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Research Article

The effect of *Rhus coriaria* L. methanolic extract on cytotoxicity of *Cladophora glomerata* L. Kützing methanolic extract against human breast carcinoma MCF-7 cell line

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ABSTRACT
The present study aimed to evaluate the cytotoxicity of the methanolic extract of *Cladophora glomerata* macroalga against human breast carcinoma MCF-7 cell line and natural cells (Vero cells) as well as evaluating the combined effect of *Rhus coriaria* (sumac) methanolic extract. We performed the preliminary phytochemical screening of *C. glomerata* methanolic extract, then we evaluated the cytotoxicity of *C. glomerata* methanolic extract against MCF-7 and Vero cell lines using MTT assay with determining the value of Half maximal inhibitory concentration (IC_{50}) of *C. glomerata* methanolic extract. According to the results *C. glomerata* methanolic extract contains alkaloids, flavonoids, tannins, saponins, glycosides, triterpenoids, and sterols while it is free of phenols. The *C. glomerata* methanolic extract has a high cytotoxicity activity against MCF-7 cells compared with Vero cells, the IC_{50} of the methanolic extract of *C. glomerata* was 116.7 and 120.4µg/ml against MCF-7 cells and Vero cells respectively. The isobologram analysis results showed that there is antagonism between *C. glomerata* methanolic extracts and sumac methanolic extracts. The results of the DNA fragmentation analysis showed that there was no degradation of the DNA of MCF-7 cells by using any concentrations of both *C. glomerata* methanolic extracts and sumac methanolic extracts. We concluded that *C. glomerata* methanolic extract may be of interest in the cancer treatment field, and we also concluded that the use of sumac methanolic extract for combination did not improve the efficiency of *C. glomerata* methanol extract.

Keywords: *Cladophora glomerata*, Cytotoxicity, MCF-7 cell line, *Rhus coriaria*

INTRODUCTION
Cladophora glomerata macroalga is a widely distributed algae in freshwater environments around the world [1]. A branched green filamentous alga is frequently present in the marginal shallows of nutrient-enriched lakes [2], and also in deeper water [3]. *Cladophora glomerata* has many bioactive properties and is placed among the most important medicinal and pharmaceutical macroalgae as well as other uses in cosmetics and nutrition, many researchers have proven its effectiveness because it has an antibacterial [4, 5, 6], antifungal [5, 7], antiparasitic [8], and anti-inflammatory effects [9, 10]. It also has efficacy against diabetes [11], as an antiulcer [12], has an antioxidant [10, 12, 13], and an anti-cancer [10, 13, 14, 15]. *C. glomerata* is rich in active compounds such as flavonoids, alkaloids, phenols, and tannins [16], fatty acids [17], sterols, and terpenoids [18].
Rhus coriaria (in the middle east countries called sumac) has medical and pharmaceutical properties, sumac word comes from summaq, meaning dark red in Arabic and Syriac [19]. Globally, Sumac is used as a spice that grows in temperate and subtropical areas, especially in the Mediterranean and Middle East regions [20], sumac was used as a traditional cancer treatment by mixing it with other substances [19]. Besides its nutritional properties, Sumac has many biological properties, including antibacterial properties [21, 22, 23, 24], also, antioxidant effects [25, 26], and it has cytotoxicity and anticancer properties [19, 20, 27, 28, 29]. Sumac is a rich source of many active compounds such as flavonoids, phenols, and tannins [30], also it has gallic acids, anthocyanins, quercetin, terpenoids, iso quercetin, myricitrin, and kaempferol, etc. [31, 32, 33, 34].
Due to the lack of studies on the methanolic extract of *C. glomerata* as an anti-cancer and also, it has medical and pharmaceutical importance, we evaluate the effectiveness of the

methanolic extract of C. *glomerata* against cancer cells also, we evaluate the methanolic extract of sumac to improve the effectiveness of C. *glomerata* cytotoxicity.

MATERIAL AND METHODS

Samples collection and extraction

C. *glomerata* samples were collected in April 2019 from several ponds in Basrah, Iraq. While sumac fruit samples were collected in July 2019 from some areas of northern Iraq. Samples were transferred to the laboratory, after washing them, they have dried at room temperature away from direct sunlight, then ground and stored in a container at 18 °C. All samples were identified at the University of Basrah, Basrah, Iraq.

According to [35] the methanolic extracts of C. *glomerata* and sumac samples were prepared by dissolving 40 g of dry samples in 200 ml of methyl alcohol (95%), after the mixture was left for 72 h with stirring every 24 h, filtered using filter paper (Whatman No.1), the extract concentrated at 40°C using a rotary evaporator, then left to dry at room temperature and stored in the dark and sterile container at 4°C.

Preliminary phytochemical screening test

Many chemical tests have been performed to determine the main chemical groups of C. *glomerata* methanolic extract, which are: alkaloids, phenols, triterpenes, and sterols tests according to [36], tannins test according to [37], saponins test according to [38] and glycosides and flavonoids tests according to [39].

Cytotoxicity assay

The cytotoxicity of methanolic extracts was evaluated using MCF-7 and Vero cell lines using MTT assay, the cell lines were obtained from the Iraqi Center for Cancer and Medical Genetics Research, Al-Mustansiriya University, Baghdad, Iraq, and grown in the tissue culture laboratory, Department of Biology, College of Education for Pure Sciences, Basrah University. According to [40], 1×10^4 cells were seeded using the culture EME medium with 10% serum in a 96 well flat-bottom culture plate and incubated at 37°C, 5% CO_2 for 24 h. After incubation, cells were exposed in five replications to the different concentrations of extracts along with control (only culture medium), then incubated for 48 h at 37°C, 5% CO_2. The medium was removed and replaced by a fresh medium along with 100 µg of 2 mg/ml MTT stain solution (3-[4,5-dimethylthiazol-2-yl] -2,5-diphenyl-tetrazolium bromide) in each well, then incubated for 4 h at 37°C, 5% CO_2. For each well 100 µl of Dimethyl sulfoxide (DMSO) was added and left with shaking for 20 min. The absorbance was measured using a Microplate reader at 550 nm. The viability percentage was determined for each concentration according to the following equation: viability % = the absorbance of treated / the absorbance of control × 100.

Fragmentation analysis

7×10^5 cells of Mcf-7 cells were seeded on plate 6 well, the cells were separately exposed to the following combined concentrations, sumac extract (15 µg/ml) + C. *glomerata* extract (90 µg/ml); sumac extract (25 µg/ml) + C. *glomerata* extract (95 µg/ml) and sumac extract (35 µg/ml) + C. *glomerata* extract (115 µg/ml). Each plate was covered with adhesive paper and incubated for 24 h at 37°C, 5% CO_2. After incubation, the DNA extraction of treated MCF-7 cells was achieved using a cell culture protocol (Geneaid) according to the manufacturer's instructions. The extracted DNA was quantified by NanoDrop. Promo phenol blue dye has already been added to 5 µl of each DNA sample or 6 µl of Bioneer DNA ladder (100-2000 PB), the mixture has been loaded with an agarose gel in the appropriate wells (1.5 %), the gel has been run and 85 volts for 65 min, the DNA in the agarose gel has been visualized using a transilluminator UV.

The combination test

The combined effect was achieved according to [41] by determined the combination index (CI) values using Compusyan software version 1. Three combined concentrations were used including sumac extract (15 µg/ml) + C. *glomerata* extract (90 µg/ml); sumac extract (25 µg/ml) + C. *glomerata* extract (95 µg/ml) and sumac extract (35 µg/ml) + C. *glomerata* extract (115 µg/ml). The exposure time was 72 h.

Cell morphological study

According to [42], 7×10^5 (cells/cover) of Mcf-7 cells were seeded on coverslips within plate 6 well after the confluent monolayer was formed. The cells were exposed separately to IC_{50} concentrations of C. *glomerata* and sumac methanolic extracts, and the other coverslips were untreated as a control, each plate was covered with adhesive paper and incubated for 24 h at 37°C, 5% CO_2. After incubation, one replicates from each treated coverslip was stained in fluorescent acridine orange-ethidium bromide. A fluorescence microscope was used to examine the cells.

Statistical analysis

The IC_{50} value was determined using the Graph Pad Prism software 6.04 and the data were statistically analyzed using the chi-square (χ^2) test via IBM SPSS Statistics ver. 19 software and $P \leq 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Preliminary phytochemical screening

The preliminary phytochemical screening results were shown in Table 1, many of the bioactive compounds including alkaloids, flavonoids, tannins, saponins, glycosides, triterpenoids, and sterols were found in the methanolic extract of *C. glomerata* and it was free of phenols.

Alcohol is known to be a polar solvent capable of dissolving many chemical compounds and elements, such as fats, polysaccharides, minerals, peptides, and others [43]. Many previous studies are in line with the current results, including [16], found that the *C. glomerata* extract has flavonoids, alkaloids, and tannins, also [44] confirmed that the *C. glomerata* extract contains tannins, flavonoids, saponins, glycosides, and terpenoids, these results were also agreed with [45] that the *C. glomerata* methanol extract contains alkaloids, tannins, flavonoids, saponins, terpenoids, sterols and without phenols. The presence of these active compounds in the methanolic extract of *C. glomerata* indicates its importance for use in different fields, such as medical and pharmaceutical.

Cytotoxicity results

The cytotoxicity results showed that *C. glomerata* methanolic extract has a high cytotoxicity activity against MCF-7 cells compared to Vero cells (Table 2). Six algal extract concentrations were used in the test, all of which affected both cell lines in different degrees, the highest viability percentage of Vero cells was 98.1% at 90 µg/ml, while the viability percentage of MCF-7 cells at the same concentration was 57.09%, but the lowest viability percentage of Vero cells at 175 µg/ml concentration was only 18% compared with 43.31% of MCF-7 cells at the same concentration, other concentration results are shown in Table (2). The IC_{50} values of *C. glomerata* methanolic extract to MCF-7 cells and Vero cells were 116.7 and 120.4 μg/ml respectively. The results of the statistical analysis showed that there are no significant differences ($P \leq 0.05$, $P = 0.669$) in all the different concentrations between the viability of the MCF-7 cell line. It also showed that there is a significant difference ($P \leq 0.05$, $P = 0.000$) in all different concentrations between the viability of the Vero cell line (Table 2).

The cytotoxicity of *C. glomerata* methanolic extract may be due to its possession of many effective compounds, such as alkaloids, tannins, flavonoids, saponins, glycosides, triterpenoids, and sterols, this was confirmed by the current results. Chemicals such as vitamins, carotenoids, terpenoids, flavonoids, polyphenols, alkaloids, tannins, saponins, pigments, enzymes, and minerals are developing anti-cancer activities, blocking various hormone actions and metabolic pathways associated with cancer development [46]. Numerous global studies have shown that these active compounds are important for influencing cancer cells, such as the study of [47] which examined the effectiveness of all types of terpenoids and their roles as anticancer agents. [48] have also demonstrated the role of saponins and tannins as anticancer agents. On the other hand, alkaloids have efficacy against different cancer cell types [49]. The study of [50] has verified that glycosides demonstrate anticancer activity. Some types of flavonoids have been beneficial compounds in various carcinogenesis stages [51].

In the present study, the *C. glomerata* methanolic extract caused the toxic effect in MCF-7 cells depending on the concentration, while the results of the DNA fragmentation analysis showed that MCF-7 cells had no apoptosis, accordingly, the mechanism of action may be related to epigenetic and signal transduction pathways in the cell [43], or to cell cycle effect and cell replication inhibition [52], or other causes.

The combination results

The combination results showed that the viability of MCF-7 cells at 15 µg/ml of sumac + 90 µg/ml of *C. glomerata* was 55.89% while the viability of MCF-7 cells was 57.09 % when using 90 µg/ml of *C. glomerata* alone, also when using the concentration of 25 µg/ml of sumac + 95 µg/ml of *C. glomerata*, the viability of MCF-7 cells was 55.37%, whereas the viability of MCF-7 cells was 55.56% when using 95 µg/ml *C. glomerata* alone. At 35 µg/ml of sumac + 115 µg/ml of *C. glomerata* the viability of MCF-7 cells was 49.56%, whereas the viability of MCF-7 cells was 53.72% when using 115 µg/ml *C. glomerata* alone (Tables 2,3).

Although the results indicate a relative decrease in the viability of MCF-7 cells when *C. glomerata* extract combined with sumac extract, the antagonism effect between sumac and *C. glomerata* at all three concentrations (15 µg/ml of sumac + 90 µg/ml of *C. glomerata;* 25 µg/ml of sumac + 95 µg/ml of *C. glomerata;* 35 µg/ml of sumac + 115 µg/ml of *C. glomerata*) was shown according to the CI results in the compusyan isobologram software (1.31701, 1.66021 and 2.3133) respectively (Table 4)(Figure 1).

The combination process did not improve the efficacy of *C. glomerata* methanolic extract, as there is no previous study reporting a synergistic effect of any materials with *C. glomerata* methanolic extract.

DNA fragmentation analysis and cell morphological changes

The results of the DNA fragmentation analysis showed that the MCF-7 cells were exposed separately to three concentrations (15 µg/ml of sumac + 90 µg/ml of *C. glomerata*; 25 µg/ml of sumac + 95 µg/ml of *C. glomerata*; 35 µg/ml of sumac + 115 µg/ml of *C. glomerata*) that did not degrade the cell's DNA (Figure 2).

The fluorescent staining of Mcf-7 cells showed that the Mcf-7 cells treated with the IC_{50} of *C. glomerata* methanolic extract were unaffected, with the same result being seen in Mcf-7 cells treated with the IC_{50} of sumac methanolic extract except for a few apoptosis cells. This usually means that there was no apoptosis in the Mcf-7cells (Figure 3).

According to the DNA fragmentation analysis results, the use of any concentration of both *C. glomerata* and sumac methanolic extracts did not degrade cell DNA, these results were agreed with the results of cell morphological changes, showing approximately the absence of apoptosis in cells treated with *C. glomerata* methanolic extract and sumac methanolic extract, Therefore, inhibition of cells as a result of cell cycle arrest rather than apoptosis in response to DNA damage promoting a G2 / M DNA damage checkpoint, where cells suffer DNA damage during the cell cycle, the typical response seen is cell cycle arrest to allow DNA repair. Cells damaged during S or G2 typically arrest at the G2/M boundary [52].

CONCLUSION

In the present study, we concluded that *C. glomerata* methanolic extract may be of interest in the cancer treatment field, and we also concluded that the use of sumac methanolic extract for combination did not improve the efficiency of *C. glomerata* methanol extract.

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Conflict of interest: The authors declare that there is no conflict of interest.

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Table 1: Preliminary phytochemical screening of C. glomerata methanolic extract.

Chemical compounds	Test	Result
Alkaloids	Wagner's test	+
Tannins	Lead acetate hydrate 1%	+
Saponins	Mercuric chloride 5%	+
Glycosides	Benedict's reagent	+
Flavonoids	Alcoholic KOH 5N	+
Phenols	Ferric chloride 1%	-
Triterpenes	Sulphuric acid	+
Triterpenes & Sterols	Liebermann-Burchard reagent	+

+: present; -: not present

Table 2: The cell viability of the cell lines treated with C. glomerata methanolic extract.

Concentration μg/ml	Cell lines viability %	
	MCF-7	Vero
90	57.09	98.1
95	55.56	89.9
115	53.72	72.12
135	48.96	22.07
155	46.43	20.87
175	43.31	18
χ^2	3.203	128.596
P value	0.669	0.000

Table 3: The cell viability of MCF-7 cell line treated with the combination of C. glomerate and sumac methanolic extracts.

Concentration μg/ml	Cell viability %
Sumac (15) + C. glomerata (90)	55.89

Sumac (25) + C. glomerata (95)	55.37
Sumac (35) + C. glomerata (115)	49.56

Table 4. CI values of the combination of C. glomerate and sumac methanolic extracts.

Concentration μg/ml	Effect	CI
Sumac (15) + C. glomerata (90)	0.558	1.31701
Sumac (25) + C. glomerata (95)	0.553	1.66021
Sumac (35) + C. glomerata (115)	0.495	2.3133

CI: combination index

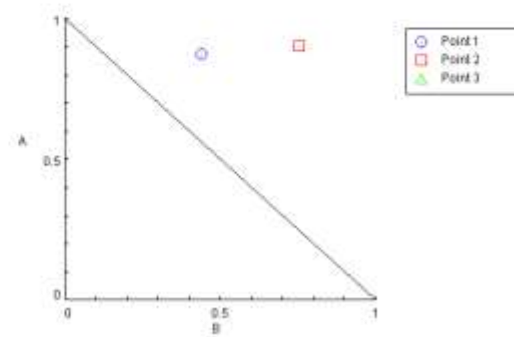


Fig.1: The isobologram of the interaction between sumac and C. glomerata methanolic extracts. A: Sumac extract con. μg/ml; B: C. glomerata extract con. μg/ml; point 1: sumac extract (15 μg/ml) + C. glomerata extract (90 μg/ml); point 2: sumac extract (25 μg/ml) + C. glomerata extract (95 μg/ml); point 3: sumac extract (35 μg/ml) + C. glomerata extract (115 μg/ml).

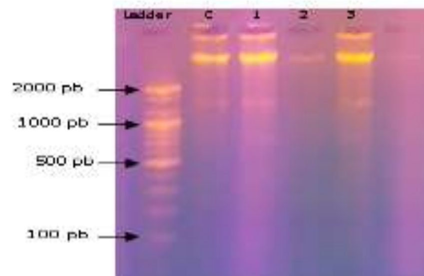
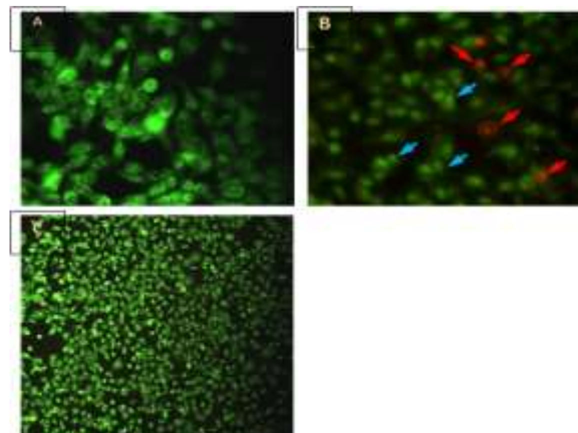


Fig.2: DNA fragmentation analysis of MCF-7 cell line. C: control; 1: sumac extract (15 μg/ml) + C. glomerata extract (90 μg/ml); 2: sumac extract (25 μg/ml) + C. glomerata extract (95 μg/ml); 3: sumac extract (35 μg/ml) + C. glomerata extract (115 μg/ml).



Figur3. Mcf-7 cells stained with acridine orange-ethidium bromide. A: cells treated with IC50 of C. glomerata methanolic extract (200 X), B: cells treated with IC50 of Sumac methanolic extract, the red arrows refer to apoptosis cells blue arrows refer to unaffected cells (200 X), C: untreated cells (100 X).