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ISOLATION AND CHARACTERIZATION OF BERBERINE HCl AND CYTOTOXIC EFFECT ON THE CELL LINE OF BREAST CANCER

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ABSTRACT

In this research Berberine HCl was isolated from roots of Berberis aristata by ethanol using soxhlet apparatus. Identification of berberine was done by TLC and compound was determined by m.p., UV and FT-IR Spectra. This study was planned to assess the cytotoxicity effect of berberine HCl on breast cancer cell line and the activity was carried out by using MCF-7 cell line. HPTLC profiling of the compound confirmed about the presence of Berberine and standard drug was compared with berberine. FT-IR was performed and compared with standard as well as isolated berberine. UV spectra and melting point was performed for isolated berberine. The cytotoxicity of standard and extracted berberine HCl was carried out by MTT assay and the results of extracted berberine were compared with standard berberine. It was found on the basis of UV, m.p., HPTLC and FT-IR that isolated compound is berberine HCl. FT-IR of standard and isolated berberine HCl were compared and found to be similar spectra. The % cytotoxicity of standard and extracted berberine at concentration of 12µM was found to be 82.19 % and 72.56 %. In cell viability assay, % cell viability of standard and extract Berberine HCl at concentration of 12µM was found to be 15.78% and 28.16%. The Berberine HCl was isolated and showed significant effect on MCF-7 cell line. In future prospect, in vivo study in animals models need to be performed to confirm the anticancer effect of Berberine HCl.

KEYWORDS: Berberis aristata, cytotoxicity, Berberine HCl, Breast cancer.

INTRODUCTION

A number of plant-derived agents are currently successfully used in cancer treatment, such as vinca alkaloid, etoposide, taxanes paclitaxel, etc., whereas some are currently under investigation. Berberis aristata (Fam. Berberidaceae) is one of the herbs mentioned in all ancient scriptures of Ayurveda, Charaka and Susruta have mentioned its different properties along with various uses for the treatment of numerous illnesses. Root of plant Berberis aristata contains alkaloid which are berbamine, Berberine, oxycanthine, epiberberine, palmatine, dehydrocaroline, jatrorhizine, columbamine,[1,2] karachine,[3] dihyrokarachine, taximaline,[4] oxyberberine, aromoline.[5] Four alkaloids, pakistanine, 1-O-methyl pakistanine, pseudopalmatine chloride and pseudoberberine chloride were also isolated from Berberis aristata.[6] The plant is found to possess various pharmacological activities such as hepatoprotective, anti-depressant, inotropic, immunomodulatory, influence on t-cell mediated immunity, hepatic amoebiasis, anti-carcinogenic, anti malarial, anti inflammatory, diabetes mellitus and hepatic ameobiasia.[7] The present study is focused on isolation of Berberine HCl from root of Berberis aristata and assesses cytotoxcity effect on breast cancer cell line.

MATERIALS AND METHOD

Collection of plant material

Root powder of plant was purchased from courtesy of Maharishi Ayurveda. Root powder was passed through 40 mesh sieve.

Isolation of Berberine HCl from Berberis aristata[8]

Powdered crude drug was extracted with ethanol in a Soxhlet apparatus for 12 hours. The collected extract was dried using vacuum, the residue dissolved in sufficient amount of water and filtered hot to separate resinous mass. Concentrated HCl was added to the filtered solution and kept in a cool dry place to allow formation of crystals of Berberine HCl; separated by filtration and were recrystallized in alkaline hot water made by addition of few drops of 10% NaOH solution. Acetone was added to the solution and diluted with equal amount of water. The mixture was cooled overnight at 5ºC. The crystalline powder obtained was filtered off and the product was recrystallized by using the mixture of alcohol: chloroform (9:1) at 5ºC.

Experimental

TLC

2.5 mg of standard drug was dissolved in 5 ml ethanol with vigorous shaking to form standard solution and 100mg of extract was dissolved in 4 ml of ethanol with vigorous shaking to form test solution. Mobile phase was prepared by mixing n- butanol, ethyl acetate, formic acid and water in a ratio of 4:4:1:1. With the help of separate capillaries, standard drug and test drug solutions were applied on the silica gel coated plates and labeled properly. The loaded plate was transferred to the developing chamber. After running through 80% of the plate it was taken out and air dried.

UV Analysis for drug identification

5 mg of the extract was weighed and dissolved in 5 ml of ethanol resulting in a concentration of 1mg/ ml. It was further diluted to get a concentration of 100 µg/ml of the drug. 4 ml of solution was transferred to the cuvettes and scanned between wavelengths of 200 to 600 nm using a UV spectrophotometer.

FT-IR spectroscopy

FT-IR absorption spectra of the drug were obtained with a Bio-Rad FTS spectrometer, using potassium bromide (KBr) disk technique. 5 mg extracted sample of Berberine HCl was mixed with 100 mg dry KBr, grounded into fine powder and compressed into disk under a hydraulic press at 10,000 psi. Each KBr disc was scanned at 4 mm^{-1} at a resolution of 2 cm over a wave number ranged from 400-4000 cm^{-1} using Win-IR software. The obtained FT-IR spectrum was compared with reference spectrum of Berberine HCl.

High Performance Thin-Layer Chromatography (HPTLC)[9]

The stock solution of Berberine HCl was prepared by dissolving 2.5 mg of Berberine HCl in 5 ml of methanol resulting in a concentration of 500µg per ml. The drug was vortexed to dissolve the drug completely. 100 mg of the extracted sample of Berberine HCl was weighed and dissolved in 4 ml of methanol resulting in a concentration of 25mg per ml.

Mobile phase was prepared by mixing n- butanol, ethyl acetate, formic acid, & water in a ratio of 4:4:1:1. With the help of a Camag Linomat V syringe (application rate, 150 nl/s; space between each band, 16.0 mm; slit dimension, 5.00 mm × 0.45 mm; scanning speed, 20 mm/s) 1 µl/spot of sample solution was applied in triplicate on the pre-activated, pre-coated silica gel aluminium plate 60 GF254 using a band width of 6.0 mm. The length of each

chromatogram run was 8 cm. Densitometric scanning (Camag TLC Scanner 3) was done at λmax 254nm & 366nm, operated by WINCATS software (V 1.2.6, Camag). All spots showed fluorescence under UV light.

Cytotoxic activity on the cell line of Breast Cancer[10,11,12]

Cytotoxic Cell Lines

MCF-7 Breast cancer cell lines were gifted from Department of biochemistry, AIIMS, New Delhi.

Preparation of Medium[13]

Preparation of DMEM (Cell culture) media[14]

13.5gm of sterile powdered DMEM along with 3.7 gm of $NaHCO_3$ was dissolved in 700 ml of autoclaved distilled water. The medium was dissolved thoroughly and the pH was adjusted to 7.2 by using 0.1M HCl and 0.1M NaOH. The final volume was adjusted to 1000ml by using autoclaved distilled water. Then the media was filtered by Millipore disc filter (0.22 µm). Filtered medium was kept overnight in CO_2 incubator to check the growth of any contamination.

Preparation of Antibiotic solution (Pen-Strep)

1ml of 100x Pen-Strep solution was pipette out, dissolved in 9 ml autoclaved distilled water and stored at -20°C.

Preparation of complete cell culture media

8.9 ml of DMEM media is added to the falcon tubes.1 ml of fetal bovine serum is also added to the DMEM media.0.1 ml of Antibiotic solution is added to the DMEM media completing the Cell culture media. Aliquots of 10ml each of Serum were prepared to prevent regular freezing & thawing of it and were stored at -20°C.

Preparation of Phosphate Buffer Saline, 1x (PBS)

Weigh 8 gm of Sodium Chloride (NaCl), 0.2 gm of potassium Chloride (KCl), 1.44 gm of Sodium hydrogen phosphate (Na_2HPO_4) and 0.24 gm of Potassium di-hydrogen phosphate (KH_2PO_4).All the salts were dissolved in distilled water. Then pH was adjusted to 7.4 using 0.1M HCl before making-up the final volume to 1000ml.Solution was sterilized by autoclaving.

PBS was used to wash the cells during the process of trypsinisation to maintain their isotonicity.

Maintenance of cell lines

MCF-7 cell lines were grown under aseptic conditions at 37°C, 5% CO_2 using DMEM medium containing 10% FBS and 1% Penicillin-Streptomycin solution supplemented with 3.7 gm $NaHCO_3$. Cells were examined daily using the inverted microscope for signs of bacterial or fungal contamination. If there were any colour change in the media it was replaced with the fresh DMEM. Cultured cells were sub cultivated when the cell concentration exceeds 2 X 10^6 per ml.

MCF-7 human breast cancer cell lines become confluent in 30hrs after seeding. The media in the flask was discarded as the MCF- 7 cells attached themselves onto the surface of the flasks. The flasks were rinsed using 5.0 ml of PBS (pH 7.4) then 1.0 ml of 0.25 % trypsin-EDTA was added into the tissue culture flasks so that the cells would detach themselves from the surface of the flasks. The cells were incubated at 37^o C for 1-2 minutes in 5.0% CO_2 incubator or till they rounded up. Cells were treated for not more than 2-3 mins to prevent over trypsinization. Activity of trypsin can be checked by looking at the treated cells under inverted microscope. Once fully detached the activity of trypsin was neutralized by adding complete DMEM.Cells was centrifuged at 1500 rpm for five minutes. Supernatant in the eppendroff tube was discarded and the pellet of MCF 7 cells was resuspended in 2.0 ml 10.0% supplemented DMEM medium. Cells were counted using haemocytometer and the medium was adjusted to give a plating density of 1 X 10^6 cells. Then the flasks were incubated in 5.0 % CO_2 incubator at 37 ^{o} C. the doubling rate for MCF- 7 cells. Serum inactivates trypsin. Trypsin solution was prepared in PBS (pH 7.4).

Cell Counting

Cell counting is an important procedure which helps in the determination of the number of cells present in the suspension. It is useful during processes like storage, revival and also for experiments like cell cytotoxicity assays. The concentration of a cell suspension may be determined by placing the cells in an optically clear chamber under a microscope. The cell number within a defined area of known depth is counted and the cell concentration is derived from the count. Cell counting was performed by using a hemocytometer.

Analysis

Calculated the average of two counts and derived the concentration of sample by using the formula

$C = n / v$

where,

c - Cell concentration (cells/ml)

n - Number of cells counted, and

v - Volume counted (ml)

For the improved Neubauerhemocytometer, the depth of the chamber is 0.1 mm and the central area 1 mm^2; therefore v is 0.1 mm^3or 1 x 10^{-4}ml. The formula then becomes:

$c = n/10^{-4}$ or $c = n \times 10^4$

Observed cell concentration

$c = n / v$

$n = 178/2 = 89$

Therefore, $c = 89 \times 10^4$/ml.

Hence, the concentration of cells was found out to be 89×10^4/ml.

Cell Proliferation assay

Measurement of cell viability and proliferation forms the basis of In-vitro assays of a cell population in response to an external factor. The MTT cell proliferation assay measures the cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis, the reduction in cell viability.

Analysis

The trypan blue was used to detect any dead cells. Faint or dark blue cells within the grid were counted as dead cells.

Count total number of cells and total number of viable (unstained) cells. Percent viable cells as follows:

% viable cells = (number of unstained cells / total number of cells) x 100

RESULTS AND DISCUSSION

Isolated Berberine HCl was found to be yellow colored needle like crystals which were sparingly soluble in cold water, freely soluble in hot water and ethanol. Melting point of

compound was 197^{0}C. In the UV analysis, the resultant peaks were obtained at 227.76, 262.92 and 344.24 nm in ethanol (Figure No.1). HPTLC profile at 254 & 366 nm is shown in Figure No. 2. The R_f value of standard and isolated drug was found to be 0.52 and is shown in (Figure No. 3 and 4). In FTIR analysis, different peaks were obtained for different functional groups in the molecular structure and it helped in the confirmation of the compound. The standard as well as extracted Berberine HCl was analyzed, the spectra are shown in (Figure No.5 and 6) and when compared to the standard, it was found to be similar. The cell line was maintained and cultured in the laboratory by using DMEM media. Once the cell line confluency reaches 70% the cells were trypsinised and subcultured. Before performing MTT, the cell counting was carried out as less than 200000 cells/ml were required. The total number of cells was found to be 89 x 10^4 cells/ml. The cytotoxicity of standard and extracted berberine HCl was carried out by MTT assay and the results of extracted berberine were compared with standard berberine. The % cytotoxicity of standard and extract Berberine HCl at a concentration of 12µM was found to be 82.19%, 72.56% and 71.42% (Table 1) and comparison (Fig. 7). In cell viability assay, % cell viability assay of standard and extracted Berberine HCl at a concentration of 12µM was found to be 15.78%, 28.16% & 25.02 % (Table 2) and comparison (Fig. 8). The results of extracted Berberine HCl when compared with standard Berberine HCl were found significant.

ILLUSTRATIONS

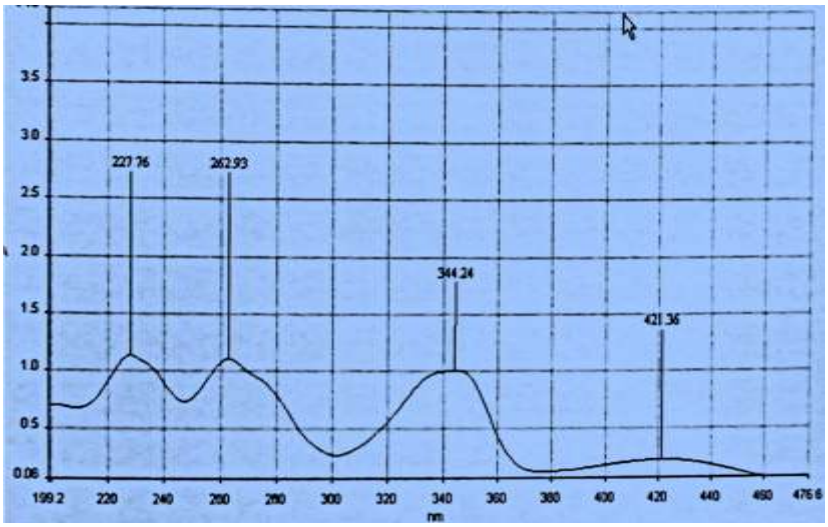

Fig.1: UV Spectra of Isolated Berberine

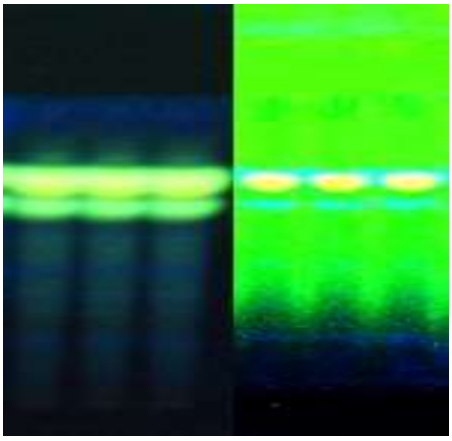

Fig. 2: HPTLC Fingerprinting of Berberine at 366nm & 254nm respectively

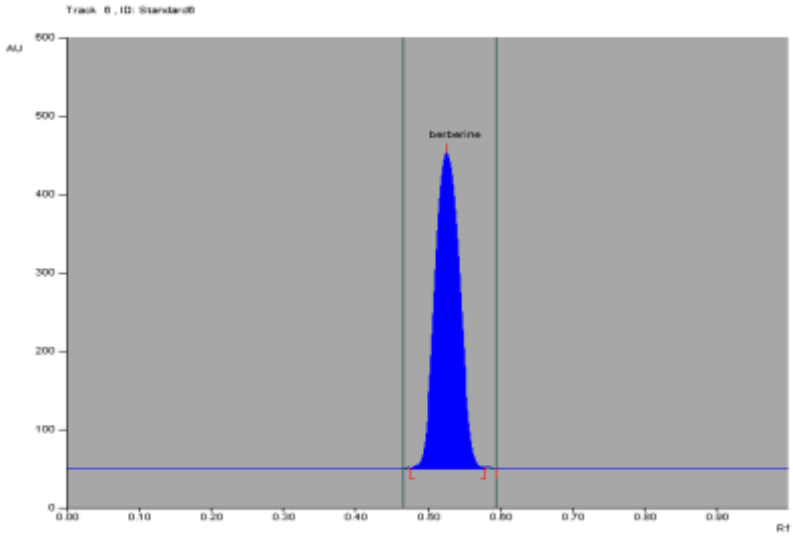

Fig. 3: HPTLC profile of Standard Berberine HCl

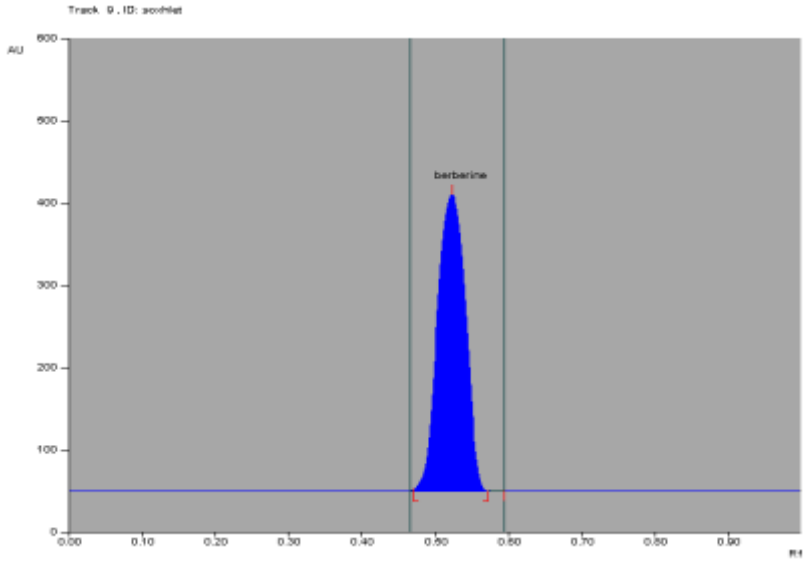

Fig.4: HPTLC Profile of Isolated Berberine HCl

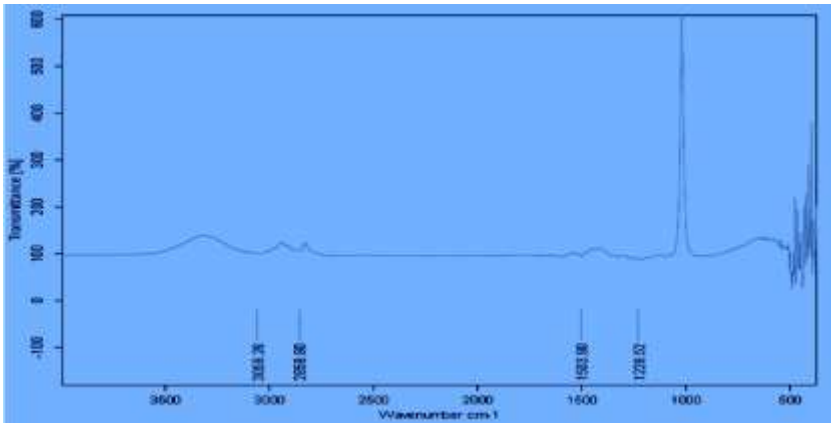

Fig. 5: FT-IR spectra of Isolated Berberine HCl

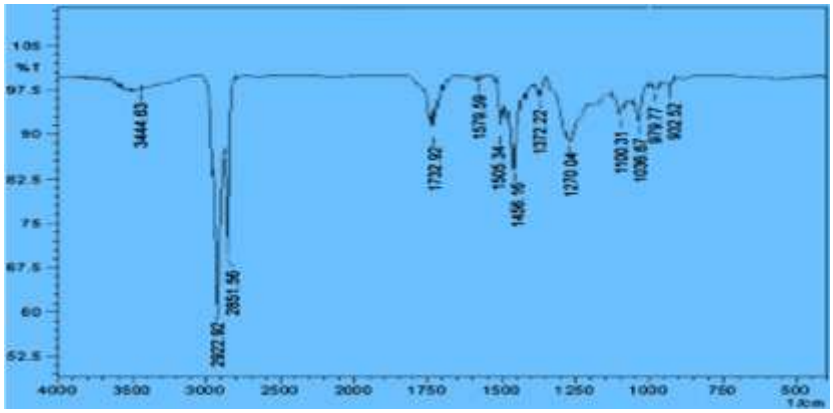

Fig. 6: FT-IR spectra of Standard Berberine HCl

Table 1: % Cytotoxic effect of Standard and Extracted Berberine HCl.

S. No.	Concentration (µM)	% cytotoxicity (Standard)	% cytotoxicity (Extract)
1.	2	30.71	15.14
2.	4	47.39	29.47
3.	6	59.49	36.15
4.	8	67.43	47.39
5.	10	77.37	61.54
6.	12	82.19	72.56

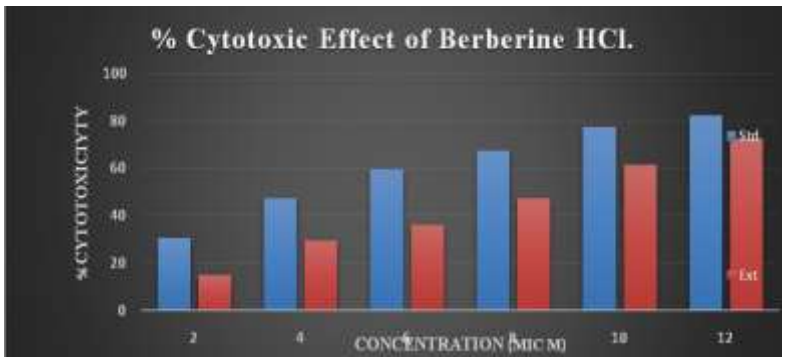

Fig. 7: % cytotoxicity effect of Standard and Extracted Berberine HCl

Table 2: % Cell viability of Standard and Extracted Berberine HCl

S. No.	Concentration (µM)	% Cell Viability (Standard)	% Cell viability (Extract)
1.	2	71.84	89.25
2.	4	65.36	72.11
3.	6	45.82	59.25
4.	8	33.25	42.48
5.	10	21.36	33.79
6.	12	15.78	28.16

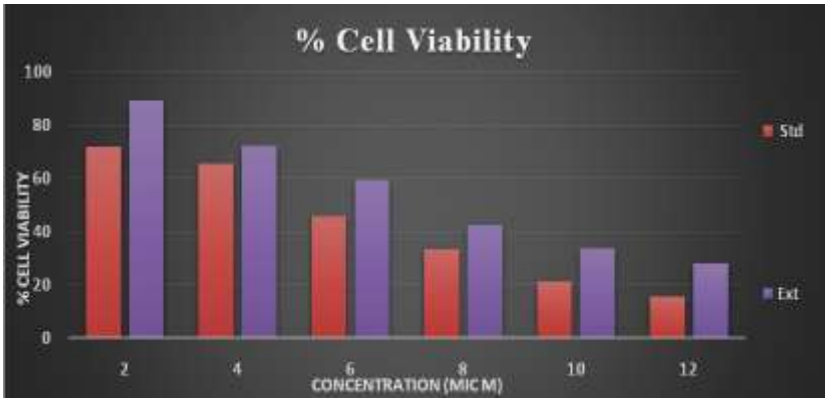

Fig. 8: % Cell viability Standard and Extracted Berberine HCl

CONCLUSION

The Berberine HCl was extracted from Berberis aristata by using Soxhlet apparatus. The extractive yield was found to be 63.08%. After the extraction of Berberine for the confirmation, TLC was also performed to confirm the R_f value to be 0.52. Successful use of an indigenous Berberine is an indication, suggesting a promising use in the treatment of a challenging disease like breast cancer. Therefore an elaborate study of the effect of Berberine HCl on various specific breast cancer cell lines may be carried out to prove the potential of this plant which can produce evidences. Also In vivo study in animals models need to be performed to confirm the anticancer effect of Berberine HCl.

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