°c to give a crude extract [41].

Extraction Method No.2

one hundred gram of powdered stems of *C. procera* were extracted by reflux apparatus for 10 hours with 600 ml of 70% ethanol, then the ethanolic extract was filtered and the clear filtrate evaporate to dryness under reduced pressure by using rotatory evaporator at temperature didn't exceed 40°c to give a crud extract [42]. The phytochemical investigation carried out by using Dragendroff's reagent for alkaloids, alkaline reagent test for flavonoids, terpenoid's test for terpenoids, foam test for saponins, and Fehling's reagent for reducing sugar [43].

Antibacterial Activity

The antibacterial activity of *C. procera* stems extracts can be determined by disc diffusion method (DD) as qualitative assay. The agar disk diffusion method developed in 1940, which is the official method that used in many microbiological laboratories for the routine antimicrobial susceptibility test. The complete assay takes time for about five days. Four types of bacteria were used in this research, two gram (+) bacteria which are *Basillus subtilis* and *Staphylococcus aureus*, and two gram (-) bacteria which are *Escherichia coli* and *Pseudomonas aeruginosa*, [44] all bacteria brought from from Al-Hussein Medical City at Karbala.

Culture Media and Material

The Culture media that are used to generate bacteria were achieved in the sterilized nutrient broth (NB) for 16-18 hour at 37 °C. Muller Hinton (MH, 20 g/L), and Nutrient Broth (NB, 8 g/L) were dissolved in distilled water. The glasses (Z-rode, pipettes, beakers, and tubes), solutions (NB, MH) and filter paper discs (6 mm in diameter) were sterilized in autoclave at 121 °C for 2.5 hour.

The concentration of culture and bacteria, was prepared by comparing it with McFarland solution (0.05 ml of BaCl2 solution 1 % in broth, and 9.95 ml of H2SO4 solution 1 % in broth) equivalent to $150x10^6$ colony-forming unit (CFU)/ml. Crude extracts (1800 µg/ml) were prepared by dissolving 3.6 mg in 0.5 ml of DMSO.

Disc Diffusion Method

Two crude extracts were investigated by disc diffusion method for antibacterial activity according to the published report [45] with some modifications. First of all, 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 by use Z-glass rod on Muller Hinton agar (MH) medium, after that, two paper discs were individually impregnated with 20 µl of extract (1800 µg/mL), two blank discs (with DMSO only), standard disc of streptomycin sulfate (10 µg/disc) for bacteria was arranged and placed on MH Petri dish .Finally 37°C for 24 h.

GCMS Analysis Technique

GC. MS analysis technique for Ethanolic extracts was carried out by using GC spectrometry instrument at the Regional Center for Food & Feed (RCFF) that established in 1980 under the name of Central Laboratory for Food & Feed (CLFF) with the cooperation of the government of

Denmark under the authority of the Egyptian Agricultural Organization within Ministry of Agriculture. The GC system was a GC (Agilent Technology 7890A) interface with the mass-selective detector (MSD, Agilent 7000) equipped with a polar Agilent HP-5ms (5% phenyl methyl poly siloxane) capillary column (30 m × 0.25 mm i. d. and 0.25 µm film thickness).

Helium was the carrier gas with linear velocity of 1ml/min. The temperature of detector and injector was 250º C and 200º C, respectively. The injected volume of sample was 1µl. The MS operating parameters were as follow: acquisition mass range 50–800, ionization potential 70 e V, and interface temperature 250º C. The compounds identification depend on the comparison obetween their retention time and mass spectra with those of the authentic compounds and by computer matching with those NIST and WILEY library as well as by comparison fragmentation pattern of mass spectral data with those in literature [46].

Results

The *C. procera* extraction methods of stems give percentage yields of the crude extracts so method No.2 give 4.57% while extraction method No.1 give 8.34% which was higher than that obtained from extraction method No.1 as shown in Table :1.

Table 1: Percentage yield of stem crude extracts of *C.procera*, obtained from extraction methods No.1, and No.2

Extraction methods	% yield of stems crude extract of *C. procera*
Method No.1	4.57
Method No.2	8.34

The preliminary phytochemical investigation showed the presence of flavonoids, terpenoids, alkaloids, reducing sugar, and saponins in plant crude extracts but they are differ in concentrations as shown in Table: 2.

Table 2: Phytochemical investigation of flavonoids, terpenoids, alkaloids, reducing sugar, and saponins

Test name	Stems
Flavonoid test	+++
Reducing sugar test	+
Alkaloid test	+++
Terpenoid test	+
saponins test	++

Table 3: Antibacterial activity of stems of *C. procera* ethanolic extracts by disc diffusion method on Muller-Hinton agar

Bacteria species	Ethanolic extract 70% DD (mm)	Ethanolic extract 100% DD (mm)	Control (Streptomycin) DD(mm)
Staphylococcus aureus	11.5 ± 0.7	11.5 ± 0.7	34.5 ± 3.5
Basillus subtilis	10.5 ± 0.7	11.0 ± 1.4	21.0 ± 1.4
Escherichia coli	6.0 ± 0.0	6.0 ± 0.0	22.5 ± 0.7
Pseudomonas aeruginosa	6.0 ± 0.0	6.0 ± 0.0	19.0 ± 1.4

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

Both methods were analyzed by GC. MS technique to determine about forty different compounds in the *C. procera* ethanolic extract depending on the extraction method as shown in Figures (2-3) and recorded in Table 4.

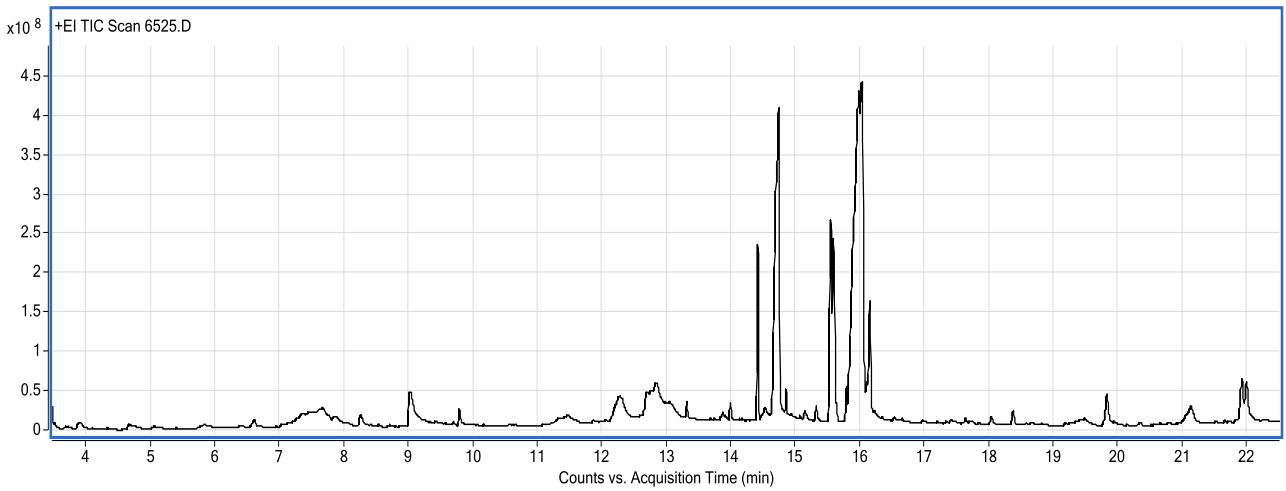


Fig.2: GC-MS chromatograms of phytochemicals of C. procera stem Ethanolic Extract 100%

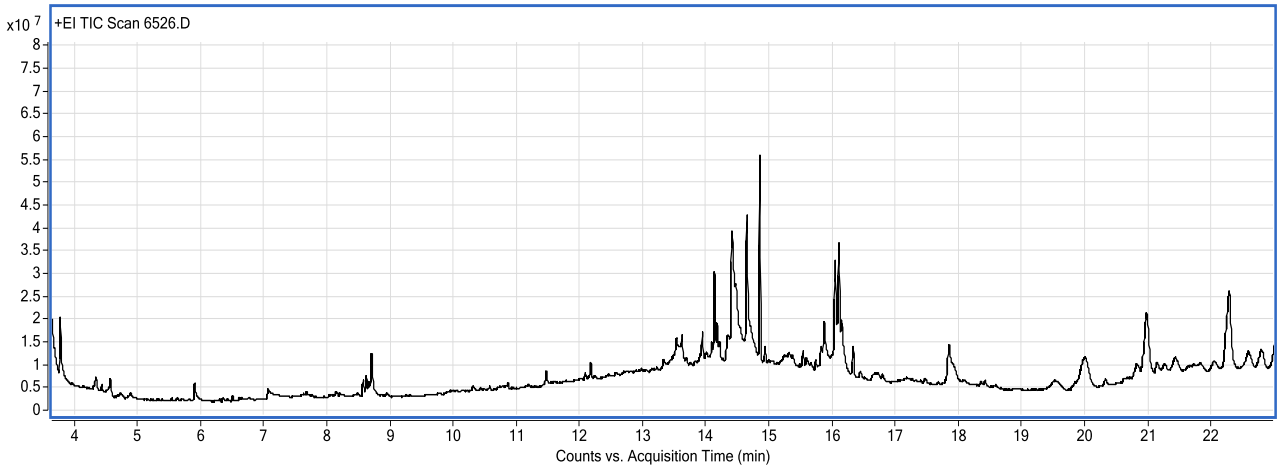
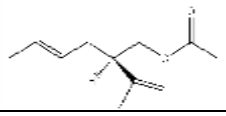
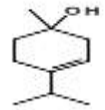
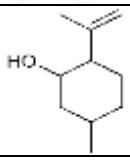
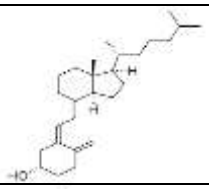
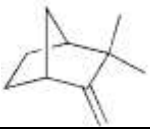
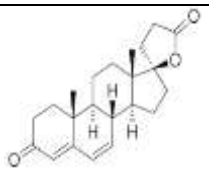
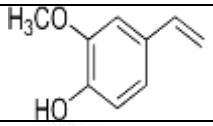
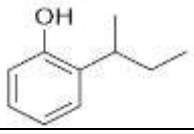
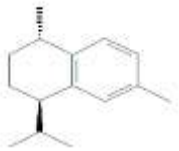
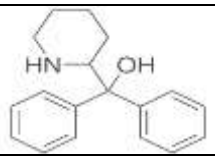
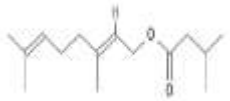
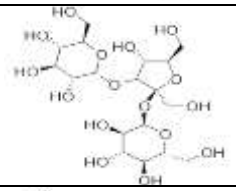
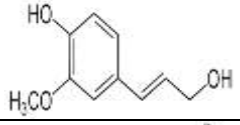
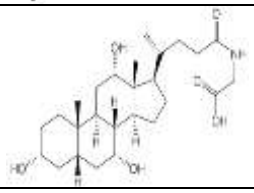
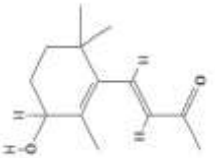
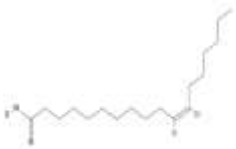
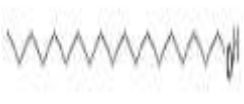
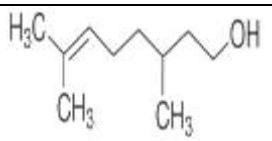
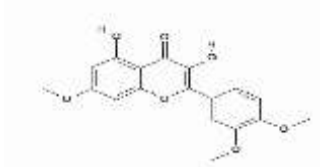
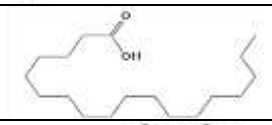
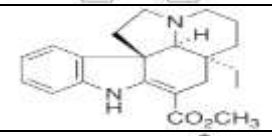
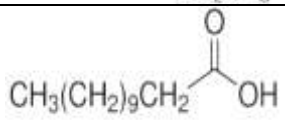
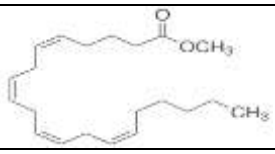
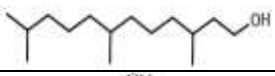
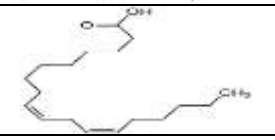
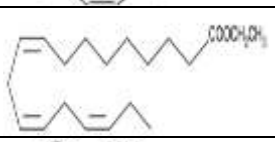
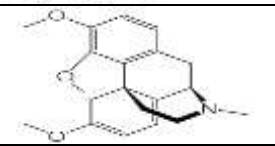
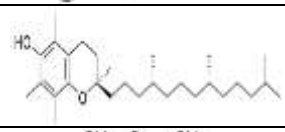
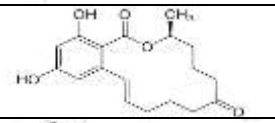
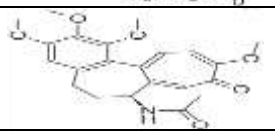
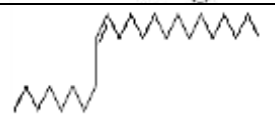
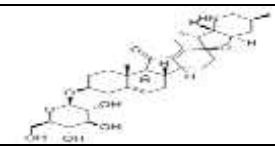


Fig.3: GC-MS chromatograms of phytochemicals of C. procera stem Ethanolic Extract 70%

Table: 4: Compounds in ethanolic extracts of *C. procera* stems plant identified by GC.MS technique

No.	RT (min)	Compound	Area sum % in 100% stems Ethanolic extract	Area sum % in 70% stems Ethanolic extract	Structure
1	7.03	(R)-lavandulyl acetate	0.52	1.13	
2	8.12	1-Terpinenol	0.44	0.55	
3	8.24	Isopulegol	0.65	0.54	
4	8.59	3-Carene	0.42	0.70	
5	8.61	Cholecalciferol	-	0.77	
6	8.71	Camphene	0.4	1.87	
7	9.051	Hydrocoumarin	2.96	0.62	
8	9.38	Canrenone	0.4	0.92	
9	9.76	2-Methoxy-4-vinylphenol	0.44	1.04	
10	9.85	O-sec-butyl-phenol	0.54	0.64	

11	10.25	trans-calamenene	0.58	0.78	
12	10.8	Pipradrol	0.69	0.68	
13	11.4	Geranyl isovalerate	1.01	0.56	
14	11.59	Melezitose	1.23	0.76	
15	11.8	Coniferol	0.54	0.91	
16	12.07	Glycocholic acid	-	1.05	
17	12.16	1-Eicosene	3.47	0.59	
18	12.77	4-Hydroxy-ß-ionone	4.43	0.78	
19	13.29	Tetrahydrospirilloxanthin	0.47	1.57	
20	13.5	Minovine	0.68	0.65	
21	13.6	cis-Vaccenic acid	0.64	0.76	
22	13.93	Phytol	0.44	0.73	
24	14.13	Nonadecanol	0.47	0.86	

25	14.2	ß-Citronellol	0.42	3.16	
26	14.33	3',4',7-Trimethylquercetin	-	0.96	
27	14.72	n-Hexadecanoic acid	0.43	0.63	
28	14.8	Octadecanoic acid	20.33	3.03	
29	14.91	(-)-Vincadifformine	-	17.13	
30	15.32	Dodecanoic acid	0.4	13.97	
31	15.55	Methyl arachidonate	0.66	9.01	
32	15.72	Hexa-hydro-farnesol	6.16	0.84	
33	15.92	Gamolenic acid	1.04	2.22	
34	16.1	Linolenic acid, ethyl ester	39.59	1.18	
35	16.33	Thebaine	3.01	3.56	
36	17.8	(+)-α-Tocopherol	0.72	6.66	
37	18.01	Zearalenone	0.82	4.29	
38	18.39	Colchicine	0.93	2.6	
39	18.56	Z)-9-Tricosene	0.52	2.3	
40	19.46	Pseudojervine	0.74	2.2	

Discussion

The results exhibit the best extraction method of *C. procera* stems was method no.2 which gives percentage yield 8.34% of crude extract. This differences could belong to the extraction method no.1 performed by soxhlet apparatus which is preferred for soft plant structure like leaves, while extraction method no.2 done by reflux apparatus which is preferred for hard plant parts like stems which was used in this study, direct heat source facilitate the active compounds to dissolve in solvent and then extracted, and the differences in solvent polarity .The preliminary phytochemical investigation exhibit that C. *procera* stems contain highest percent of active compounds like alkaloids, flavonoids, saponins, terpenoids, and reducing sugar.

The ethanolic stems extracts were investigated for antibacterial activity against *S. aureus, E. coli*, *p. species* and *B. subtilis.* Which determined by using disc diffusion methods [47] .The results revealed that ethanol 100% was the best extractive solvent of *C. procera* stems for antibacterial properties because it gave the widest zone of inhibition 11.5±0.7mm against *S. aureus* and 11.0±1.4mm against *B. subtilis*.

Alcoholic extracts exhibit mild antibacterial activity against clinically isolated pathogenic microbial strains in comparison to the standard, streptomycin. The observations were listed in Table 3. The antibacterial activity was showed because of the presence of bioactive components. The growth of the previous four bacterial isolates were inhibited by the two ethanolic extracts except *P. aeruginosa* and *E. coli* that were more resisted to the ethanolic extracts of stems of *C.procera*. The chemical compositions of ethanolic extract of *C. procera* stems which investigated by GC.MS is tabulated in Table 4. Forty compounds are the compositions of 70% and 100% ethanolic extracts ranging from aldosterone antagonist, terpenes, essential oils, alkaloids, fatty acids, protein, phenolic compounds, steroids, coumarines, vitamins, esters and others. The predominant compounds were hydrocoumarin,1-Eicosene, Methyl arachidonate, colchicine, Octadecanoic acid, Z)-9-Tricosene, 4-Hydroxy-β-ionone, (+)-α-Tocopherol, pesudojervine, Phytol, Hexadecanoic acid, Rhodopsin, Citronellol, (-)-Vincadifformine, Dodecanoic acid, Hexa-hydro-farnesol, (+)-α-Tocopherol, Gamolenic acid, Linolenic acid ethyl ester, Thebaine, and Zearalenone. The highest percent in 100% ethanolic stems extract belongs to Octadecanoic acid 20.33%, Hexa-hydro-farnesol 6.16%, and Linolenic acid ethyl ester 39.59 while the highest percent in 70% ethanolic stems extract belongs to (-)-Vincadifformine 17.13%, Dodecanoic acid 13.97%, Methyl arachidonate 9.01%, and (+)-α-Tocopherol 6.66%. The others chemical compounds percent range from about 5%-0.3%. These differences in percent belong to the rule of like dissolve like, so the more polar compound could appear in 70% ethanolic extract and vice versa in case of 100% ethanolic extract.

Conclusion

The preliminary phytochemical investigation revealed that flavonoids and alkaloids compounds were present in large quantities compared to the other active constituents. The high percent of the phenolic compounds in *C. procera* stems give it benefit in cancer treatment because of antioxidant activity of flavonoids and other phenolic compounds. Isolation and identification of these active components are very important to discover a new drug from *C. procera* plant because of very little researches about it, so further researches are required for other parts of this plant in Iraq.

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