Cytotoxicity of Xanthium Strumarium against Breast Cancer Cell Lines

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Abstract

Objective: This study aimed to investigate the possible in vitro cytotoxicity of Xanthium strumarium. Methods: The dried leaves powder was extracted with chloroform. Extract alone and combined with Doxorubicin have been evaluated by MTT assay for cytotoxicity against AMN3, AMJ13, MCF7 breast cancer cell lines and normal REF cell line. Results: Chloroform extract has potent cytotoxicity in a dose dependent manner, against AMN3, AMJ13, MCF7 cell lines and less toxic effect on normal REF cell line; where IC50 (2.93µg/ml), (2.99µg/ml), (2.67µg/ml), and (15.84µg/ml) respectively. The cytotoxicity of extract combined with Doxorubicin showed a synergistic activity on AMN3, AMJ13, MCF7 cell lines, and dose dependent effect on normal REF cell line (synergism at 3.125-6.25µg/ml, addition at 12.5µg/ml, and antagonism at 25-100µg/ml). Conclusion: Chloroform extract has potent cytotoxicity against breast cancer cell lines and less toxic effect on normal REF cell line. In addition to synergistic activity in combination with Doxorubicin on breast cancer cell lines and dose dependent effect on REF cell line.

Keywords: Chloroform extract, Xanthium strumarium, Cytotoxicity, MTT assay, cell line.

Introduction

The incidence of cancer is increasing worldwide and it is the single most common cause of death in both developed and developing countries. In 2008 approximately 12.7 million cancers were diagnosed and 7.6 million people died of cancer worldwide [1]. Cancers as a group account for approximately 13% of all deaths each year [2]. Herbs derived products have been used by about 80% of populations particularly in developing countries [3]. Although chemotherapeutic drugs and radiation are more powerful maneuvers for treatment of malignancy, but they are associated with serious adverse effects [4].

Another challenge in the treatment of cancer is the development of resistance against therapy [5]. Hence, the major goals of herbs derived products in treatment of malignancy are; prevention of malignancy through induction of unsuitable environment for cancer cells growth, prevention of recurrence of malignancy, and stimulating immunity [6].

Xanthium strumarium is a well known herb which has been used since ages as a cure for various ailments like leucoderma, biliousness, epilepsy, salivation, fever and poisonous bites of insects [7]. The most important chemical constituents of Xanthium strumarium include phenolic compounds such as thiazolidinediones, chlorogenic acids, ferulic acids, 1,3,5-tri-O-caffeoyl quinic acid, 1,5-di-O caffeoyl quinic acid, caffeic acid, as well as isoprenoids such as strumaristerol, β-sitosterol, also monoterpenes, sesquiterpene hydrocarbons, triterpenoid, sesquiterpene lactones and saponins [8], [9], [10].

Most of the pharmacological effects can be explained by the constituents like phenols, sesquiterpene lactones, glycoside, and poly sterols present in all parts of the plant. The objective of this study was to investigate the in vitro cytotoxicity of chloroform extract of Xanthium strumarium.
Materials and Methods

Extraction

Fresh leaves of Xanthium strumarium were collected in August 2016 from farms (Iraq - Baghdad –Salamiyat county) and authentication was done in Pharmacognosy and medicinal plants department - college of Pharmacy / Al-Mustansiriya University prior of purchasing. The leaves were cleaned and shade dried at room temperature, then grinded into fine powder. The total of 600g powder was divided into four equal parts and extracted via chloroform in a ratio of 1:4 W/V (150g powder /600ml solvent), for 24hrs by shaking water bath at 40 °C, then Whitt man no.1 filter paper used for isolating the pure extract. Vacuum rotary evaporator was used for concentrating and obtaining the final crude extract that dried under stream of cold air, and weighed to determine the yield. Extraction process repeated three times and the extract kept in desiccators at room temperature prior to the experiment [11].

Cell Lines

Breast cancer cell lines (AMN3, AMJ13, and MCF7) and normal REF cell line, were supplied by tissue culture unit / ICCMG (Iraki Centre for Cancer and Medical Genetic Researches), Baghdad, Iraq. These cells were maintained in RPMI-1640 media (Roswell Park Memorial Institute -1640 medium) with 10% fetal calf serum and incubated at 37 °C in the humid atmosphere of 5% CO2.

3-(4, 5-dimethylthiazol-2-yl)- 2, 5-diphenyltetrazolium Bromide (MTT) Assay

Cell line proliferation measured according to Mosmann method [12]. The cells were between passages 4-7. The cells were treated with serial concentrations of chloroform extract of Xanthium strumarium for 72 hrs. MTT was prepared by adding 5mg/ml in PBS (phosphate buffer saline). 20µl of MTT was used per well and the plates were incubated at 37 °C, in 5% CO2 for 5hrs. The plates were removed from the incubator and the supernatant was aspirated. DMSO (200µl) was added to each well.

The plates were shaken vigorously for one minute at room temperature to dissolve the dark blue crystals. The absorbance reading was taken at 570nm and the reference at 650nm by using micro-plate reader. The absorbance of cells cultured in control media was taken to represent 100% viability. The viability of treated cells was determined as a percentage of that for the untreated control. Each concentration was tested in triplicate, and the experiment was repeated twice. The concentration of the cells in each well was 1x10⁴. The percentage of cell line inhibition was determined as the mean ± SD using the following equation.

\[ 1-(A0-A1)/(A2-A1) \]

\[ A0 = \text{Absorbance of sample} \]
\[ A1 = \text{Absorbance of blank} \]
\[ A2 = \text{Absorbance of control} \]

IC50 values were calculated by the logarithmic correlation equation.

Synergistic Study of Extract Combination with Chemotherapy (Chou–Talalay Analysis)

The median effect doses (ED50) were calculated for the drug and extract for each cell line. For synergism determination, extract and Doxorubicin were studied as a non-constant ratio.

To analyze the combination of extract and Doxorubicin, Chou–Talalay combination indices (CI) were calculated using Compu Syn software (Combo Syn, Inc., Paramus, NJ, USA). Non-fixed ratios of extract and Doxorubicin, as well as mutually exclusive equations, were used to determine the CIs. A CI between 0.9 and 1.1 is considered additive, whereas CI < 0.9 and CI > 1.1 indicate synergism and antagonism, respectively [13].

Experimental Design and Analysis of Data

The experiment design used for this study was Rationalized Complete Block Design (RCBD). The results were reported as means ± standard deviation (SD). One way analysis of variance (ANOVA) followed by Tukey test comparison t-test (2-tailed) was used to compare between treatments groups. The differences between the means are considered significant at the 5% confidence level. The statistical analysis was carried out by using SSPS 16.0, the level of significance was set at P<0.05.

Results

Cytotoxicity against AMN3 Cell Line

Figure 1 (A and B) shows the in vitro cytotoxicity of chloroform extract on AMN3 cells, which were in passage 4. The results
showed a dose dependent inhibition on the cell growth after 72hr. The extract concentrations used were (100, 50, 25, 12.5, 6.25, and 3.125µg/ml), with each concentration in triplicate and the experiments were repeated twice. The data is represented as the mean ±SD.

The percentages of AMN3 cell proliferation inhibition (IR %) were (86.43±0.015%, 85.37±0.023%, 84.3±0.004%, 83.77±0.039%, 81.91±0.053%, and 80.45±0.011%) at each mentioned concentration respectively. The IC50 value of chloroform extract was (2.93µg/ml), calculated by linear regression equation $\gamma=1.558\ln(x)+79.39$, where $\gamma$ is percentage of inhibition and $x$ is concentration. Doxorubicin was used as a positive control, and had IC50 equal (2.12µg/ml). Figure 1 (C and D) shows effect of the combination of extract and Doxorubicin that showed a dose dependent inhibition on the cell growth after 72hr. An equal concentrations for both extract and Doxorubicin used in combination were (100, 50, 25, 12.5, 6.25, and 3.125µg/ml), with each concentration in triplicate and the experiments were repeated twice. The data is represented as the mean ±SD.

The percentages of AMN3 cell proliferation inhibition were (98.5 ±0.001%, 97.9 ±0.001%, 96.97 ±0.001%, 95.92 ±0.002%, 95.35 ±0.001%, and 93.97 ±0.003%) at each mentioned concentration respectively. Figure 2 (A) shows that the combination in serial concentrations produce AMN3 cell growth inhibition higher than that when Doxorubicin and chloroform extract used each alone. (B) Isobologram analysis shows synergism between chloroform extract and Doxorubicin at serial concentrations as confirmed by combination index data for each dose in table (C), and dose–effect curve (D).
Cytotoxicity against AMJ13 Cell Line

Figure 3 (A and B) shows the *in vitro* cytotoxicity of chloroform extract on AMJ13 cells, which were in passage 4. The results showed a dose dependent inhibition on the cell growth after 72hr.

The extract concentrations used were (100, 50, 25, 12.5, 6.25, and 3.125µg/ml), with each concentration in triplicate and the experiments were repeated twice. The data is represented as the mean ±SD. The percentages of AMJ13 cell proliferation inhibition (IR %) were (84.24±0.003%, 83.05±0.013%, 82.42±0.038%, 80.99±0.014%, 80.04±0.019%, and 79.61±0.04%) at each mentioned concentration respectively.

The IC50 value of chloroform extract was (2.99µg/ml), calculated by linear regression equation $y=1.385 \ln(x) +77.74$, where $y$ is percentage of inhibition and $x$ is concentration. Doxorubicin was used as a positive control, and had IC50 equal (2.41µg/ml). Figure 3 (C and D) shows effect of the combination of extract and Doxorubicin that showed a dose dependent inhibition on the cell growth after 72hr. An equal concentrations for both extract and Doxorubicin used in combination were (100, 50, 25, 12.5, 6.25, and 3.125µg/ml), with each concentration in triplicate and the experiments were repeated twice. The data is represented as the mean ±SD. The percentages of AMJ13 cell proliferation inhibition were (97.12±0.004 %, 95.76±0.001%, 95.18±0.003%, 94.92±0.001 %, 93.48±0.007%, and 91.22±0.011%) at each mentioned concentration respectively.

Figure 4 (A) shows that the combination in serial concentrations produce AMJ13 cell growth inhibition higher than that when Doxorubicin and chloroform extract used each alone. (B) Isobologram analysis shows synergism between chloroform extract and Doxorubicin at serial concentrations as confirmed by combination index data for each dose in table (C), and dose–effect curve (D).
Figure 3: Cytotoxicity of chloroform extract and its combination on AMJ13 cell line. (A) Cell proliferation inhibitory effect of serial concentrations of chloroform extract on AMJ13. (B) Cytotoxic effect of 100µg/ml of chloroform extract on AMJ13 cells, showed as cell detachment and nuclear condensation referring to cell injury and necrosis. (C) Cell proliferation inhibitory effect of serial concentrations of the combination of extract and Doxorubicin on AMJ13. (D) Cytotoxic effect of 100µg/ml of the combination of extract and Doxorubicin on AMJ13 cells, showed as cellular detachment indicating cellular injury and necrotic damage. (E) Control (untreated AMJ13 cells). (F) Cytotoxic effect of 100µg/ml of Doxorubicin as positive control on AMJ13 cells, showed as wide cells damage and necrosis.

Figure 4: Cytotoxicity of the combination of chloroform extract and Doxorubicin on AMJ13 cell line. (A) Comparison between effects of Doxorubicin, chloroform extract, and combination on AMJ13 growth inhibition. (B) Isobologram analysis shows synergism. (C) Combination index. (D) Dose-effect curve.
Cytotoxicity against MCF7 Cell line

Figure 5 (A and B) shows the *in vitro* cytotoxicity of chloroform extract on MCF7 cells, which were in passage 4. The results showed a dose dependent inhibition on the cell growth after 72 hr. The extract concentrations used were (100, 50, 25, 12.5, 6.25, and 3.125 µg/ml), with each concentration in triplicate and the experiments were repeated twice. The data is represented as the mean ±SD.

The percentages of MCF7 cell proliferation inhibition (IR %) were (84.17±0.017%, 83.25±0.023%, 82.5±0.016%, 80.94±0.006%, 79.06±0.005%, and 78.22±0.042%) at each mentioned concentration respectively. The IC50 value of chloroform extract was (2.67 µg/ml), calculated by linear regression equation $y = 1.808 \ln(x) + 76.16$, where $y$ is percentage of inhibition and $x$ is concentration. Doxorubicin was used as a positive control, and had IC50 equal (2.81 µg/ml). Figure 5 (C and D) shows effect of the combination of extract and Doxorubicin that showed a dose dependent inhibition on the cell growth after 72 hr. An equal concentrations for both extract and Doxorubicin used in combination were (100, 50, 25, 12.5, 6.25, and 3.125 µg/ml), with each concentration in triplicate and the experiments were repeated twice. The data is represented as the mean ±SD. The percentages of MCF7 cell proliferation inhibition were (97.27±0.002%, 95.35±0.001%, 94.75±0.002%, 91.79±0.006%, 90.89±0.006%, and 90.28±0.012%) at each mentioned concentration respectively.

Figure 6 (A) shows that the combination in serial concentrations produce MCF7 cell growth inhibition higher than that when Doxorubicin and chloroform extract used each alone. (B) Isobologram analysis shows synergism between chloroform extract and Doxorubicin at serial concentrations as confirmed by combination index data for each dose in table (C), and dose – effect curve (D).

Figure 5: Cytotoxicity of chloroform extract and its combination on MCF7 cell line. (A) Cell proliferation inhibitory effect of serial concentrations of chloroform extract on MCF7. (B) Cytotoxic effect of 100 µg/ml of chloroform extract on MCF7 cells, showed as cell detachment and nuclear condensation referring to cell injury and necrosis. (C) Cell proliferation inhibitory effect of serial concentrations of the combination of extract and Doxorubicin on MCF7. (D) Cytotoxic effect of 100 µg/ml of the combination of extract and Doxorubicin on MCF7 cells, showed as cellular detachment indicating cellular injury and necrotic damage. (E) Control (untreated MCF7 cells). (F) Cytotoxic effect of 100 µg/ml of Doxorubicin as positive control on MCF7 cells, showed as wide cells damage and necrosis.
Cytotoxicity against REF Cell line

Figure 7 (A and B) shows the in vitro cytotoxicity of chloroform extract on normal REF cells, which were in passage 4.

The results showed a dose dependent inhibition on the cell growth after 72hr. The extract concentrations used were (100, 50, 25, 12.5, 6.25, and 3.125µg/ml), with each concentration in triplicate and the experiments were repeated twice. The data is represented as the mean ±SD.

The percentages of REF cell proliferation inhibition (IR%) were (65.03 ±0.004%, 62.99 ±0.002%, 53.7 ±0.002%, 49.62 ±0.002%, 47.08 ±0.012%, and 28.03 ±0.02%) at each mentioned concentration respectively.

The IC50 value of chloroform extract was (15.84µg/ml), calculated by linear regression equation y=9.761 ln(x) +23.03, where y is percentage of inhibition and x is concentration. Doxorubicin was used as a positive control, and had IC50 equal (3.32µg/ml).Figure 7 (C and D) shows effect of the combination of extract and Doxorubicin that showed a dose dependent inhibition on the cell growth after 72hr. An equal concentrations for both extract and Doxorubicin used in combination were (100, 50, 25, 12.5, 6.25, and 3.125µg/ml), with each concentration in triplicate and the experiments were repeated twice. The data is represented as the mean ±SD.

The percentages of REF cell proliferation inhibition were (67.7 ±0.003%, 65.67 ±0.002%, 64.39 ±0.001%, 61.84 ±0.006%, 59.28 ±0.003%, and 58.19 ±0.001%) at each mentioned concentration respectively.

Figure 8 shows the cytotoxicity of the combination of chloroform extract and Doxorubicin on normal REF cell line.

(A) Shows that the combination at lower concentrations (3.125- 6.25µg/ml) produce cell growth inhibition higher than that when Doxorubicin and chloroform extract used each alone; while at higher concentrations (25-100µg/ml), it produce lower cell growth inhibition.

(B) Isobologram analysis shows that chloroform extract and Doxorubicin have synergism at lower concentrations (3.125-6.25µg/ml), addition at (12.5µg/ml), and antagonism at (25-100µg/ml) as confirmed by combination index data for each dose in table (C), and dose–effect curve (D).
Figure 7: Cytotoxicity of chloroform extract and its combination on normal REF cell line. (A) Cell proliferation inhibitory effect of serial concentrations of chloroform extract on REF. (B) Cytotoxic effect of 100µg/ml of chloroform extract on REF cells, showed as some cell detachment and little nuclear condensation referring to cell injury and moderate necrosis. (C) Cell proliferation inhibitory effect of serial concentrations of the combination of extract and Doxorubicin on REF. (D) Cytotoxic effect of 100µg/ml of the combination of extract and Doxorubicin on REF cells, showed as cellular detachment indicating cellular injury and necrotic damage. (E) Control (untreated REF cells). (F) Cytotoxic effect of 100µg/ml of Doxorubicin as positive control on REF cells, showed as cells damage and necrosis.

Figure 8: Cytotoxicity of the combination of chloroform extract and Doxorubicin on normal REF cell line. (A) Comparison between effects of Doxorubicin, chloroform extract, and combination on REF growth inhibition. (B) Isobologram analysis shows synergism, addition, and antagonism. (C) Combination index. (D) Dose-effect curve.
Discussion

In the present study, sequential extraction was performed to get most of the active components that have been isolated based on their polarity to be used latterly [14]. In order to assess their pharmacological activity either they have ant proliferative or cytotoxic activities. The extraction procedure used in the present study called maceration method or cold method, in order to avoid possible destruction or loss of the leaves components that result from exposure to high temperature [15].

Several factors influencing the amount of extract; such as shaking speed, maceration period, solvents types and concentrations, water temperature in the water bath that contain leaves powder of Xanthium strumarium; the previous factors explain the variability in the percentage of extract amount [16]. Moreover, literature reviews shows high concentrations of several chemical constituents which may have a potent cytotoxic and ant proliferative activities such as phenolic compounds [17], saponins [18], and sesquiterpene lactones [10]. In vitro cytotoxicity of chloroform extract of Xanthium strumarium was evaluated with AMN3, AMJ13, MCF7 breast cancer cell lines and REF transformed cell line to determine if the extract has any cytotoxic effect against breast cancer cell lines.

The selective cytotoxic effect is favorable property of a new candidate antitumor drug [19]. The results showed that chloroform extract had a significant cytotoxic effect (P<0.05) in a dose dependent manner after 72hrs exposure period, on the breast cancer cell lines and less toxic effect on normal REF cell line. According to National Cancer Institute (NCI), the extract with IC50 < 20μg/ml considered cytotoxic [20].

The extract of Xanthium strumarium exerted a cytotoxic effect on Chinese hamster ovary (CHO) cells in both dose-time dependent manners, IC50 value of extract on CHO cells was 20.5μg/ml. Also it can induce in vitro DNA damage at cytotoxic concentrations [21].

The combination of chloroform extract and Doxorubicin had significant cytotoxic effect (P<0.05) in a dose dependant manner after 72hrs exposure period, on the breast cancer cell lines and lower effect on normal REF cell line. Numerous studies detected that the combination of certain natural compounds with chemotherapy improving anticancer activities through elevation of intracellular drug accretion.

The combination of Vitis vinifera pro-anthocyanidin with doxorubicin suppressing hepatocarcinoma development. The proposed mechanism of doxorubicin induced apoptosis may attribute to accumulation of doxorubicin intracellularly [22]. This study showed that chloroform extract have synergistic effect with Doxorubicin on breast cancer cell lines (AMN3, AMJ13, and MCF7), and dose dependent effect on normal REF cell line (synergism at lower doses, and antagonism at high doses); Decker and Sausville (2005) described in vitro results in more than one cell line is important in the evaluation of potential combination regimens [23].

Conclusion

The results showed that chloroform extract of Xanthium strumarium has potent cytotoxicity against breast cancer cell lines and less toxic effect on normal REF cell line. In addition to synergistic activity in combination with Doxorubicin on breast cancer cell lines and dose dependent effect on REF cells.

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References


