

Assessment the Cytotoxic Effect and Phytochemical Constituents of Ethyl Acetate Fraction of Iraqi *Cassia glauca* on Esophagus Cancer Cells

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ABSTRACT

Background: Cell lines obtained from cancer cells are usually used in experiments, such as use as a representative to cancer and to come up with a new treatment. The aim of the study: To explore the chemical constituents and assessment the anticancer activity against esophageal adenocarcinoma cell line of *Cassia glauca* (steams, leaves, flowers) parts where no phytochemical study had been done in Iraq on this plant.

Method: (Steams, leaves, flowers) parts of *C. glauca* defatted by maceration in *n*-hexane. The defatted plant parts were extracted by cold method, the aqueous ethanol 85% used as a solvent for extraction and the extract fractionated by chloroform, ethyl acetate and *n*-butanol. The ethyl acetate fraction then analyzed by High Performance Liquid Chromatography (HPLC) to investigate its constituents. The MTT assay used to evaluate the anticancer activity by employment 96 well plates, the cell lines implanted by (1×10^4) cells/well. And 24 h later different concentration had been used to treat the SK-GT₄ cell lines. By removing the medium after 72 h of treatment the cell viability was measured, 28 μ L of MTT solution of concentration (2 mg/mL) were added and then the cells were incubated for 1.5 h at 37°C. When the MTT solution where removed any crystals stucked on the walls by addition of 130 μ L of DMSO (Dimethyl Sulphoxide) were solubilized, then another incubation at 37°C with shaking for 15 min. On a micro plate reader at 492 nm (test wavelength) the absorbance where determined.

Conclusion: Plant extract from *C. glauca* ethyl acetate fraction showed very acceptable anticancer activity because it inhibits tumor progression particularly in human esophagus adenocarcinoma cell line. Our results encourage that this plant extract fraction is hopeful as anticancer reagent.

Key words: *Cassia glauca*, Anti-cancer activity, SK-GT-4, HPLC, Phytochemical

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INTRODUCTION

In 2018, the number of esophageal cancer worldwide estimated as 572000 new cases which include 85000 (15%) cases of Esophageal Adenocarcinoma (EAC) [1]. And the burden from EAC is going to increase specially in the high income countries, chiefly among men and by 2030, particularly in the United Kingdom and the Netherlands 1 in 100 are probable to have EAC during their lifespan [2]. And the esophageal cancer is known by its aggressiveness, due to the absence of serosa in the esophageal wall which is a serious factor in spreading of the cancer and makes it pervasive by many pathways including direct extension, hematogenous metastasis and lymphatic spread. Concerning the prognosis as no

anatomical barrier, the tumor diffuse readily into the vicinal organs of the thorax and neck which consist of the trachea, larynx, pericardium, thyroid gland, diaphragm and lung [3]. Thus, an important demand to evolve new, affordable and effective drugs [4]. An extracts from a number of herbal plants had been proved to have anticancer activities both *in vitro* and *in vivo* by several researches [5,6]. Due to enhancement the immune system as improved by many researches [7]. So the natural and herbal products have been rated as precious source for treatment of diseases and malignant and about 60% of drugs at present are used as a cancer medication it is source is the natural products [8,9].

The cassia is one of four large genus of the family leguminosaea (worldwide distribution family and had estimated to 16000 to 19000 species) and amongst the twenty five largest genera of dicotyledonous plants [10,11].

C. glauca is a tall tree, with glabrous branchlets. Leaves distinctly petioled, 1/2-3/4 ft; leaflets ovate, acute or sub-

obtuse, 2-4 in, flowers large, bright yellow. Pod flat; thin strap shaped 6-8 in. Long and 1/2-3/4 in broad, distinctly stalked, 20-30 seeded [12]. It has diverse common names as *C. surattensis*, *C. sulfurea* and *Senna arborescens*. It is native to East Indies, distributed from the Himalayas, in India through Ceylon and the Polynesian island to Australia [13]. Cultivated in the tropical countries in Asia such as Sri Lanka, Thailand, Burma and Malaysia [14]. And is known to have escaped into the wild, naturalizing in many of places (Irwin and Barneby, 1982). In the ayurvedic system the *C. glauca* total aerial parts used as depressant to the central nervous system, antimalarial, purgative and diuretic [15]. Leaves are used for blennorrhagia and also, in folk medicine the bark and leaves are usually utilized for the treatment of gonorrhea and diabetes (Indian medicinal plants Kirtikar and Basu, 1944). Whilst in indigenous system of medicine the seeds oil are used in the treatment of leucoderma and skin diseases [16]. In traditional Brazilian medicine it has been used for the treatment of flu, cold, fever and headache, while the decoction of the roots is commonly used to treat snake bites [17,18]. Many reports have shown the cassia species possessing anti-diabetic, anti-microbial, anti-malarial, anti-cancer and hepatoprotective [19,20]. Finally, some compounds had been isolated from *C. glauca* including steroids (*beta sitosterol*), anthraquinones (emodin, chrysophanol and physcion) and flavonoids (Apigenin, Kaempferol) [20-22].

So, investigation to esophagus anticancer activity and isolation, Identification and characterization of some pharmacological active compounds of *C. glauca* ethyl acetate fraction cultivated in Iraq will be done.

MATERIALS AND METHDOS

Plant material collection

The (steams, leaves, flowers) parts of *C. glauca* were taken from side road of Baghdad city in April 2021. The plant was specified and authenticated by Prof Dr. S Abass department of biology college of sciences university of Baghdad. The parts were washed, dried at room temperature and crushed by using a mechanical grinder to powder.

Method of work

The dried powder is weighted and defatted by n-hexane solvent to exclude the chlorophyll and waxy materials, the defatted powder is extracted by maceration with 85% ethanol for 7 days then the extract dried by rotary evaporator and weighted after that dissolved in water and successively fractionated by using separator funnel with chloroform, ethyl acetate and n-butanol solvents [23]. Each fraction is dried by rotary evaporator and weighted for further analysis.

Preliminary qualitative and phytochemical analysis

Chemical tests done by using the ethyl acetate extract of the plant and standard procedures carried out to identify the active constituents (Table 1) [24,25].

Table 1: Chemical tests used to identify the active constituents in ethyl acetate fraction.

Constituent	Test
Flavonoids	Alkaline test: 1 ml from ethyl acetate extract and few drops of sodium hydroxide solution. A yellowish precipitate indicating a positive test for flavonoids. Shinoda's test: 1 ml from ethyl acetate extract treated with magnesium and concentrated HCL. Red orange color indicate the presence of flavonoids.
Tannin and phenolic compounds	5% ferric chloride: 1 ml of ethyl acetate extract mixed with 1 ml of FeCl ₃ solution (5% w/v) to produce dark green or blue black precipitate indicates the presence of tannins. 10% lead acetate: 1 ml of ethyl acetate extract mixed with 1 ml of 10% lead acetate solution a white precipitate indicates the presence of tannins.
Alkaloids	Dragendroff's test: A small portion of ethyl acetate fraction was treated with 1% HCl on steam bath then Dragendroff's reagent (solution of potassium bismuth iodide) was added. Orange brown precipitation due to double salt formation indicates the presence of nitrogenous substances. Mayer's reagent: A small portion of ethyl acetate fraction was treated with 1% HCl on steam bath then Mayer's reagent (1.35 gm mercuric chloride in 60 ml water +5 gm potassium iodide in 10 ml water) was added, creamy precipitate due to double salt formation indicates the presence of alkaloids.
Steroids	Libermann burchard test: A small portion of ethyl acetate fraction was dissolved in 5 ml of chloroform, then the chloroform layer was dried over anhydrous sodium sulfate and later on mixed with 10 drops of acetic anhydride and 2 drops of concentrated sulphuric acid are then added, bluish green color indicates the presence of steroidal nucleus as oxidation occur in steps
Anthraquinones	Brontrager's test: A small portion of ethyl acetate fraction was shaken with 3 ml of benzene and then filtered, mixed with 3 ml of 10% ammonium hydroxide solution, pink color in the ammonical phase indicate the presence of anthraquinones
Cardiac glycoside	Keller-kiallian's test: A small portion of ethyl acetate fraction was mixed with 2 ml of glacial acetic acid and 1 drop of 0.1% ferric chloride solution then add 1 ml of H ₂ SO ₄ (drop by drop) were add, formation of green blue color indicates the presence of cardiac glycoside.

High Performance Thin Layer Chromatography (HPLC)

Standard and sample solutions (rutin, isorhmentine, quercetin and ethyl acetate F.) were dissolved in absolute methanol.

HPLC technique Waters 1525 was used to check the constituents found in the ethyl acetate fraction. The mobile phase composed of 1% aqueous acetic acid solution (B) and 100% MeOH (C). The samples elution done by this gradient: 90% B and 10% C from 0 to 6 min, 84% B and 16% C from 7 to 25 min, 72% B and 28% C from 26 to 37 min, 65% B and 35% C from 38 to 47 min, 50% B and 50% C from 48 to 64 min and 90% B and 10%

C from 65 to 70 min, to restore the initial conditions so a second sample can be injected. 0.8 mL/min the flow rate was, 5 µL was the injection volume. Hypersil gold column (150 × 4.6) at 25°C temperature. The detection of flavonoids was done by UV detector at $\lambda=278$ nm. The identification based on retention time for the compound by comparing with standards in the same conditions [26].

Chemicals and reagents

The chemicals, reagents and instruments used listed in the below Table 2.

Table 2: Chemicals, reagents and instruments used.

No.	Item	Company	Country
1	Trypsin/EDTA	Capricorn	Germany
2	DMSO	Santacruz biotechnology	USA
3	RPMI 1640	Capricorn	Germany
4	MTT stain	Bio world	USA
5	Fetal bovine serum	Capricorn	Germany
6	incubator	Cypress diagnostics	Belgium
7	Micro titer reader	Gennex lab	USA
8	Laminar flow hood	K and K scientific supplier	Korea
9	Micropipette	Cypress diagnostics	Belgium
10	Cell culture plates	Santa Cruz biotechnology	USA

Maintenance of cell cultures

SK-GT-4 cell line was preserved in MEM solution (Minimal Essential Medium) added 10% fetal bovine, 100 µg/mL streptomycin and 100 units/mL penicillin. Cells were passaged using trypsin EDTA incubated at 37°C and reseeded at 50% confluence twice a week [27].

Cytotoxicity assay

To define the cytotoxic activity, the MTT assay used which was done by 96 well plates, the cell lines implanted with (1×10^4) cells/well [28]. And after 24 h different concentration had been used to treat the SK-GT₄ cell lines. By removing the medium after 72 h of treatment the cell viability was measured, 28 µL of MTT solution of concentration (2 mg/mL) were added and then the cells were incubated for 1.5 h at 37°C. When the MMT solution where removed any crystals stucked on the walls by addition of 130 µL of DMSO (Dimethyl Sulphoxide) were solubilized, then another incubation at 37°C with shaking for 15 min [29]. On a micro plate reader at 492 nm (test wavelength) the absorbance where determined; in

triplicates the assay done. The percentage of cytotoxicity to cell growth was calculated by this equation [30].

% Cell viability=(Absorbance of treated cell/Absorbance of non-treated cell) × 100

% Cytotoxicity=100-Cell viability

Statistical analysis

Using by usage of graph pad prism 6 and an unpaired T test the data were analyzed [31]. And by the mean ± SD of triplicate measurements the values were presented [32].

RESULTS AND DISCUSSION

Phytochemical analysis

The results of phytochemical analysis are given in below Table 3.

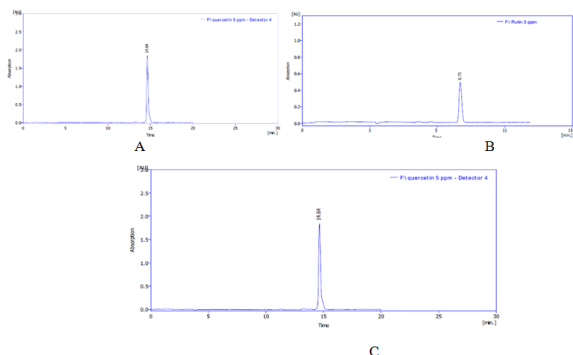
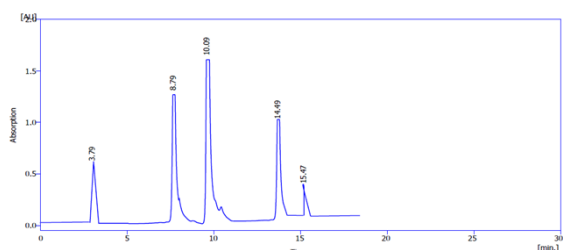
Table 3: Qualitative phytochemical analysis of *C. glauca* (steams, leaves, flowers) ethyl acetate fraction.

Tested component	Type of test	Result
Flavonoids	Alkaline	+ve
	Shinoda's	+ve
Tannin and phenolic compounds	5% ferric chloride	+ve
	10% lead acetate	+ve
Alkaloids	Dragendroff's	+ve
	Mayer's reagent	-ve
Steroids	Liebermann-Burchard	-ve
Anthraquinones	Brontrager's	-ve
Cardiac glycosides	Keller-Kiallian's	-ve

(+,-) represent presence and absence of phytoconstituents respectively.

The results of preliminary phytochemical screening of plant ethyl acetate fraction showed the presence of, flavonoids, tannins alkaloids and steroids in the (steams, leaves, flowers) parts of Iraqi species of this plant and the absence of anthraquinones and cardiac glycosides in the same plant parts.

The identification of compounds by HPLC done by comparing the obtained retention times for both standards and ethyl acetate fraction. Their chromatograms of standards (Figure 1) compared with ethyl acetate fraction chromatogram (Figure 2).

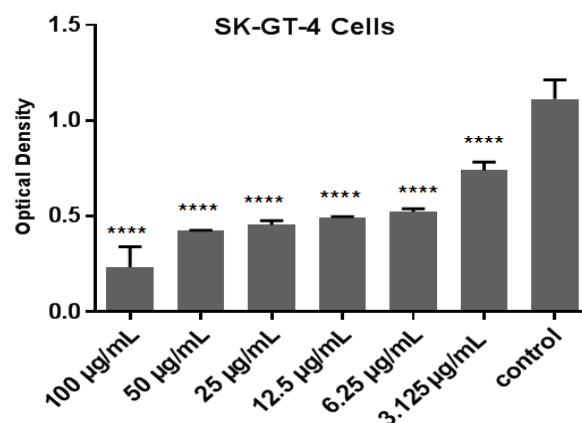
**Figure 1: HPLC chromatogram of quercetin standard (A), rutin standard (B) and isorhmentine standard (C).****Figure 2: HPLC chromatogram of ethyl acetate fraction.**

In this study, the cytotoxic effect of *C. glauca* ethyl acetate fraction against cancer cell was evaluated by using SK-GT₄ cell line. The results demonstrate that a very noteworthy cytotoxic activity against the EAC cell lines as represent (Figure 3). Below, the data showed that there is

ability in ethyl acetate fraction to curb the growth of esophagus cancer cell line and significantly, in concentration dependent manner (Figure 4).

IC₅₀=5.498 50 nm of the ethyl acetate fraction (which is the half inhibitory concentration usually use to measure of the potency of a drug to inhibit a biochemical or biological substance). It may be possible that the antioxidant activities of this fraction are likely due to the presence of flavonoids. The flavonoids can inhibit injury caused by free radicals by scavenging of reactive oxygen species, metal chelating activity, activation of antioxidant enzymes, tocopheryl radicals reduction, mitigation of oxidative stress caused by (nitric oxide, increase in uric acid) and inhibition of oxidases. Also antioxidant properties of low molecular antioxidants [33].

And in comparison of IC₅₀ of *C. glauca* ethyl acetate fraction with previous study done on the same cell line and same MTT assay but, with different concentrations for paclitaxel (FDA approved drug for gastro esophageal adenocarcinoma) the IC₅₀ was 50 nm which is higher concentration by about 10 times than *C. glauca* fraction IC₅₀ concentration [34]. Another study done also on same cell line in the same condition for *rumex acetosella* methanol extract IC₅₀=42.62 nm, so we found that our plant is much more active against EAC (Figure 5) [35].

**Figure 3: Cytotoxic effect of *C. glauca* ethyl acetate fraction in SK-GT-4 cells. IC₅₀=5.498 nM.**

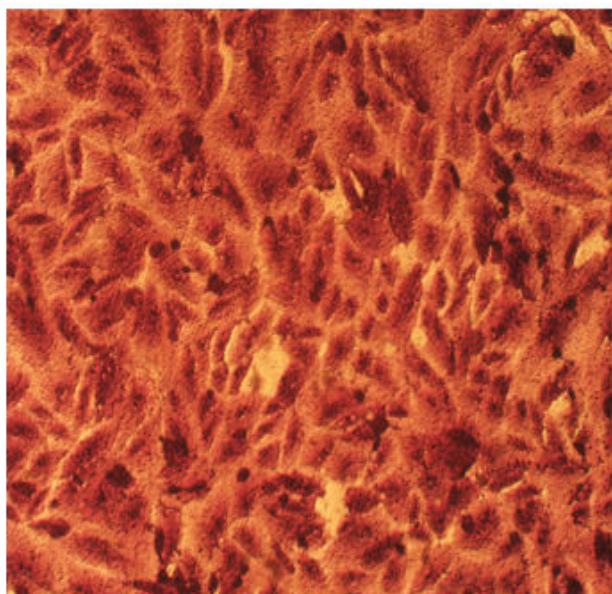


Figure 4: Control untreated SK-GT-4 cells.

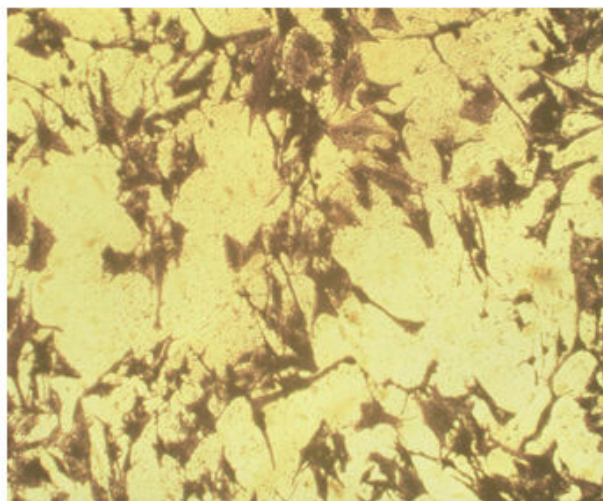


Figure 5: Morphological changes in SK-GT₄ cells after treated with *C. glauca* ethyl acetate fraction.

In spite of many compounds isolated from this plant are being precisely studied for their anticancer activity, it is becoming obvious that the benefit effects of whole plant parts use is because of the synergistic effect of the compounds which present rather than the use of single constituent alone [36,37].

CONCLUSION

C. glauca ethyl acetate fraction extract of (stems, leaves, flower) may be has promising anticancer activity against EAC. The activity may related to the flavonoids exist in the fraction in addition to synergistic effect.

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