Original Article

Ethanolic Extract of Grewia Asiatica: A Promising Anti-Cancer Agent Against HepG2 Cell Line

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ABSTRACT

Objective: To screen plant extracts for their anti-cancer potential, considering their cost-effectiveness and low side effects. Further research is needed on animals and humans to apply it to cancer treatment in the future. Cancer is one of the leading causes of death in prosperous countries. This study evaluated the anti-cancer activity of the ethanolic extract of Grewia asiatica against the HepG2 cell line. The study aimed to check the cytotoxic effect of the extract on the cancer cell line, using different viability assays such as MTT, Crystal violet, Trypan blue, and Scratch assay.

Study Design: Experimental study

Place and Duration of Study: This study was conducted at the Institute of Molecular Biology and Biotechnology (IMBB), University of Lahore from July 2021 to Dec 2021.

Methods: The cell and tissue culture test center at the University of Lahore will provide the HepG2 and normal cell lines**.** The cell lines that have been frozen in Liq. Nitrogen cylinders will be thawed from cryo vials when culturing is needed. The dried plant of Grewia asiatica will be obtained from the commercial market of Bahawalpur (The Islamia University Bahawalpur). The dried plant will be rinsed using tap water and dried in the shade.

Results: The results indicated that the ethanolic plant extract showed a significant reduction in the level of proliferation of HepG2 cells compared to untreated cells.

Conclusion: The ethanolic plant extract of Grewia asiatica has an effective in-vitro role in anti-cancer therapy. MTT assay estimated the reduction in the growth rate of rapidly dividing tumor cells, while the crystal violet and trypan blue assays showed that the plant extract elicited a significant inhibition of cell viability. The scratch assay also displayed the anti-cancer potential of the plant extract.

Key Words: cancer, normal cells, angiogenesis, apoptosis, anti-proliferation

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a life-threatening disorder and is considered a liver cancer at the primary level. People having chronic liver infections (like cirrhosis stimulated due to hepatitis B/hepatitis C) may suffer from this type of cancer, which sometimes leads to deadly situations in adults. The possibility of developing hepatocellular carcinoma is greater in people already suffering from long-lasting liver infections[(1,2)]. In 1975, a 15-year-old guy from Argentina was reported with a well-distinguished case of hepatocellular carcinoma, and a remarkable cancer cell line was obtained from his liver tissue. This liver cell line was HepG2.[(3)]. Hepatic cells are of great importance in the drug metabolism process ever since the liver is the primary organ in control for drug metabolic processes and drug-drug collaborations and the chief detoxifying organ of the human body. Therefore, these HepG2 cells are extensively used in drug development processes and testing related to toxicity.

Phalsa (Grewia asiatica) is a colorful exotic shrub and small nutritious fruit recognized for its massive medicinal use. Phalsa fruit can be handled as a revitalizing fruit or even used up fresh or in desserts, and extensively used in soft drinks throughout hot weather conditions in India [(1)]

Phalsa fruit consumes a very short lifetime of almost 1-2 days and is appropriate only for limited marketing. This fruit's nutrients include vitamin C, minerals, proteins, and amino acids. Several bioactive combinations, such as flavonoids, phenolics,

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anthocyanins, and tannins, are also enclosed in this fruit. Many parts of this plant can be used for different pharmacological purposes. Leaves are well reputed for their strong anti-microbial, anti-septic, anti-platelet, and anti-cancer effects; fruits are presumed to be used as anti-cancer, anti-oxidant, radio-protective, and anti-hyperglycemic agents; stem bark can provide pain-relieving and anti-inflammatory treatments. [1]

Regardless of its massive uses in the medicinal field, the fruit is suffering a noteworthy disrespect, which is too obvious as we could not find any literature concerning this plant [3]

METHODS

The cell and tissue culture test center at the University of Lahore will provide the HepG2 and normal cell lines**.** The cell lines that have been frozen in Liq. Nitrogen cylinders will be thawed from cryo vials when culturing is needed [4]. The dried plant of Grewia asiatica will be obtained from the commercial market of Bahawalpur (The Islamia University Bahawalpur). The dried plant will be rinsed using tap water and dried in the shade. The plant of Grewia asiatica (in dried form) will be separately milled into powder with an electrical grinder. For 3 days, powdered material will be added into hydro-ethanoic solvent (30/70 volume/volume). The mixture of solvent and plant will be agitated daily to dissolve phytochemicals in a solvent, followed by filtration and then evaporation using a rotary evaporator at 35ºC, and the result will be a dense, sticky extract that will be kept in the refrigerator at 4ºC. A dose-response study will be conducted, and optimum concentration will be determined for further assays. HepG2 cells will be treated with the optimal dose representing our treatment group. The treatment group data will be compared against the negative control (NC) group (untreated cells).

Culturing of HepG2 and normal cell lines: Thawed cells will be cultivated using T75 flasks with DMEM (Dulbecco's Modified Eagle's Medium) (Caisson's Lab from USA) (higher glucose), accompanied by penicillin-streptomycin (Caisson's Lab from USA) and 10% FBS (fetal bovine serum) (Sigma Aldrich from USA) using humidified incubator at the temperature of 37°C along with 5% CO_2. The medium will be replenished after 2-3 days, while DMEM without FBS will be applied for therapy [4].

MTT Analysis via 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide: MTT analysis will be applied to determine the cell feasibility (Sigma Aldrich, USA). The analysis will be run in triplicate for each experimental group. The MTT analysis will be used in a 96-well plate to assess the extract potential of normal and HepG2 cells. The cells will be rinsed using PBS (phosphate buffer saline) and cultured for 2 hours in 100µl of serum-free DMEM medium and 25 µl of MTT solution (with concentration 5mg/ml). The purple-colored formazan crystals will then be solubilized using 10% SDS (sodium dodecyl sulfate), and the absorbance at 570 nm will be measured. The preceding approach will be used to analyze percentage feasibility [5].

Crystal Violet Analysis: Crystal violet reagent will assess cell feasibility (Sigma Aldrich, USA). An alternative technique for assessing cell viability in cell lines is crystal violet analysis. HepG2 and normal cells from all the experimental groups will be collected within 96-well plates, the culture medium will be discarded, and PBS will be used to wash the cells. A mixture of 0.1% crystal violet reagent and 2% ethanol will be used and applied to the cells containing wells on the plate, and the cells will be incubated with dye for 15 minutes at room temperature to get stained. After incubation, the dye will be removed and cells within wells will be washed gently so they may not flow through the wells. 100 µL of 1% sodium dodecyl sulfate (SDS) will be poured within each well to solubilize the crystal violet stain for 10 minutes. The assay will be performed in triplicate for each experimental group. The absorbance of cell suspensions will be then recorded at 595 nm with the help of a spectrophotometer on a microtiter plate, as mentioned by Feoktistova et al. [6].

Live dead analysis: Trypan blue will detect living and deceased cells, and the percentage of deceased cells will be calculated. The cells from various experimental collections, treated and untreated, will be washed three times in PBS for at least 5 minutes before being incubated with trypan blue (Invitrogen Inc., USA). Then, these cells will be rinsed three times in PBS and examined with the help of a microscope. Trypan blue-stained cells will be regarded as deceased cells [4].

Scratch Analysis: Scratch assay was performed in 6 well cell culture plates by using the IC50 values of extracts following the Liang et al. protocol [8]. Images were captured at 0, 48 and 72 hours

RESULTS

Cellular Metabolic Activity by Mtt Assay: According to the MTT experiment, the conversion of tetrazolium reagent to a formazan reagent occurred in metabolically active cells.

Table No. 1: The Cell Viability Values of Untreated and Treated Hepg2 Cells

Groups & Doses	Values(±SEM)
Untreated	0.803 ± 0.179
G. asiatica ethanolic extract treatment (20µg/ml)	0.897 ± 0.236
G. asiatica ethanolic extract treatment (50µg /ml)	0.795 ± 0.0331
G. asiatica ethanolic extract treatment (100µg /ml)	0.523 ± 0.0860
G. asiatica ethanolic extract treatment (500µg /ml)	0.263 ± 0.0662

In the HepG2 treated cell line, more tetrazolium was found than the purple formazan dye, which concluded that the cell growth and proliferation are inhibited. According to the data in Figure 1, metabolic activity of the cells is reduced by increasing the drug doses 20µg/ml to 500µg/ml and by increasing the drug dose further, it causes cell inhibition and growth.

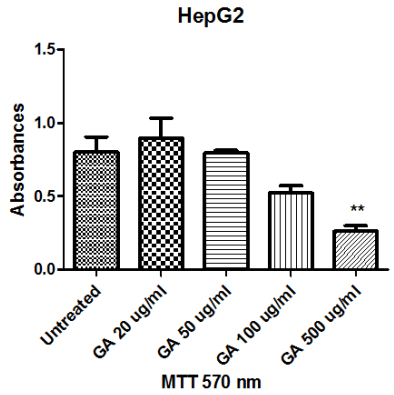

Figure No. 1: The graphical representation illustrates that plant extract significantly interfered with the primary adhesion of HepG2 cells when treated for 72 hours of experiment, and absorbance was taken at 595nm of optical density. The asterisk shows significant values between treated and untreated groups.

Trypan Blue Assay: The trypan blue dye binds to only dead floating cells based on it. The calculation was done by counting live and dead cells under IC50 concentration of plant extract. According to the data in Figure 5, the adherence was reduced with IC50 dose than the control, which means the viable and adherent cell count has been reduced by the activity of plant extract.

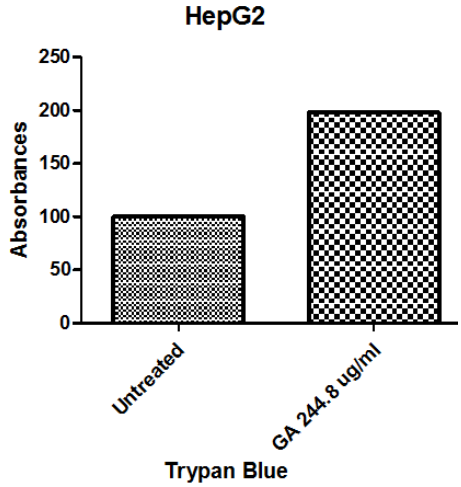

Figure No. 2: The graphical representation illustrates that plant extract significantly interfered with the primary adhesion of HepG2 cells when treated for 72 hours of experiment.

Scratch Assay discloses the migration ability of HEPG2 Cell Line. The Scratch analysis was assessed to evaluate the consequence of plant extract on the migration of the HepG2 cell line. The images were captured using bright field microscopy.

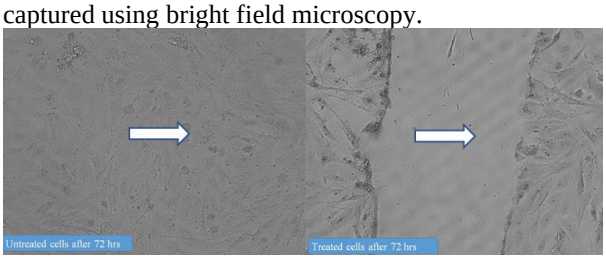

Figure No. 3: Bright field imaging of untreated and plant extract treated cells of HepG2 cells. The untreated cells had almost filled the scratch gap, depicting the metastatic.

The images of cells after 72 hours in Figure 6 show cells along with linear scratch. After 72 hours, the scratch gap is filled in the untreated group. Plant extract efficiently reduced the migratory potential of hepg2 cells, visible from the unfilled gap area.

DISCUSSION

Plant-derived natural chemicals are expected to be crucial in improving potential medications for life-threatening infections.

Nutritional disproportions cause many malignancies in our world. Therefore, looking for new anti-cancerous agents from the natural plant sources with extraordinary anti-oxidant properties is critical. According to our findings, the proliferation of HepG2 cells applied with ethanol plant extracts was dramatically inhibited.

Hepatocellular carcinoma is an aggressive tumor of malignant potential with clinical manifestation of chronic liver disease and cirrhosis frequently presented in the advanced stage. Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer. HCC occurs most often in people with chronic liver diseases, such as cirrhosis caused by hepatitis B or C infection[8].

A tumor of malignant potential known as cancer results from abnormal, uncontrolled cellular proliferation with loss of normally controlling cell growth. Cancer is an extremely heterogeneous disease comprising epigenetic, morphologic, and genetic variability. The progression and heterogeneity of cancer are explained by the following two models: [1] the cancer stem cells (CSCs) or cancer-initiating cells (CICs).[2] The stochastic or clonal evolutionary model [9].

Significant advancements have been made in cancer treatment, and many chemotherapeutic agents are available to cure and control the cancer. Though these agents are quite useful, they display several side effects like multiple drug resistance and non-specificity[10]. Globally, medicinal plants are extensively used in practice because of their healing effect on various ailments [11]

World Health Organization (WHO) has declared that medicinal plants are the beneficial reserves of important phytochemicals that could have multiple pharmacological activities. Moreover, therapeutic agents obtained from medicinal plants are relatively safe and economical compared to synthetic medicines manufactured synthetically. Therefore, there is an urgent need to focus on drugs prepared from natural plants, which are inexpensive, harmless, and effective[12]. In traditional medicine, herbal plants are broadly used for their numerous therapeutic properties, increasing researchers' interest in exploring these plants[13].

One of the leading causes of death worldwide remains cancer. Various therapies have been used, including natural products. These natural plant-derived compounds provide a new treatment with fewer side effects and sometimes even better efficacy.

New antitumorigenic compounds have extensive effects on the proliferation of cancer cells, but it remains very important to address the problematic issue of chemotherapy resistance. Natural compounds from plants are considered to become the key players in the development of potential drugs for life-threatening diseases. Many cancers in our world are due to dietary imbalances. Thus, it is very important to discover a new anti-cancer agent from a natural plant source with high anti-oxidant activities. The study's main aim was to investigate the anti-proliferative and apoptotic activity of Grewia asiatica against the HepG2 cell line. For this purpose, plants were extracted in ethanolic solvent. Our results here indicate that HepG2 cells treated with ethanolic plant extracts show a significantly reduced level of proliferation [14].

Phalsa (Grewia asiatica) is an exotic bush plant and small fruit crop well-known for its large medicinal uses. Phalsa fruit is consumed fresh, in desserts, or processed into refreshing fruit and soft drinks consumed during hot weather in India [1, 15]

Phalsa fruit has a short life span and is considered available for local marketing. Fruit provides nutrients such as proteins, amino acids, vitamins, and minerals and contains various bioactive compounds, like anthocyanins, tannins, phenolics, and flavonoids. Different parts of this plant have different pharmacological properties. Leaves are well reputed for anti-microbial, anti-cancer, anti-platelet, and antiemetic activities; fruit has anti-cancer, anti-oxidant, radio-protective, and anti-hyperglycemic properties. While stem bark provides analgesic and anti-inflammatory activities. [1, 8]

Despite its diverse use, it has suffered notable disregard, as is evident from the lack of literature on this plant.

CONCLUSION

Grewia asiatica extract has been shown to have anti-cancer properties in vitro, inhibiting the growth and proliferation of HepG2 tumor cells and reducing their viability. The extract also showed significant inhibition of cell viability in the crystal violet assay and induced cell death in the trypan blue assay. Additionally, the scratch assay showed a delayed gap closure and decreased metastatic potential of HepG2 cells. Further research is needed to explore its potential as a cost-effective and low-side-effect treatment for cancer.

Author's Contribution:

Concept & Design of Study:	Ahmad Farooq Butt
Drafting:	Rubab Aftab, Zobiah Hafeez
Data Analysis:	Maimoona Nasreen, Sara Mukhtar, Ayesha Faraz
Revisiting Critically:	Ahmad Farooq Butt, Rubab Aftab
Final Approval of version:	Ahmad Farooq Butt

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