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Research paper

The extracts of *Gentiana lutea* with potential cytotoxic effects on human carcinoma cell lines: A preliminary study

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ABSTRACT

Introduction: Cancer is a disease with increasing incidence and is the second cause of death worldwide. Phytotherapy is based on the use of plants to treat diseases and is important in the development of new therapeutic strategies such as anti-cancer drugs. The aim of this work was to perform a study on the effect of aqueous and ethanolic extracts of *Gentiana lutea* in two human cancer cell lines, human cervical cancer (HeLa) and breast adenocarcinoma (MCF-7).

Methods: The aqueous and ethanolic extracts were prepared both with MEM culture medium at different concentrations as follow: 31.25 μg/ml, 62.5 μg/ml, 125 μg/ml, 250 μg/ml and 500 μg/ml. Cells were plated in 96-multiwell culture plates at a density of 10^4 and 10^5 cells/well for each extract concentration. Untreated cancer cells acted as a control group.

Results: The results exhibited a hyperbolic relationship between growth inhibition for HeLa /and MCF-7 cell lines and ethanol extract concentration revealed the highest inhibition observed at 500 μg/ml for both cell concentrations. MCF-7 cell line was inhibited when exposed to the ethanol extract of *Gentiana lutea* (highest growth inhibition of 25%). Aqueous plant extracts exhibited a different behavior since there was an increase (15–20%) in cell growth at low extract concentrations while a cell growth inhibition (15–25%) was only observed at the highest extract concentration.

Conclusion: The present results strongly suggest growth inhibition of carcinoma cell lines by the hyperbolic relationship for both plant extracts and it will require a detailed future research to understand its molecular mechanism.

1. Introduction

According to the World Health Organization (WHO), cancer is the second leading cause of death globally, nearly 1 in 6 deaths is caused by cancer disease [1]. Lung, prostate, colorectal, stomach and liver cancer are the most common types among men whereas breast, colorectal, lung, cervix and stomach cancer occur very often in women [1]. Therefore, there is still a huge challenge for cancer prevention and therapy worldwide. Treatment options may include surgery, chemotherapy, radiotherapy, targeted therapy and immunotherapy, aiming for controlling tumor growth and prolonging survival time as well as improving quality of life [1–3].

Although these therapies target either the neoplastic cells or the mechanisms promoted by them, they present several drawbacks. Firstly they may be associated with multiple adverse effects such as risk of post-surgical complications, lymphedema, fatigue, anemia, nausea and diarrhea, hair loss, immunosuppression and increased risk of infectious diseases [2,4]. These adverse effects seem to decrease patients quality of life, limit the use of these treatments at therapeutic doses and compromise the success of treatment [2,3]. Secondly, recent drugs with targeted and immune mechanisms of action also cause side effects and are highly expensive and hence most patients cannot afford to buy them [2]. Thirdly, existing anticancer therapies do not have the desirable effectiveness and relapse from disease is a frequent event [5,6].

The history of mankind is marked by the use of medicinal plants which has been an ancient practice for millions of years. Western medicine has exploited this traditional knowledge to develop new drugs and therefore, natural products are a major source of anticancer drugs [7,8]. Some examples include the Vinca alkaloids (vinblastine and vincristine) from *Catharanthus roseus*, the terpene paclitaxel from *Taxus*

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brevifolia, camptothecin from *Camptotheca acuminata* and the semisynthetic derivatives from *Podophyllum peltatum* [5,9].

The interest of the western scientific community in plants used in Traditional Chinese Medicine (TCM) is increasing significantly as they have been recognized as a new source of new anticancer drugs [8,10,11] such as Artesunate from *Artemisia annua* and Curcumin from *Curcuma longa* [5,9,12–14]. As far as medicinal plants are concerned, only about 15% have been studied for their chemical composition and biological actions and therefore, there is a great need for further studies regarding screening of other medicinal plants for their biological activities. *Gentiana* is the largest genus of Gentianaceae family which have been used for medicinal uses such as anti-rheumatic, anti-inflammatory, analgesic, antipyretic, hypoglycemic and diuretic [15,16]. *Gentiana lutea* is used both in western traditional herbal medicinal and in TCM [17,18]. The active substances are classified as bitter constituents and belong to the class of secoiridoid glycosides, with gentiopicroside (also known as gentiamarine and gentiopicrine). Although several reports have been published in the literature on medicinal properties of this plant [15,16], there are no reports on the use of either extracts or isolated active compounds from this plant for cytotoxic action on human carcinoma cell lines.

Therefore, the present work involves the investigation of in vitro cytotoxic activity of aqueous and ethanolic extract from *Gentiana lutea* on two human carcinoma cell lines [epitheloid cervix carcinoma (HeLa) and breast adenocarcinoma (MCF-7)].

2. Methods

2.1. Chemicals

Minimum Essential Medium Eagle (MEM) culture medium, fetal bovine serum (FBS), L-Glutamine, gentamicin, non-essential amino acid solution, phosphate-buffered saline solution (PBS), trypsin-EDTA solution (0,25%), 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) and dimethyl sulphoxide (DMSO) were purchased from Sigma-Aldrich (Germany). All other reagents and chemicals used were of analytical grade.

2.2. Plant material

The dry root of *Gentiana lutea, species voucher nº BCN* 24893 was purchased at Magnolien Apotheke, Germany, Karlsruher Str. 14, 69126 Heidelberg.

2.3. Preparation of crude aqueous and ethanolic extracts

Ethanolic extract was prepared by adding 125 g of gentian to 625 ml of ethanol (70%, v/v). The extract was obtained by maceration at room temperature for 5 days with mild shaking. Subsequently the suspension was filtered and centrifuged for 1 h at 15,000 rpm to discard the sediment. The supernatant was evaporated under reduced pressure to yield 40.42 g of dry crude extract. A stock solution of this extract was prepared with DMSO, filtered with 0.20 µm and all tested solutions were prepared with MEM culture medium as follows: 31.25 µg/ml, 62.5 µg/ml, 125 µg/ml, 250 µg/ml and 500 µg/ml. The final concentration of DMSO in the culture medium was < 0.1% (v/v) [19].

Aqueous extract was prepared by adding 125 g of gentian to 625 ml of distilled water and the extract was obtained by mixing it for 30 min at room temperature and boiling it for another 30 min. Subsequently the suspension was filtered and centrifuged for 1 h at 15,000 rpm to discard the sediment. The supernatant was evaporated under reduced pressure to yield 35.26 g of dry crude extract. A stock solution of this extract was prepared with distilled water, filtered with 0.20 µm and all tested solutions were prepared with MEM culture medium as follows: 31.25 µg/ml, 62.5 µg/ml, 125 µg/ml, 250 µg/ml and 500 µg/ml.

2.4. Tissue culture

Two human carcinoma cell lines were used to investigate the cytotoxic activity of these plant extracts. Epitheloid cervix carcinoma (HeLa) and breast adenocarcinoma (MCF-7) were grown in MEM culture medium supplemented with 10% FBS, 2 mM of glutamine, 50 mg/l of gentamicin and 1% of non-essential amino acids at 5% CO2 and 37 °C for several days. Growth curves were carried out for these cell lines by trypsin treatment and viable cells were determined by trypan blue and MTT assays [20–22].

2.5. MTT reduction assay

To evaluate effects of the different concentration of aqueous and ethanolic plant extracts on cell viability, cells were plated in 96-multiwell culture plates at a density of 10^4 and 10^5 cells/well. Twenty four hours after plating, testing concentration were added (31.25 µg/ml, 62.5 µg/ml, 125 µg/ml, 250 µg/ml and 500 µg/ml) as well as 0.1% DMSO as negative control. After 72 h of incubation with plant extracts, MTT was added at a final concentration of 0.5 mg/ml and incubated for 2 h. Then, the medium was removed, and the formazan crystals were dissolved in isopropanol. Absorbance was measured at 570 nm and 655 nm in a microplate reader. 655 nm absorbance background was subtracted from the 570 nm absorbance. The MTT colorimetric assay is based upon mitochondrial conversion of tetrazolium salt (MTT) into formazan crystals, and thus alterations in the number of viable cells can be detected by measuring formazan crystals optical density [20–22]. The results were expressed as the percentage of cell inhibition relative to control cells concerning the untreated cancer cells which was carried out in quadruplicate.

2.6. Statistical analysis

All experiments were carried out in quadruplicate and the results are presented as mean value ± standard deviation (SD). Correlation and regression analyses were performed with the Excel software 2013 package. Correlations were considered statistically significant at $p < 0.05$ according to Tukey HSD and Scheffé test.

3. Results

In order to test the effect of *Gentiana lutea* ethanolic and aqueous extracts on cell viability, two human cancer cell lines, Hela and MCF-7 were used. The assay was performed on exponentially growing cells. Each cell line was incubated for 72 h with extracts at different concentrations (i.e. 31.25, 62.50, 125, 250 and 500 µg/ml), and then cell viability was evaluated by MTT assay. The results were presented as a percentage of cell inhibition compared with control cells which were also incubated during 72 h at same conditions but without any extract.

3.1. HeLa cell line

The data in Fig. 1A exhibited cell viability during the incubation time of HeLa human carcinoma cell line, at two cell concentrations per well, when exposed to the ethanol extract of *Gentiana lutea*. There is a hyperbolic relationship between cell growth inhibition and ethanol extract concentration and the highest inhibition (i.e. about 100% inhibition) was observed at 500 µg/ml for both cell concentrations (Fig. 1B). However, aqueous plant extracts exhibited a different behavior since there was an increase in cell growth (i.e. about 15–20% increase) at low extract concentrations and cell growth inhibition (i.e. about 15–25% inhibition) was only observed at the highest extract concentration (Fig. 2). This behavior was apparently similar for both cell concentrations tested (Fig. 2).

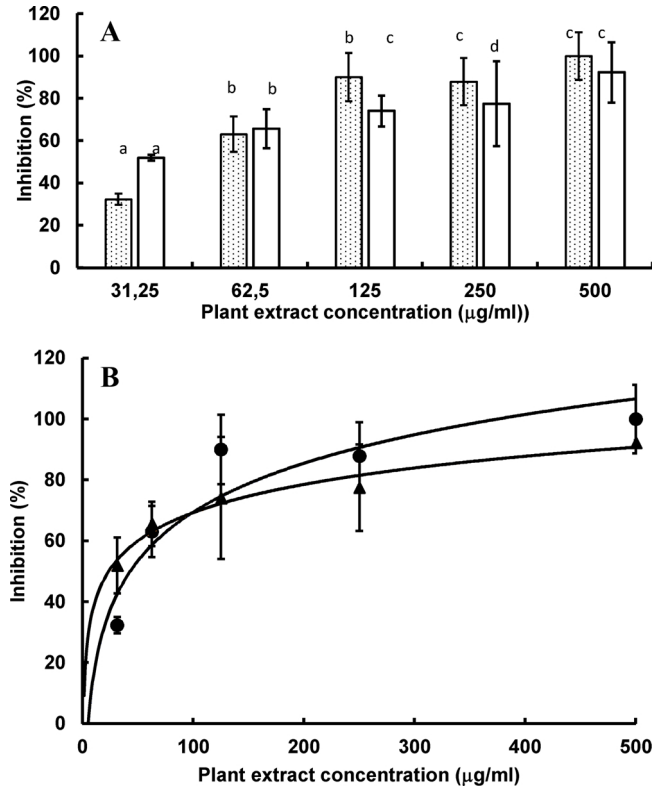

Fig. 1. (A) Effect of ethanol extract on growth of HeLa human carcinoma cell line which was incubated with different concentrations of plant extract as described in the Materials and Methods. Dotted columns - (1×10^4 cells / well) and open columns - (1×10^5 cells / well). Values with different letters were significantly different ($p < 0.05$) according to Tukey HSD and Scheffé tests. (B) Hyperbolic relationship between growth inhibition of HeLa human carcinoma cell line and increasing ethanol extract concentrations as described in Materials and Methods. 1×10^4 cells / well – ● and 1×10^5 cells / well - ▲.

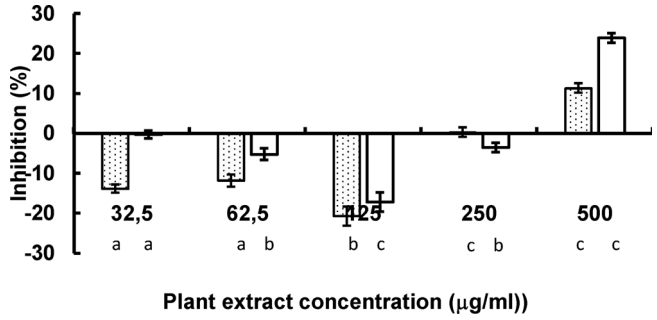

Fig. 2. Effect of aqueous extract on growth of HeLa human carcinoma cell line which was incubated with different concentrations of plant extract as described in the Materials and Methods. Dotted columns - (1×10^4 cells / well) and open columns - (1×10^5 cells / well). Values with different letters were significantly different ($p < 0.05$) according to Tukey HSD and Scheffé tests.

3.2. MCF-7 cell line

The data presented in Fig. 3 revealed cell viability during the incubation period of MCF-7 human carcinoma cell line, at two cell concentrations per well, when exposed to the ethanol extract of *Gentiana lutea*. There is a bell-shape relationship between growth inhibition and plant extract concentration and the highest inhibition was observed at 125 µg/ml for both cell concentrations (Fig. 3). Moreover, the highest growth inhibition of this cell line was only about 25% as opposed to 100% inhibition observed for HeLa cell line in the presence of ethanol extract (Fig. 1). However, aqueous plant extracts (Fig. 4A) exhibited a

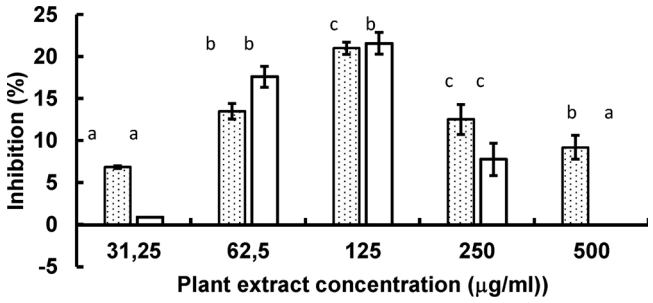

Fig. 3. Effect of ethanol extract on growth of MCF7 human carcinoma cell line which was incubated with different concentrations of plant extract as described in the Materials and Methods. Dotted columns - (1×10^4 cells / well) and open columns - (1×10^5 cells / well). Values with different letters were significantly different ($p < 0.05$) according to Tukey HSD and Scheffé tests.

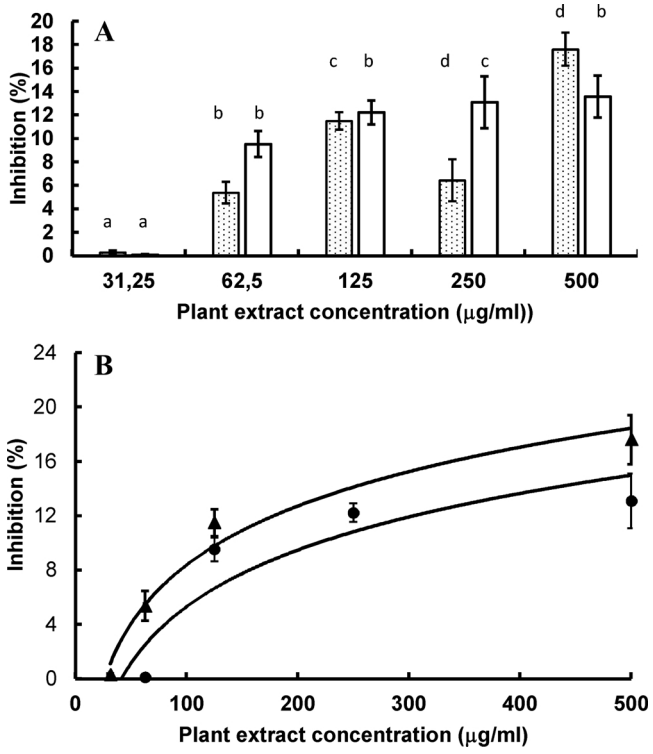

Fig. 4. (A) Effect of aqueous extract on growth of MCF7 human carcinoma cell line which was incubated with different concentrations of plant extract as described in Materials and Methods. Dotted columns - (1×10^4 cells / well) and open columns - (1×10^5 cells / well). Values with different letters were significantly different ($p < 0.05$) according to Tukey HSD and Scheffé tests. (B) Hyperbolic relationship between growth inhibition of MCF7 human carcinoma cell line and increasing aqueous extract concentrations as described in Materials and Methods. 1×10^4 cells / well – ● and 1×10^5 cells / well - ▲.

different behavior on cell growth since there was a hyperbolic relationship between cell growth inhibition of this cell line and aqueous extract concentrations used (Fig. 4B). Moreover, the highest growth inhibition for this cell line was only about 15–20% which were similar to values obtained for HeLa cell line for aqueous extracts. This behavior was apparently similar for both cell concentrations tested (Fig. 4A). These data strongly suggest that both ethanol and aqueous extracts contained different secondary metabolites which exhibited growth inhibition of this human carcinoma cell line but to a lesser extent than HeLa cell line.

4. Discussion

Phytotherapy plays a major role in the development of new therapeutic strategies against cancer cells. The aim of this work was to investigate the effect of aqueous and ethanolic extracts of *Gentiana lutea* on two human cancer cell lines (i.e HeLa and MCF-7). The methodology of extract preparation of medicinal plants plays a major role for potential cytotoxic effects on human carcinoma cell lines in vitro [23]. In the present work, preliminary experiments were carried out with ethanol and water as solvents for plant extract preparation both at room temperature and at high temperature (i.e boiling). The preparation of aqueous plant extract at room temperature did not exhibit inhibition of human carcinoma cell line which may be due to the absence of secondary metabolites with inhibitory activity (data not shown). Therefore, the aqueous extract was boiled in order to extract secondary metabolites with inhibitory activity which were present in the plant material. Since the present work involves the use of roots of *Gentiana lutea*, aqueous extract preparation should be carried out at high temperature which is in agreement with literature report [24]. Although the boiling procedure may degrade some thermolabile compounds present in plant material, boiling has been widely used in the literature for aqueous extraction of secondary metabolites from traditional Chinese medicinal plants [25].

Ethanol extract was found to inhibit the proliferation of HeLa human carcinoma cell line by exhibiting a hyperbolic relationship. This hyperbolic behavior was reported for several plant extracts (i.e *Eclipta alba* and *Asteriscus graveolens*) due to the presence of various secondary metabolites, respectively [26,27]. However, aqueous extract exhibited an increase in cell growth of HeLa cell line at low extract concentrations whereas cell growth inhibition was only observed at highest cell concentration. These data strongly suggest that ethanol extracts contained secondary metabolites which exhibited growth inhibition of human carcinoma cell line at all extract concentrations whereas aqueous extracts had secondary metabolites that activated cell growth at low extract concentrations. As far the literature is concerned, no work has been reported about the effect of *Gentiana lutea* plant extracts on human carcinoma cell lines. However, several reports have been published in the literature about various therapeutics properties exhibited by the family of Gentianaceae, which contained relevant and useful secondary metabolites. Secoiridoidal glycosides are the most important bitter compounds which belong to Gentiana genus. Gentiopicroside and amarogentin are the secoiridoid which have been isolated from *G. lutea*'s root [28]. Secoiridoidal glycosides isolated from different Gentiana species exhibited several important biological activities since amarogentin and amaroswerin have revealed the strongest gastroprotective effects among the other secoiridoidals [29]. Gentiopicrin and xanthone isogentisin and mangiferin isolated from leaves and flowers of *G. lutea* exhibited considerable antimicrobial activities [30]. Gentiana roots also revealed the hepatoprotective activities due to the presence of sweroside, swertiamarin and gentiopicrin compounds [31].

As far as *MCF-7 cell line* is concerned, the present work exhibited a bell-shape relationship between growth inhibition and ethanol extract concentration. However, aqueous plant extracts exhibited a different behavior on cell growth since there was a hyperbolic relationship between cell growth inhibition of this cell line and aqueous extract concentrations used. It was mentioned previously that similar reports have been described in the literature about hyperbolic relationship for plant extracts (i.e *Eclipta alba and Asteriscus graveolens*) [26,27]. However, there is a striking difference between these two cell lines since aqueous extracts exhibited activation of cell growth for HeLa cell line whereas there was growth inhibition for MCF-7 cell line at low extract concentrations. These data strongly suggest that both ethanol and aqueous extracts contained different secondary metabolites which exhibited growth inhibition of this human carcinoma cell line but to a lesser extent than HeLa cell line. As far literature is concerned, there is no work reported about the effect of *Gentiana lutea* extracts on human carcinoma cell lines.

The new findings of the present study strongly suggest cell growth inhibition of human carcinoma cell lines in the presence of either aqueous or ethanol extracts. The implications of these findings will require a systematic investigation of several factors affecting inhibition of cell growth in vitro. The perspective for this study are very interesting in order to devise a novel therapeutic strategy for cancer treatment.

It must be stressed that the solvents (i.e water and ethanol) used in the present work have different polarity and dielectric constants and therefore water is more polar than ethanol which means that the former has a higher capacity to dissolve polar or hydrophilic compounds whereas ethanol can dissolve non-polar or hydrophobic compounds. Another issue that must be pointed out is due to the fact that crude plant extracts were used in these experiments which contained many secondary metabolites that may act either synergistically or opposing activity on human carcinoma cell lines *in vitro*. In fact, there is also some evidence that the presence of various secondary metabolites in plant extracts have the ability to buffer the toxic effects of a single component [32].

However, further work is required which involves fractionation of the crude extract in order to separate several secondary metabolites by GC–MS and to analyse their effects on human carcinoma cell lines. Moreover, secondary metabolites responsible for growth inhibition must be identified by analytical techniques such as FTIR, NMR and GC–MS. And finally, the molecular mechanism responsible for growth inhibition of human carcinoma cell lines in vitro must be analysed in detail.

5. Conclusions

There is a hyperbolic relationship between growth inhibition for HeLa cell line and ethanol extract concentration and the highest inhibition was observed at 500 μg/ml. However, aqueous plant extracts exhibited a different behavior since there was an increase in cell growth (i.e. about 15–20% increase) at low extract concentrations and cell growth inhibition (i.e. about 15–25% inhibition) was only observed at the highest extract concentration. On the other hand, MCF-7 human carcinoma cell line exhibited inhibition when exposed to the ethanol extract of *Gentiana lutea*, showing a bell-shape relationship between growth inhibition and plant extract concentration. Moreover, the highest growth inhibition of this cell line was only about 25% as opposed to 100% inhibition observed for HeLa cell line in the presence of ethanol extract. However, aqueous plant extracts exhibited a different behavior on MCF-7 cell growth since there was a hyperbolic relationship between growth inhibition of this cell line and aqueous extract concentrations used. And the highest growth inhibition for this cell line was only about 15–20% which were similar to values obtained for HeLa cell line for aqueous extracts. The data presented in this work will stimulate more detailed research work in this field in order to understand the molecular mechanism of these plant extracts on cytotoxic effects of human carcinoma cell lines.

Author's contribution

AK designed the research plan, CR performed all the experiments and AK, CR and JM wrote the MS.

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Conflict of interest

The authors declare there are no conflicts of interest.

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