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In-vitro Cytotoxic Screening of *Glycyrrhiza glabra* L. (Fabaceae): A Natural Anticancer Drug

Rathi SG, Suthar M[1], Patel P, Bhaskar VH, Rajgor NB

M.P Patel College of Pharmacy, Department of Pharmaceutics, Jeevanshilp Campus, Kapadwanj, Dist-Kheda-387 620, Gujarat; [1]S.K Patel College of Pharmacy, Education and Research, Ganpat University, Kherva, Gandhinagar-Mehsana Highway, Gujarat, India

Address for correspondence: *Mr. Sanjesh Rathi; E-mail: rathi_sanjesh@yahoo.co.in*

ABSTRACT

During the last few decades, chemo preventive and chemotherapeutic compounds against various types of cancer have been isolated from various plants. Glycyrrhezic acid (2-20%), Glycyrrhizin, is present as a major chemical constituent of natural anticancer compound *Glycyrrhiza glabra* L. (Fabaceae). The present work is aimed to investigate the in-vitro cytotoxic screening of standard 18 β-glycyrrhetic acid and also for natural anticancer drug *Glycyrrhiza glabra* L. (Fabaceae) using three different extracts (chloroform, methanol and water) of the drug through MTT method. Cell viability of previously identified 18 β-glycyrrhetic acid in three different extracts of *Glycyrrhiza glabra* L. (Fabaceae) was determined by two fold trypan blue method using two different cell lines MCF7-cancerous and Vero-normal cell line and it was quantified through HPTLC method. The results of the HPTLC study indicated that the amount of 18 β-glycyrrhetic acid into three different extracts (chloroform, methanol and water extract) of *Glycyrrhiza glabra* L. (Fabaceae) was 26.6, 12.5 and 5.6 µg/g, respectively. The percentage viability of two different cell lines was 45.71% for Vero-normal cell line and 78.78% for MCF7-cancerous cell line. IC 50 values of standard 18 β-glycyrrhetic acid was 0.412±0.004 µM and those for the three different extracts (chloroform, methanol and water)of *Glycyrrhiza glabra* L. (Fabaceae) on MCF7 cell line were 0.4485±0.001, 0.9906±0.001 and 1.288±0.005 µM, respectively. From the above result, it can be said that 18 β-glycyrrhetic acid could be considered as a potential source of natural anticancer component and the percentage of which was higher in the chloroform extract.

Key words: 18 β-glycyrrhetic acid, cell viability *Glycyrrhiza glabra*, HPTLC, MTT method

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INTRODUCTION

During the last few decades, chemo preventive and chemotherapeutic compounds against various types of cancer have been isolated from various plants. The saponin glycoside, Glycyrrhezic acid (2-20%**)** is present as a major chemical constituent in *Glycyrrhiza glabra L.* (Fabaceae).[1-3] 18 β-glycyrrhetic acid is an outstanding example of natural anticancer compound, which is ahydrolyzed derivative of Glycyrrhezic acid extracted from *Glycyrrhiza glabra L.* (Fabaceae).[4,5] Glycyrrhizin and other *Glycyrrhiza glabra L.* (Fabaceae) components appear to possess anti-carcinogenic properties. They inhibit abnormal cell proliferation as well as tumor formation and growth in breast, liver and skin cancer.[6-8]

Some of the complications occur during *in vivo cytotoxic screening*, that is intravenous administration of chemotherapeutic drugs cause significant individual differences in biotransformation and biodistribution.[9]

To overcome this problem, *in vitro* cytotoxic screenings are used in which the effect of chemotherapeutic drug is being studied on the tumor cells in culture outside the body. There are two basic types of *in vitro* cancer screening method - (a) chemosensitivity and (b) chemo resistance.[10] Chemosensitivity refers to the ability of an agent to kill target cells. Chemo resistance refers to the ability of target cells to withstand exposure to the drug. MCF7 (cancerious) and Vero (normal) cell-line are used for the *in-vitro* screening of natural anticancer agent- *Glycyrrhiza glabra L.* (Fabaceae). The MCF7 cell line retains several characteristics of differentiated mammary epithelium including ability to process estradiol via cytoplasmic estrogen receptors and the capability of forming domes. Growth of MCF7 cells is inhibited by tumor necrosis factor alpha (TNF alpha). [11-12]

Common basic steps of in Vitro cytotoxic screening include: (1) isolation of cells, (2) incubation of cells with drugs, (3) assessment of cell survival and (4) interpretation of the result. The trypan blue dye exclusion assay is the most commonly accepted method for the measurement of cell viability. It relies on the alteration in membrane integrity as determined by the uptake of dye by dead cells, thereby giving a direct measure of cell viability.[13] MTT method is a sensitive, quantitative and reliable colorimetric assay that measures viability, proliferation and activation of cells. The assay is based on the capacity of mitochondrial dehydrogenase enzymes in living cells to convert the yellow water-soluble substrate 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) into a dark blue formazan product, which is insoluble in water as shown in Figure 1.[14]

MATERIALS AND METHODS

Reagents

Methanol, Chloroform, Toluene, Ethyl acetate, Glacial acetic acid, Anisaldehyde-sulfuric acid(AR grade),Trypan blue, Triton X100, DMSO cell culture grade, Sodium bicarbonate, HYQ® Antibiotic/Antimycotic solution (100X), EDTA, HYQ® DPBS/modified 1X, 0.25% Trypsin 1X ,18 β-glycyrrhetic acid standard (Sigma-Aldrich LTD, Mumbai), *Glycyrrhiza glabra L.* (Fabaceae) powder (LVG, Ahmedabad), Silica gel 60 F254 HPTLC plates (E. Merck).

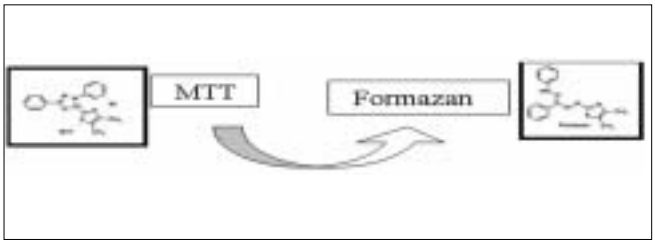

Figure 1: Conversion of 3-(4, 5-dimethylthiazol-2- yl)-2, 5-diphenyl tetrazolium bromide (MTT) into formazan

Media

DMEM, RPMI1640,FBS (Fetal Bovine Serum, South American origin, 500 ml), HYQ® SFM HEK-293™, Thioglycollate medium (TGM), Tryptone soya broth (TSB), Cell proliferation kit (MTT) 2500 tests.

Microbial and fungal culture

Candida albicans, Bacillus subtilis, Candida sporogenes, Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh.

Cell lines

VERO (Normal cell line); MCF7 (Cancerious cell line) All cell lines purchase from National Centre for Cell Science (NCCS), Pune.

Determination of bacteria and fungi in VERO and MCF7 cell lines

Cell line was cultured in the absence of antibiotics at NCCS, Pune. Cell suspension was prepared by scrapping attached cells with the use of a cell scraper and maintained the pH 7.5-8.0. In 1.5-ml cell suspension, 2-ml thioglycollate medium (TGM) and 2-ml tryptone soya broth (TSB) were added and inoculated with two different strains; *Candida albicans* (0.1ml) *Bacillus subtilis* (0.1ml). Then in 1.5-ml cell suspension, 1-ml TGM was added and inoculated with 0.1-ml *Candida sporogenes*. It was found that 2-ml (TGM) and 2-ml (TSB) were left uninoculated as negative controls. Broths were incubated at 32ºC.Test and Control broths were examined for turbidity after 14 days.[15]

Determination of cell viability by two fold Trypan blue

Hemocytometer and cover slip were cleaned and wiped with 70% methanol before use. Coverslip was placed on hemocytometer. The sample solution was prepared by mixing 0.1 ml of cell suspension with 0.1-ml trypan blue. Sample was drawn into a Pasteur pipette after being mixed. Cell suspension was drawn to fill the chamber of hemocytometer. Using a fluorescence inverted microscope in 20X objective, the number of viable and nonviable cells in both halves of the chamber was counted. PDT (population doubling time), the time require for doubling the cell population was calculated using the following formula.[11-15]

PDT = 1/r, Multiplication rate (r) = 3.32 (Log NH – Log NI) / (t_2-t_1)
Where,
t_1 = Initial time
t_2 = time of cell harvested
NH = No. of cells harvested at the end of growth period
NI = No. of cells inoculated

Estimation of 18 β-glycyrrhetic acid in Glycyrrhiza glabra L. (Fabaceae) by HPTLC

Preparation of standard solution
Standard stock solution of 18 β-glycyrrhetic acid (1 µg/µL) was prepared in methanol: chloroform (1:1).

Preparation of sample solution
Glycyrrhiza glabra L. (Fabaceae), powder (1 gm obtained from the root of *Glycyrrhiza glabra L*. (Fabaceae)) was refluxed for 5 h with 20 ml of 5 M of hydrochloric acid and extracted with 3 x 15 ml of different solvents such as water, methanol and chloroform. The different extracts were evaporated to dryness and the remaining residues were dissolved separately in 10-ml methanol: chloroform (1:1) solution.

Chromatography
Chromatography was performed on silica gel 60 F254 HPTLC plates (E. Merck). Before use, the plate was washed with methanol and activated at 110°C. The amounts of 18 β-glycyrrhetic acid working standard applied to the plate were 2, 4, 6, 8 and 10 µg as sharp bands with a Linomat IV sample applicator. The amount of different sample solutions applied to the plates was 6, 7 and 8 µl for chloroform, methanol and water extract respectively. Development to a distance of 8 cm was performed at room temperature in a 20×20 cm twin-trough chamber, with half an hour chamber saturation. Toluene: ethyl acetate: glacial acetic acid (12.5:7.5:0.5) (v/v), was used as mobile phases. After development, plates were dried in a current of hot air and scanned immediately with a Camag Scanner III. Quantification was performed at the wavelengths of maximum absorption at 18 β-glycyrrhetic acid λ = 254 nm in reflectance–absorbance mode. 18 β-glycyrrhetic acid was eluted at RF~ 0.5, WinCATs software was used for data interpretation; peak areas were measured and plotted against amount of sample to obtain the calibration plot.[16]

Cytotoxicity assay
The cells were preincubated at a concentration of 1× 10^6 cells/ml in culture medium for 3 h at 37°C and 6.5% CO_2. Then, the cells were seeded at a concentration of 5× 10^4 cells/well in 100-µl culture medium and at various concentrations 12.5, 25, 50, 75, which is followed by the addition of 100 µM of standard 18 β-glycyrrhetic acid and three different extracts of *Glycyrrhiza glabra L*. (Fabaceae) (chloroform, methanol and water) into microplates (tissue culture grade, 96 wells, flat bottom) and incubated for 24 h at 37°C and 6.5% CO_2. Then, 10 µl MTT labeling mixture was added and incubated for 4 h at 37°C and 6.5% CO_2. Each experiment was done in triplicates. Then 100 µl of solubilization solution was added into each well and incubated for overnight. The spectrophotometric absorbance of the samples was measured using a microplate (ELISA) reader. The wavelength to measure absorbance of the formazan product in between 550 and 600 nm according to the filters available for the ELISA reader was used. The reference wavelength should be more than 650 nm. IC50, the concentration of compound required to inhibit 50% cell growth, was determined by plotting a graph of Log (concentration of compound) *vs* **%** cell inhibition. A line drawn from 50% value on the *Y* axis meets the curve and interpolate to the *X* axis. The X axis value gives the Log (concentration of compound). The antilog of that value gives the IC 50 value.[10]

$$\% \text{ Cell survival} = \frac{(At - Ab)}{(Ac - Ab)} \times 100$$

Where,
At = Absorbance of Test,
Ab= Absorbance of Blank (Media),
Ac= Absorbance of Control (cell line)

% Cell inhibition = 100 - % Cell survival

RESULTS AND DISCUSSION

Total bacterial and fungal count

The examination of the test and control broths after 14 days incubation revealed the absence of turbidity Absence of turbidity in the test broth means that there was no evidence of bacterial and fungal contamination.

Cell viability

In the present study, the cell viability of the two different cell lines was determined using the two fold trypan blue method. The cell viability result of different cell lines shows that % cell viability of VERO was lower than that of MCF7cell line. The population doubling time for normal cell of VERO cell line was higher as compared to the cancerous cell of MCF7 cell line as shown in Tables 1 and 2.

Table 1: PDT and other parameters of VERO cell line

Day	1	2	3	4
Appearance of cells	Small round	Small round	Small round	Small round
Density (cells/cm^2)	14,000	20,400	58,000	75,600
PH	7.5	7.5	7.5	7.5
Total cell count (hemocytometer)	35	51	145	189
% viability	45.71%	62.74%	82.75%	88.88%
Cells/ml	7×10^4	1.02×10^5	2.9×10^5	3.78×10^5
Viable cells/ml	3.2×10^4	6.4×10^4	2.4×10^5	3.36×10^5
PDT(Hrs)	29.61			

Table 2: PDT and other parameters of MCF7 cell line

Day	1	2	3
Appearance of cells	Small round	Small round	Small round
Density (cells/cm^2)	39,600	1,02,400	1,73,600
PH	7.5	8.0	8.0
Total cell count (hemocytometer)	99	256	434
% viability	78.78%	82.42%	97.00%
Cells/ml	1.98×10^5	5.12×10^5	8.68×10^5
Viable cells/ml	1.56×10^5	4.22×10^5	8.42×10^5
PDT(Hrs)	22.52		

Table 3: Assay of 18 β-glycyrrhetic acid in different extracts of *Glycyrrhiza glabra* L. (Fabaceae)

Different extracts of *Glycyrrhiza glabra* L. (Fabaceae)	18 β-glycyrrhetic acid/gm of extract (mg) ± S.D	% w/w of 18 β-glycyrrhetic acid
Chloroform	40.1 ± 0.004	4.01
Methanol	37.6 ± 0.001	3.76
Water	31.3 ± 0.001	3.13

Table 4: IC 50 values (µM) of standard 18 β-glycyrrhetic acid and three different extracts of *Glycyrrhiza glabra* L. (Fabaceae) on two cell lines

Different extracts of *Glycyrrhiza glabra* L. (Fabaceae)	IC 50 values (µM) of cell lines	
	MCF7	VERO
Chloroform extract	0.4485 ± 0.001	> 100
Methanol extract	0.9906 ± 0.001	> 100
Water extract	1.288 ± 0.005	> 100
Standard 18 β-glycyrrhetic acid	0.412 ± 0.004	> 100

Estimation of 18 β-glycyrrhetic acid in *Glycyrrhiza glabra* L. (Fabaceae) by HPTLC

Identification and quantification of 18 β-glycyrrhetic acid was done in three different extracts (chloroform, methanol and water extract) of *Glycyrrhiza glabra L.* (Fabaceae) with the comparison of different concentrations of standard 18 β-glycyrrhetic acid by HPTLC method as shown in Table 3. Separation of 18 β-glycyrrhetic acid was shown in the standard (Tracks 1–5) and three different extracts (chloroform, methanol and water) of *Glycyrrhiza glabra L.* (Fabaceae) powder (Tracks 6-8) in U.V. 254 nm and after spraying of anisaldehyde sulfuric acid [Figure 2]. Chloroform extract showed higher amount of 18 β-glycyrrhetic acid compared to the other two extracts (methanol and water extract) as shown in Figures 3 and 4.

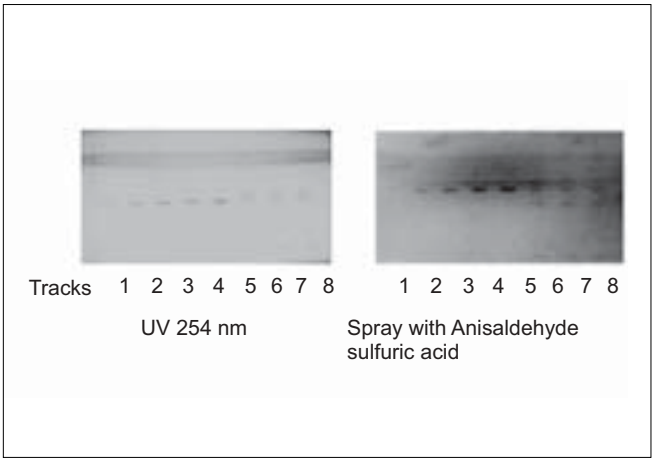

Figure 2: Separation of 18 β-glycyrrhetic acid in the standard (Tracks 1–5) and three different extracts (Chloroform, Methanol and Water) of *Glycyrrhiza glabra* L. (Fabaceae) (Tracks 6-8) in U.V. Visible Spectrophotometer with 254 nm waveslength

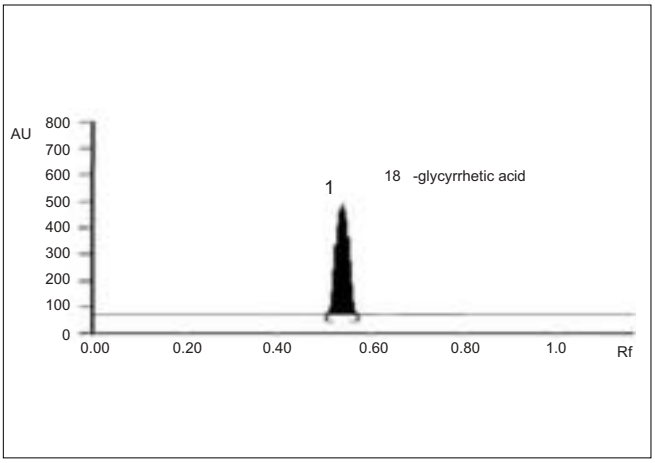

Figure 3: Chromatogram of Standard 18 β-glycyrrhetic acid (4 µg/ Spot) and Rf: 0.5 at 254 nm

Cytotoxicity assay

From the graph of Log Dose *vs* % Cell inhibition, the IC 50

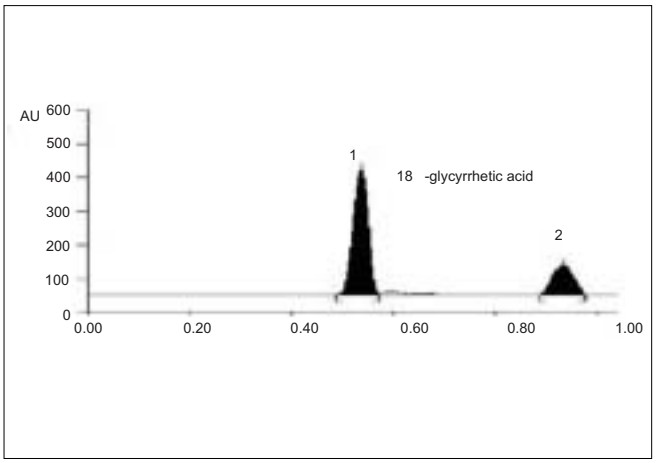

Figure 4: Chromatogram of chloroform extract of *Glycyrrhiza glabra* L. (Fabaceae) (6 µg/Spot) and Rf: 0.5 at 254 nm

value of standard 18 β-glycyrrhetic acid and three different extracts of *Glycyrrhiza glabra L*. (Fabaceae) was determined as shown in Table 4. It could be determined that the standard 18 β-glycyrrhetic acid and chloroform extract inhibited abnormal cell proliferation more effectively than the other two extracts (methanol, water) of *Glycyrrhiza glabra L*. (Fabaceae)

CONCLUSION

The present results provide clear evidence that 18 β-glycyrrhetic acid is present in higher amounts in the chloroform extract of *Glycyrrhiza glabra L*. (Fabaceae) than in the other two extracts of *Glycyrrhiza glabra L*. (Fabaceae) by HPTLC method. MTT method for cytotoxicity study shows that reference standard 18 β-glycyrrhetic acid possesses higher cytotoxicity compared with the other three different extracts of *Glycyrrhiza glabra L*. (Fabaceae). Chloroform extract of *Glycyrrhiza glabra L*. (Fabaceae) shows good cytotoxicity against cancerous MCF7 cells (Human Breast cancer) than the other two extracts of *Glycyrrhiza glabra* L. (Fabaceae) because it contains higher amount of standard 18 β-glycyrrhetic acid. Chloroform extract of *Glycyrrhiza glabra L*. (Fabaceae), which is active against MCF7 cells, is the most potent having IC 50 value of 0.4485 µM.

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