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Evaluation of the cytotoxic effects of the colchicine compound isolated from the leaves of *Calotropis procera* (Ait) against MCF-7 and SK-GT-4 cancer cell lines.

***Muna A.Y. Al-Mussawii*[1] *Emad Y.A.AL-Sultan*[2]* *Maytham A.AL-Hamdani*[2]**

[1]Department of Pharmacognosy and Medicinal Plants, Pharmacy College, University of Basrah, Iraq.
[2]Department of Biology , College of Education for pure science, University of Basrah, Iraq.
*Corresponding author: emad.awed@uobasrah.edu.iq
E-mail addresses: muna.yahya@uobasrah.edu.iq , maytham.abdulkadir@uobasrah.edu.iq

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Abstract:

Alkaloids are regarded as important nitrogen-containing chemical compounds that serve as a rich source for discovering and developing new drugs where most plant-origin alkaloids have antiproliferation effects on different kinds of cancers. Alkaloids' continence of *Calotropis procera* leaves are detected by two biochemical alkaloid reagents. Also GC-MS analysis for leaf alkaloid extract was done that showed the existence of one type of alkaloid compound at retention time12.8min detected as colchicine ($C_{22}H_{25}N0_6$) by comparing it with colchicine standard reference (Sigma Aldrich) with M.wt 399g/mol and percentage area 7.1%**.** Furthermore, identification, separation, and purification for purified colchicine compound were conducted by HPLC technique that gave one main peak at RT reached 2.5min compared with the standard reference. Evaluation of the anticancer activity of purified colchicine on two (MCF-7 &SK-GT-4) cell lines revealed significant cytotoxicity on the MCF-7 cell line that was superior to its cytotoxicity on the SK-GT4 cell line. With calculated IC**50** reached 55.33µg/ml &522 µg/ml respectively.

Keywords: Anticancer activity, *Calotropis procera* Leaf, Colchicine, Extraction and purification, GC-MS, HPLC.

Introduction:

More than 80% of the world's population still depends on traditional medicines for many diseases, and natural products have been used worldwide for medicinal purposes for along years [1]. As a result, this is a new era of natural product discovery. Thus, there is an urgent need to discover modern therapeutic phytochemical agents with different chemical structures and also rare mechanisms of actions are needed for new and emerging infectious diseases [2-3]. Alkaloids can be found in Flowers, roots, fruits, leaves, seeds and non-flowering plants, such as paclitaxel,[4]. *Calotropis procera* (Family: Asclepiadaceae) is a cultivable wild xerophytes' shrub found across Africa, Asia, and South America [5]. *Calotropis spp.* is a small genus of shrubs or small trees that can be found in the tropical and sub-tropical regions of Asia, Africa, and central and southern America. This species was found in India in two species, *C.procera* and *C.gigantean* L, which resemble each other in structure and uses, that is, fiber used as fuel [6]. *C.procera* is used to reduce Filarial symptoms alleviated by attaching a red thread to the afflicted area[7]. There is a plethora of literature available that demonstrates the blossoms of this plant have hepatoprotective properties when taken in conjunction with paracetamol to protect the liver. Different plant parts, particularly the latex, are tested against various cancer cell lines [8,9]. alkaloid compounds founded in *C. procera* leaf will be isolated ,purified, and tested their anticancer effect on MCF-7 and SK-GT-4 cell lines.

Materials and method:

Plants collection

Calotropis procera was collected on March 2020 from Basrah governorate, Southern of Iraq. Identification of the field collected plants Fig. 1 was authenticated as *Calotropis procera* by plant Taxonomist Prof Dr. Sahar Abd Al-abaas Malik, College of Science, Department of Biology, University of Basrah.

A Classification of *Calotropis procera*

Kingdom: Plantae

Phylum: Spermatophyta

Class: Magnoliopsida

Order: Gentianales

Family: Asclepiadaceae

Genus: *Calotropis*

Species: *Calotropis procera* Ait on R.Br.

Figure 1. Plant of *Calotropis procera* Ait on R. Br.

Preparation of selection plants extraction

After plant's classification, the leaves of plant were thoroughly washed by using tap water to remove any contaminates and then shade dried at 40 ° Cand the dried leaves were ground to a fine powder through a mechanical grinder and then stored in tight plastic bags labeled for study.

Preliminary Biochemical test for alkaloid compounds detection

Extraction for leaves of *C. procera* was done by using methanol (Hot continuous extraction) method for detection of alkaloid compounds[10].

Dragendorffs reagent: 1ml of a reddish-brown or orange precipitate formed when the reagent was added drop by drop to the extract shows the presence of alkaloids.

Mayer reagent: 1 ml of drop-by-drop reagent is applied to the extract and a creamy precipitate is formed as a result.

Extraction of total Alkaloids

Alkaloid compounds were extracted by method of Harborne[10]. After 24 hrs. of continuous extraction with 80 percent Ethanol, the soxhlet apparatus 250 ml volume was filtered and the filtrate was concentrated under vacuum at 45Cº until the solution reached 10ml and transferred to a separate funnel where 2 N HCl was gradually added to make it reach (pH=2), then the extract was washed with 10 ml chloroform three times, the pH value of the extract was increased to reach (pH=10) using NH4 OH that partitioned with 10 ml chloroform 3times. The chloroform portion dried to obtain the total alkaloid extract, that kept in a clean container at 4ºC for later research.

***Screening* for alkaloid compounds by using Gas chromatography-mass spectrum (GC-Mass) analysis**

GC-MS analyses were done in Nihranbinomar-Laboratory- Basra oil company. Leaf Methanolic alkaloid extract of *C.procera* for detecting their alkaloid types and structure was done by using a modified method by GC-.MS analysis[11]. Spectrometer Agilent gas chromatograph equipped and coupled to a mass detector Agilent 5977A spectrometer with an HP- 5MS (5% Phenyl methyl siloxane), 30m ×0.25mm × 0.25 mm ID of the capillary column. The temperature of the injector was 40 C maintained for 5 min then raised gradually to 300 ºC at a rate of increment 10\min. Helium gas 99.99% used as mobile phase at a flow rate of 1ml\min. an injection volume of 1 µL. Mass spectra were taken at 70 ev; a scan-interval of 4 min. and fragments from 45 to 450 Da. The solvent delay was 4min. and the total GC-MS running time was 45min. The samples were injected in (Split mode) 50:1 Mass spectral scan range was set at 45 to 650 m/z .

Identification , Separation & Purification of alkaloid compounds by using the High Performance Liquid Chromatography (HPLC) technique

HPLC analysis was carried out using a modified method[12], LC-W100A HPLC (USA) system connected to LC-UV100 plus UV detector with manual injectors. Data interfered using PC with (les (x86) \HPLC SYSTEM) separation was performed through Exformma technologies Column Arcus EPC18 5um, 4.6 x 250mm, isocratic mobile phase used for the analysis of leaf methanolic alkaloid extract consist of (acetonitrile75: water:25), with 1ml/min flow rate at 25 ºC, and pressure of 100 p.s.i, injection volume was 10 µL. 0.05 mg\ml sample of extract that was filtered by 125mm filter paper before injection, the run time was for…delete for 10 min for each run, and detection was conducted at 300 nm wavelength. Thus, a modified method was used to separate and purify the

colchicine compound by HPLC [12-13]. The eluted mobile phase during the appearance of the identified peak of colchicine with recorded retention time by HPLC at R.T reached 2.5min. under the same conditions. Isolated mobile phase portions then undergo HPLC analysis to confirm the purity of the isolated.

***In vitro* anticancer activityMTT assay against the (SK-GT-4 and MCF-7) cell cultures**

Maintenance of cell cultures

To maintain the cancer cell lines,of **MCF-7 (breast adenocarcinoma derived from metastatic site: pleural effusion)** and **SK-GT-4** (esophagus **adenocarcinoma derived from metastatic site: pleural effusion)** (the IRAQ Biotech Cell Bank Unit in Basrah) provided cancer cell lines, and RPMI-1640 supplemented with 10% Fetal bovine serum, 100 units/mL penicillin, and 100 g/mL streptomycin was used. Reseeded at 70 percent confluence twice a week, the cells were kept at 37 C^{o} and 5% CO2 for three weeks [14].

Cytotoxicity Assays

C. procera's colchicine was used in the MTT cell viability assay on 96-well plates to investigate the cytotoxic effect of purified colchicine.10^4 cells/well were used to seed cell lines. Cells were treated with (colchicine) at varying concentrations 100, 250, 500, 750, 1000 of µg/µl after 24 hours or after a confluent monolayer had formed. A solution of 2 mg/mL MTT (after incubating the cells for 2 hours at 37°C) was used to evaluate cell viability after 72 hours of treatment. To dissolve the residual crystals, 10 µL of DMSO (Dimethyl Sulphoxide) was added to the wells and incubated for 15 minutes at 37 °C [15], The assay was carried out in triplicate and the absorbency was measured using a microplate reader at a test wavelength of 620 nm. The proportion of cytotoxicity (cell growth inhibition) was estimated as follows: If A is the mean optical density in untreated wells and B is the mean optical density in treated ones, then PR = B/A*100 and IR = 100 - PR [16-19].

Acridine Orange/Ethidium Bromide (AO/EB) staining

Ethidium bromide and Acridine Orange 100µg/ml both were added to the cells and kept in dark at room temperature. The morphological changes were observed using a fluorescence microscope [20].

Results and discussion:

Biochemical tests for alkaloid compounds' detection

A positive test appeared for methanolic leaf extract of *C. procera* as orange and white precipitate for Dragendorff and Mayer's reagent respectively, as shown in Table 1. Methanol was used as a strong solvent for bioactive extraction for the leaf of *C.procera.* An alkaloid compounds found in large amounts in the leaf of *C. procera* [15].

Table 1. Biochemical tests for alkaloid compounds detection.

Hot continuous extraction	Alkaloid test	
	Dragendorff reagent	Mayer Reagent
Leaf Methanolic extract	+	+

GC-MS analysis for the leaf methanolic alkaloid extracts

As shown in Table 2 and Fig. 2 the results of GC-MS analysis for leaf's methanolic extract proved the existence of one type of alkaloid compound that is colchicine by comparison with its actual retention time to the retention time of colchicine standard reference (Sigma Aldrich) at R.T(12.8) min with area percentage reached 7.1% with molecular weight 399g/mol, that was in line with the study of Naser *et al.*[15], that revealed the presence of colchicine compound in leaf methanolic extract of *C. procera* by GC-MS analysis in Iraq that, *C.procera* appeared to produce many bioactive compounds, such as alkaloids [21-25].

Table 2. GC-MS Analysis for leaf Methanolic alkaloid compounds in *C. procera.*

Name of alkaloid compound	Retention.Time min	Formula	M wt. g/mol	Area percentage %
Colchicine	12.8	$C_{22}H_{25}N0_6$	399	7.1%

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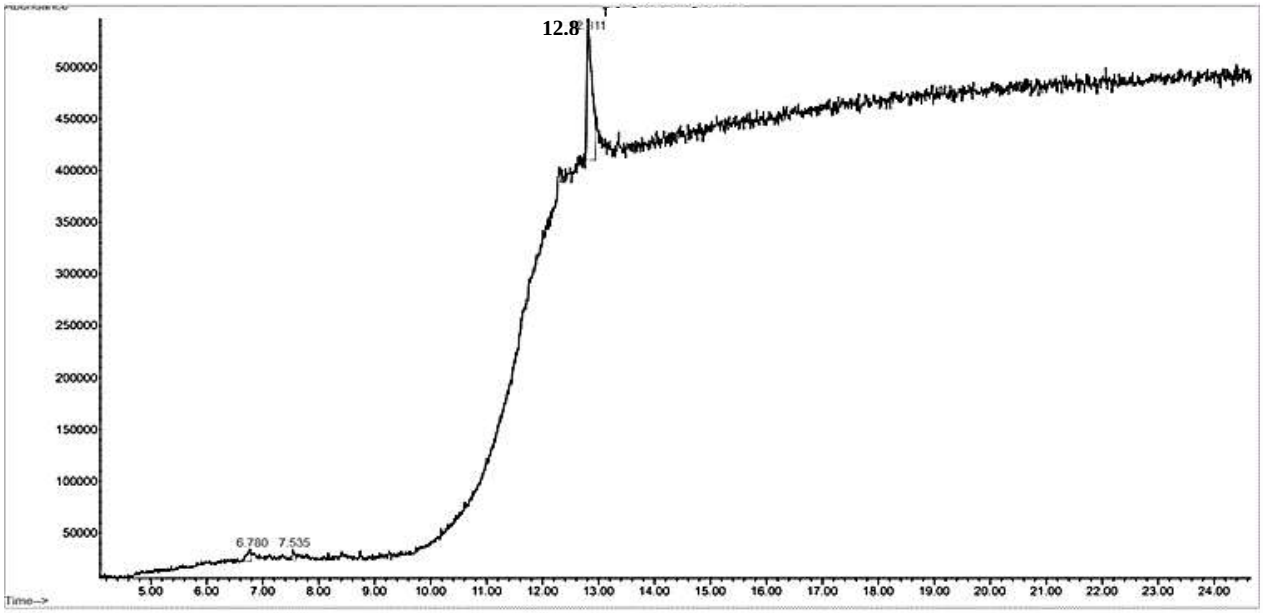

Figure 2. GC-MS Chromatogram for leaf Methanolic alkaloid compound

Isolation, Identification, and Purification of colchicine compound by HPLC technique

HPLC analysis for leaf methanolic alkaloid extract of *C procera* as shown in Fig. 3, resulted in the identification, separation, and purification of colchicine that was identified by comparison with its actual retention time to the retention time of colchicine standard reference (Sigma Aldrich)as seen in Fig. 4 , at chromatographic conditions, where the two peaks corresponded in their retention time were reached 2.5min. [26-27].

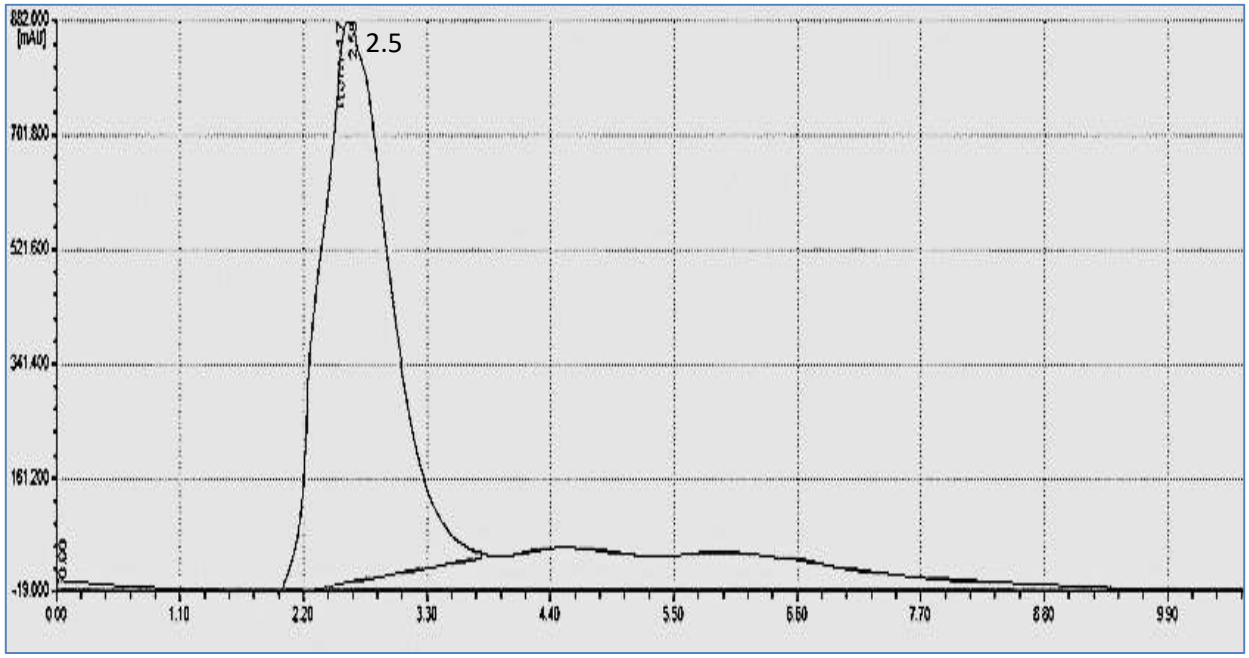

Figure 3. HPLC chromatogram for purified colchicine founded in leaf methanolic alkaloid extract in *C. procera*.

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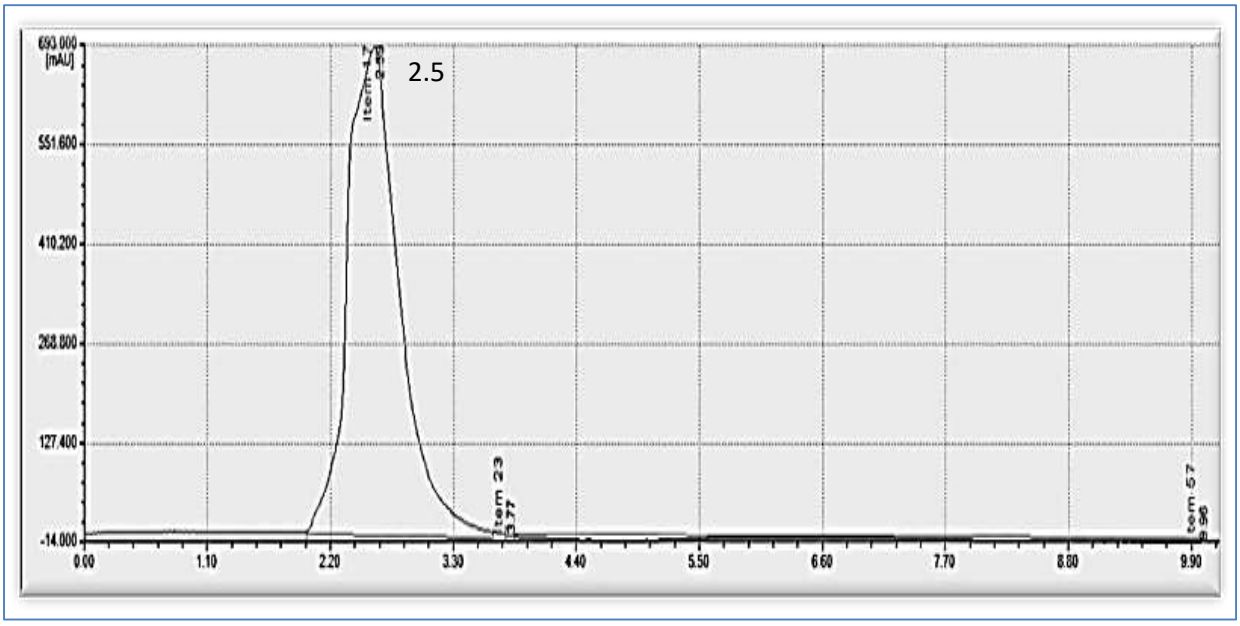

Figure 4. HPLC chromatogram for colchicine standard.

***In vitro* anticancer activity of studied alkaloid compounds**

Cytotoxicity study of the colchicine compound against (MCF-7) and (SK-GT-4) cell lines.

The cytotoxic effect of the purified alkaloid compound colchicine was tested by studying their ability as anti-proliferative against human cancer cells (MCF-7&SK-GT-4) which showed that the colchicine compound has significant cytotoxic activity on the MCF-7 cell line that is superior to that against the SK-GT-4 cell line. Assessment of anti-cancer activity using MTT assay is a colorimetric assay that correlates between cell activity and the number of viable cells by measuring the absorbance of a specific wavelength to determine the cytotoxic effect of the drugs or substances[16]. Study of Krishnasamy *et al* [19] proved anticancer activity of colchicine compound that purified from *Indigofera aspalathoids* against another type of cell lines that was (Hep-B) cell line with a value of IC_{50}% reaching 344 µg/ml. Also as seen in study of Lu *et al*.[28] and Zhang *et al*. [29] colchicine has antiproliferative effects on both two human gastric cancer cell lines (i.e., AGS and NCI-N87) by induced apoptosis,Thus , colchicine that purified from *C.procera* could be a good suggestion for the treatment of breast cancer cell line MCF-7 and esophagus cancer cell line SK-GT-4.

Calculation of the IC50 value of the colchicine compound against SK-GT-4 &MCF-7 cell lines.

As shown in Figs. 5, 6, the concentrations of colchicine tested plotted against viable cell percentage for both the MCF-7&SK-GT-4 cell lines and the IC**50** value was calculated and found to be 55.33 µg/ml &522 µg/ml respectively and that differences of IC_{50} Value for colchicine towards two different cell lines Because every cell line has its own "cell specific response" where , Each cell line has a completely unique system, with its individual biological characteristics even when cell lines were established from the same tissue[30,31].

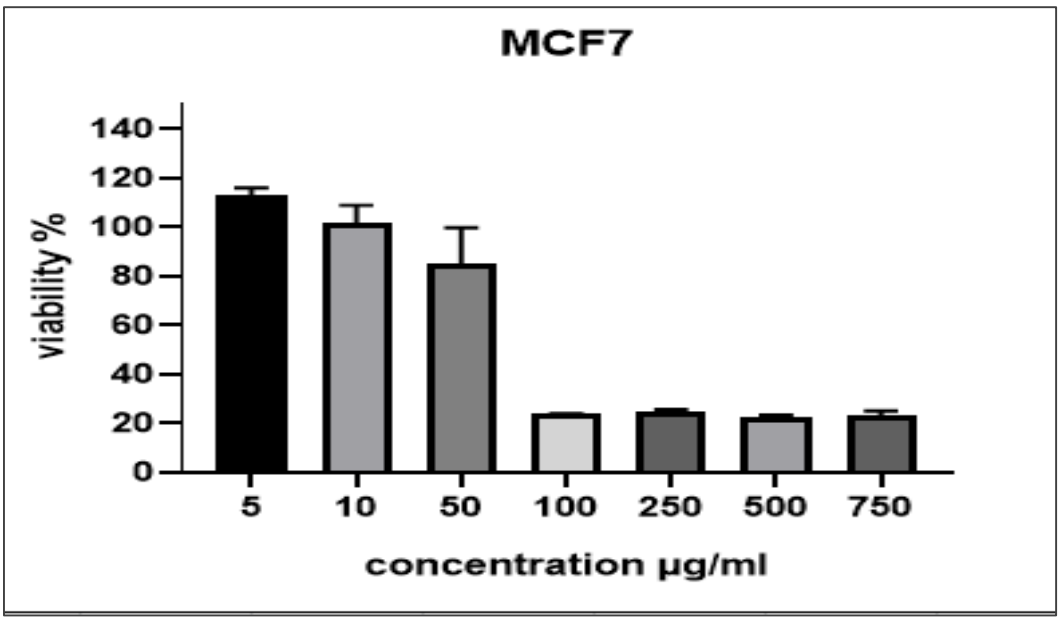

Figure 5. Viability of MCF-7 cell line for seven concentrations of colchicine with 50% inhibitory concentration (IC_{50}) 55.33µg/ml.

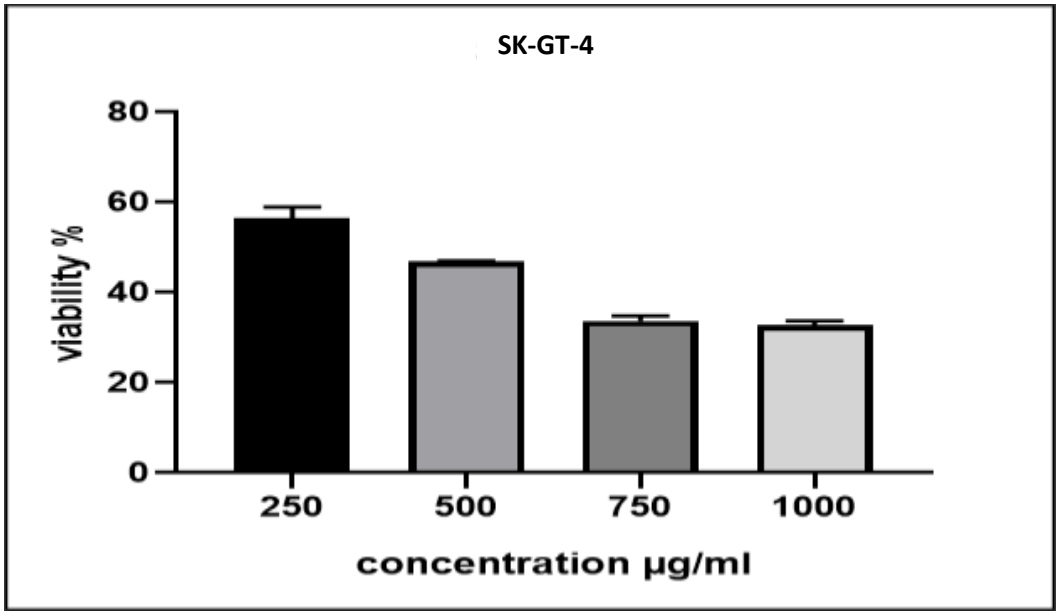

Figure 6. Viability of SK-GT-4 cell line for four concentrations of purified colchicine with 50% inhibitory concentration (IC50) 522 µg/ml.

Microscopic View of cell lines

Double labeling of MCF-7 cells with Acridine Orange and Ethidium Bromide (AO/EB) revealed morphological changes following colchicine treatment at various time points. Viable cells show green fluorescence after 48 hours of incubation Fig. 7, while late apoptotic cells show reddish or orange fluorescence after 48 hours of incubation. According to the examples provided Fig. 8 [32-34]

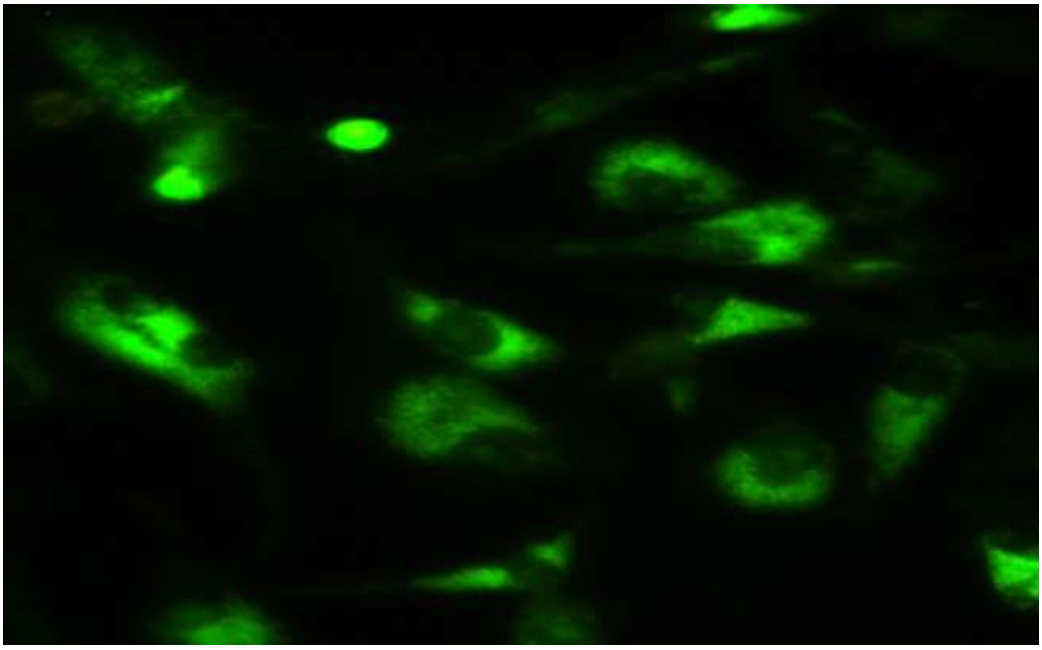

Figure 7. Control un-treated MCF-7 cells.

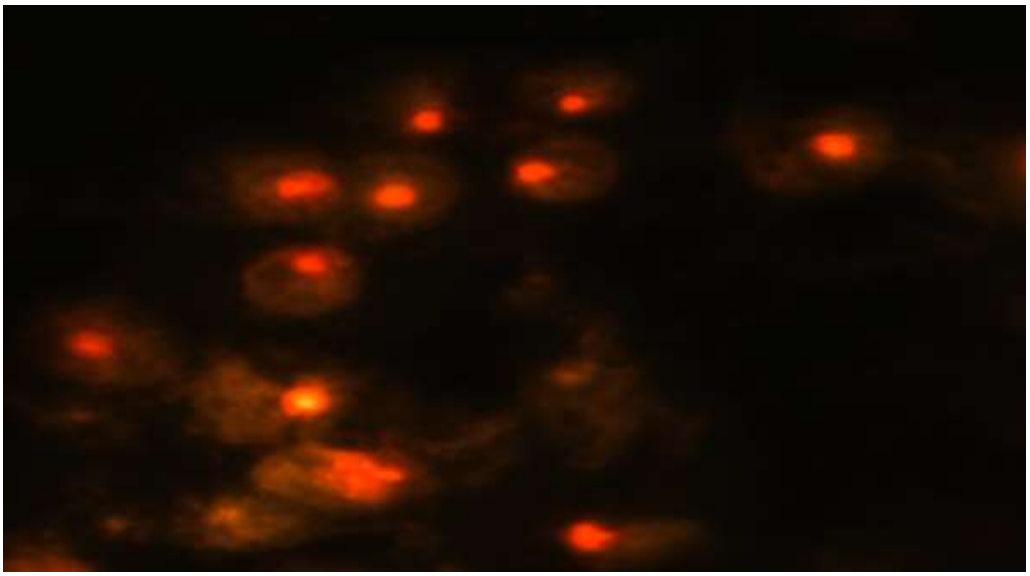

Figure 8. Morphological changes in MCF-7 cells after treated (48h) with purified colchicine compound at IC_{50} concentration (55.33 µg/ml).

Conclusion:

It is clear from the results of the current study that the leaves of a plant *C. procera* contain a chemical compound namely (Colchicine) with high efficacy against some cancerous lines, and that it shows high purity and distinctive anti-cancer efficacy that enables it to be a promising treatment after completing other studies on it to know its side effects. Also the MTT assay which is used to study the cytotoxic effect of the Colchicine compound showed significant anti-cancer activity against both MCF-7 and SK-GT-4 cell lines and The plant *C. procera*, especially its leaves, is an important source of this compound, which has anti-carcinogenic activity In turn, it is considered a promising plant in the treatment of this disease, after extensive studies of this compound, and the study is the first of its kind for purification this compoundby HPLC technique with high purity compared with standard fromthe leaves of this important plant.

Authors' declaration:

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are mine ours. Besides, the Figures and images, which are not mine ours, have been given the permission for re-publication attached with the manuscript.
- Ethical Clearance: The project was approved by the local ethical committee in

University of Basra /College of Education for pure sciences.

Authors Contribution:

M A.Y.AM :Contributed collection and preparation of plant,extraction of crude alkaloid compound ,detection ,identification and purification of Colchicine alkaloid compound from *C.procera* root.

EY.A.AS:Contributed language writing of the article and proofreading and editing the research in its final form, and the first official responsible for publishing (Corresponding author) in addition to Statistical analysis of the article data.

MA.AH: Contributed to conducting analyzes of anti-cancer tests and determining the concentrations of the purified compound colchicine towards the cell lines under study against MC-F7 and SK-GT-4 cell lines.

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تقييم التأثيرات السمية الخلوية لمركب Colchicine المعزول من أوراق نبات (*Calotropis procera* Ait) ضد خطوط الخلايا السرطانية MCF-7 و SK-GT-4.

منى عبد المطلب يحيى الموسوي[1] عماد يوسف عواد السلطان[2] ميثم ايوب الحمداني[2]

[1]قسم العقاقير والنباتات الطبية , كلية الصيدلة, جامعة البصرة, العراق.
[2]قسم علوم الحياة ,كلية التربية للعلوم الصرفة, جامعة البصرة, العراق.

الخلاصة:
تعتبر القلويدات من المركبات الكيميائية المهمة الحاوية على النايتروجين والتي تعتبرمصدر غني لاكتشاف وتطوير عقاقير جديدة حيث تمتلك معظم القلويدات ذات الاصل النباتي تأثيرمضاد للسرطان. تم تحديد وجود القلويدات في اوراق نبات *Calotropis procera* باستخدام نوعين من الكواشف الكيميائية للقلويدات.تم اجراء تحليل GC-MS للمستخلص القلويدي لأوراق هذا النبات حيث بينت النتائج ظهور مركب قلويدي واحد عند وقت احتجاز بلغ 12.8دقيقة وبوزن جزيئي مقداره 399 وبتركيز 1.7% الذي تم تشخيصه كمركب الكولجسين ($C_{22}H_{25}N0_6$) وذلك بالاعتماد على وجود المركب المعياري للكولجسين .كذلك تم فصل وتشخيص وتنقية مركب الكولجسين وذلك باستخدام تقنية كروماتوغرافي السائل عالي الاداء HPLC لمستخلص القلويدات للأوراق حيث اظهرت النتائج وجود مركب الكولجسين عند وقت احتجاز2.5 دقيقة وذلك بمطابقته مع المركب المعياري له. تم تقييم الفعالية المضادة للسرطان لمركب الكولجسين المنقى على نوعين من خطوط الخلايا السرطانية MCF-7 وSK-GT-4 حيث بينت النتائج وجود فروق معنوية للفعالية السمية لمركب الكولجسين ضد الخط الخلوي MCF-7 على تلك التي ضد الخط الخلوي SK-GT-4 كذلك تم احتساب التركيز نصف المثبط IC_{50} لكلا الخطين والتي بلغت55.33و522مايكروغرام|مل على الترتيب.

الكلمات المفتاحية: الفعالية الضد سرطانية،اوراق نبات *Calotropis procera* ،استخلاص وتنقية Colchicine، GC-M، HPLC.