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Original article

In vitro cytotoxic potential of *Solanum nigrum* against human cancer cell lines

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ABSTRACT

Plants have natural products which use to possess antiproliferative potential against many cancers. In the present study, six isolated fractions (ethyl acetate, petroleum ether, chloroform, n-butanol, ethanol and aqueous) from *Solanum nigrum* were evaluated for their cytotoxic effect on different cell lines. Hepatic carcinoma cell line (HepG2), cervical cancer cell line (HeLa) and baby hamster kidney (BHK) used as normal non-cancerous cells were evaluated for cytotoxicity against isolated fractions. Cell viability assay was performed to evaluate the cytotoxicity of all fractions on different cell lines followed by the lactate dehydrogenase and vascular endothelial growth factor assays of most active fraction among all screened for cytotoxic analysis. HPLC analysis of most active fractions against cytotoxicity was performed to check the biological activity of compounds. Results displayed the potent cytotoxic activity of ethyl acetate fraction of *S. nigrum* against HepG2 cells with IC_{50} value of 7.89 μg/ml. Other fractions exhibited potent anticancer activity against HepG2 cells followed by HeLa cells. Fractions in our study showed no cytotoxicity in BHK cells. Cytotoxic activity observed in our current study exposed high antiproliferative potential and activity of ethyl acetate fraction against HepG2 cells. The results demonstrated that *S. nigrum* fractions exhibited anticancer activity against hepatic and cervical cancer cell lines with non-toxic effect in normal cells. These results reveal significant potential of *S. nigrum* for the therapeutic of cancers across the globe in future.

1. Introduction

Hepatocellular carcinoma (malignant hepatoma; HCC) is an End-Stage Liver Disease (ESLD) followed by skin pale, ascites, blood clotting abnormalities, severe abdominal cramps, vomiting, nausea and restlessness. The majority of the cases of HCC occur in injured or virally infected hepatocytes. Other types of cells in the liver can contribute to the development of cancer, but they are relatively rare. Cancers that arise in any other body part, such as colon, breast, lung spread to the liver are known metastatic cancer other than hepatic cancer (Mathew et al., 2014). People acquire hepatic cancer in the context of chronic liver disease (cirrhosis) which heightens the risk of hepatic cancers accompanying scars. Potential risk factors that cause liver cirrhosis are chronic hepatitis B, hepatitis C, alcoholism, genetic tendency, haemochromatosis, copper toxicity and drugs abuse. HCC is found more often in men compared to women and is commonly distributed in southeast Asia and subsaharan Africa rather than in the United States. Around 700,000 people annually are identified with HCC in the world (Jabamalairaj et al., 2019). HCC is the principal source of cancer deaths around the world that might cross 600,000 mortalities every year. Hepatic cancer gradually develops from the premalignant stages due to the accumulation of a series of cellular and genetic alterations (Simon et al., 2004; Ringelhan et al., 2018). Annually, more than half a million women are diagnosed with cervical cancer over 300,000 mortalities are reported worldwide. High-risk subtypes of the human papilloma virus (HPV) are the reason of the disease in most cases (Cohen et al., 2019).

Plants have been utilized for a long time for the management of human diseases. Traditionally, many significant new drugs have

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been industrialized from compounds formerly derived from natural sources (Harvey, 2008, Han et al., 2018). The most common among those is aspirin, having a compound salicin isolated from *Salix alba*. Likewise, morphine and codeine were isolated from opium poppy. *Papaver somniferum* and quinine usually used to treat malaria from cinchona tree. Taxol extracted from *Taxus brevifolia* bark promisingly used as an anti-proliferative agent (Newman et al., 2003, Chester et al., 2019).

Solanum nigrum generally known as Maku or black nightshade belongs to Solanaceae family commonly grows in different types of soil as a weed in a moist habitat. Several disorders such as fever, pain, hepatitis and inflammation have been treated traditionally and exclusively by *S. nigrum* (Yang et al., 2021). In the oriental systems of medicine, *S. nigrum* is used for many purposes which encompasses antioxidant, anti-inflammatory, diuretic, hepatoprotective, antitumorigenic and antipyretic agent (Ali et al., 2018). These diverse activities are due to various biological compounds that have been identified extensively. Many traditional systems of medicine are dependent on *S. nigrum* worldwide for number of diseases but modern therapeutic applications have not recognized its importance so far (Nawab et al., 2012).

Currently, significant efforts have been made to developed hepatic cell lines (HepG2) with higher probability for detecting the genotoxic effects of chemicals and environmental specific to liver, which is the key target organ of chemical stimulation and detoxification processes (Shah et al., 2018). HeLa cells among the most famous cell lines are the culture of endothelial cells of the uterus. HeLa cells have universal cell surface receptors and can be used to study various cytokines. These cells can provide physiologically more relevant information in addition to being used for drug development (Ramirez et al., 2018).

Among various cell biology approaches, cell viability approach commonly known as MTT assay is a usually employed to determine cytotoxicity, cell viability and proliferation by reducing tetrazolium salt to formazan crystals. Other assays like lactate dehydrogenase (LDH) assay can determine the cell cytotoxicity (Chang and Geib, 2018). High-performance liquid chromatography (HPLC) is chromatographic technique used to split a mixture of compounds. HPLC is promising approach used to identify, quantify and purify the individual components of the mixture (Yuan et al., 2019).

Extract of *S. nigrum* cause cell arrest in G0 and G1 phase with minute toxicity among animals. Studies have revealed that total number of alkaloids isolated from *S. nigrum* alter the function and structure of the tumor cells and interrupts the DNA and RNA synthesis and transform the cell cycle events of tumor cells. (Artun et al., 2016). Experimentally it has been approved that the aqueous extract of *S. nigrum* can be employed with drugs like cisplatin and doxorubicin in chemotherapy of hepatic carcinoma patients (Khan et al., 2019).

On the basis of increased mortality and morbidity rate of cervical and hepatic cancer, Hela and HepG2 cells were used in this study. The current study aims the comparison of cytotoxic effects of six fractions obtained from the whole plant of *S. nigrum* against HepG2 and HeLa cancer cell lines and normal cell line (BHK).

2. Methodology

2.1. Sample collection and preparation of fractions

S. nigrum used in this study identified by taxonomist (GC. Herb. Bot. 3748) was collected from the Jinnah garden, Lahore, Pakistan. Collected plants as whole were shade dried and stored until further used. Around 5 kg of plant was soaked into ethanol at room temperature for 14 days followed by manual shaking and pressing. Filtered extract was subjected to rotatory evaporator at 50–55 °C for evaporation of excess solvent and was further fractionated on the basis of polarity in chloroform, n-butanol, petroleum ether, ethyl acetate and water.

2.2. Cell culture

HepG2 liver cancer cells, cervical cancer cells (HeLa) and normal (BHK) cells were procured from Institute of Molecular Biology and Biotechnology (IMBB), University of Lahore, Pakistan. Dulbecco's Minimum Essential Medium (DMEM) with 10% fetal bovine serum (FBS) and 50 μg/mL gentamicin were used for cell culturing. Cells were incubated at 37 °C with humidified atmosphere (5% CO_2 and 95% air) (Alves et al., 2016).

2.3. Cell viability assay (MTT)

MTT assay was opted to determine antiproliferative activity of different fractions of *S. nigrum*. Exponentially growing HepG2, HeLa and BHK cells were seeded into 96-well plates and permitted for their attachment to wells for 24 hrs. Different concentrations of *S. nigrum* fractions were applied for treatment of cells followed by the incubation of 72 h. 0.1% DMSO in media was used for cells in the control group. Media of treated and control cells was removed and washed with 200 μL of PBS. 25 μL of MTT reagent was added to each well and the microplate was incubated at 37 °C for 3 hrs. MTT reagent was removed from each well followed by adding 100 μL DMSO and kept for overnight incubation. Absorbance values for each treatment in triplicate were recorded using ELISA reader (xMark™ Microplate Absorbance Spectrophotometer, BioRad, United States) at 540 nm wavelength and compared with untreated cells (Bishayee et al., 2010). Dose response inhibition curves in Graph pad prism 5 were used to calculate the IC_{50} values.

2.4. Vascular endothelial growth factor (VEGF) assay

VEGF were assessed by enzyme-linked immunosorbent assay (Kumar et al., 2003). HepG2 and HeLa cells were plated in 6-well plate and treated with the IC_{50} value of biologically most active ethyl acetate fraction of *S. nigrum* for 72 h to measure the level of VEGF protein in the supernatant of HepG2 and HeLa cells with the help of Elisa kit (VEFG Human ELISA Kit, Invitrogen, USA). Absorbance values for each treatment were measured in triplicate at 450 nm wavelength using ELISA reader.

2.5. Lactate dehydrogenase (LDH) assay

Cells after 24 h of culturing and differentiation were plated in 96 well plates and treated with obtained IC_{50} values of the biologically active ethyl acetate fraction of *S. nigrum* for 72 h (Riss et al., 2019). LDH activity was measured through the supernatants of each well separately. Cell lysis solution was added to the monolayers of collected cells for 30 min at room temperature (25 °C). The absorbance values were measured for each treatment in triplicate by ELISA reader at 490 nm wavelength.

2.6. Trypan blue and crystal violet assay

Cells were plated in 96 well plates and treated with obtained IC_{50} values of the *S. nigrum* fractions for 72 hrs and mixed with equal volume of isotonic trypan blue (0.4%) to asses cell viability. Hemocytometer was used to count total cells and dye accumulating non-viable cells under light microscope compared with untreated (Patel et al., 2009). Crystal violet (0.5%) in ethanol for 10 min was used for cells staining. Cell lysis was performed using sodium dodecyl sulfate (1%) solution (Almutary and Sanderson,

2016). The data obtained was presented as means of replicates from three independent experiments. Absorbance of each treatment was recorded in triplicate using spectrophotometer (Varioskan LUX Multimode Microplate Reader, ThermoFisher Scientific, USA) at 595 nm spectrum.

2.7. Characterization of fraction for bioactive compounds

The most cytotoxic active fraction of *S. nigrum* was subjected to HPLC and separation of bioactive composites on the basis of chromatographic techniques (Sammani et al., 2013). Active cytotoxic fractions were achieved through a shim-pack column CLC-ODS(C-18) 25 cm × 4.6 mm (Shimadzu, Japan). Water and acetonitrile A (H20: AA-94:6, ph. 2.27), B (ACN100%) was used in the mobile phase. Gradient elution was performed following 0–15 min of 15% B, 15–30 min of 45% B and 30–45 min of 100% B with a flow rate of 1 ml/min. Precise quantification of HPLC separated fractions was performed using photodiode array detector (1260 Infinity II Diode Array Detector HS, Agilent Technologies, USA) at wavelength of 280 nm.

2.8. Statistical analysis

Experiments were performed in triplicate and the obtained data was represented as means ± standard deviation (SD). One-way ANOVA and bonefferoni test was employed for the analysis of

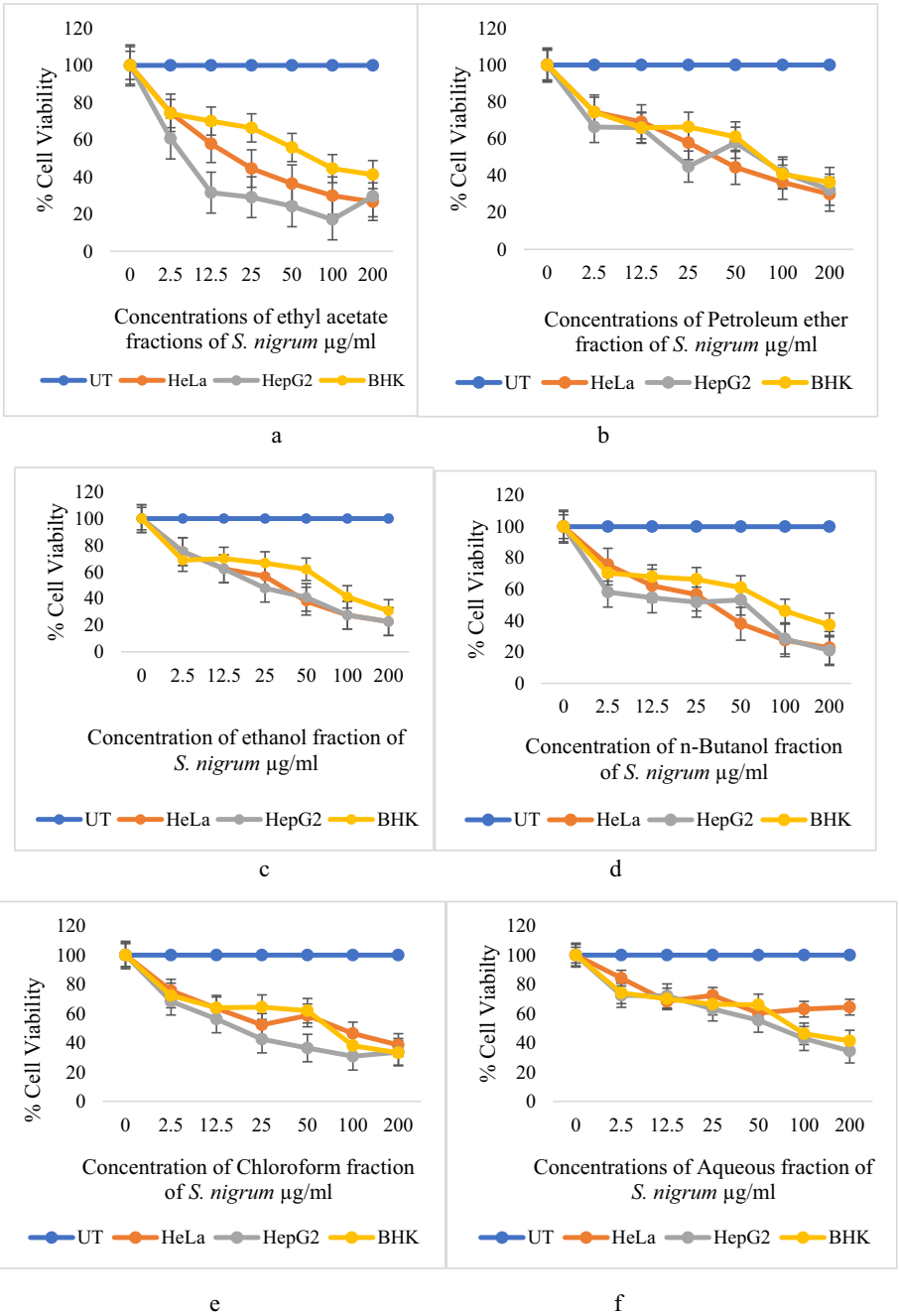

Fig. 1. Antiproliferative activity of different dose (0, 2.5, 12.5, 25, 50, 100 and 200 µg/ml) dependent fractions of *S. nigrum* on HepG2, HeLa and BHK. Graphs showing the percentage proliferative inhibition values of cell lines and cell survivals significance different from each other in all concentrations with a $p \leq 0.05$. The data was expressed as a mean value of replicates of each fraction.

Table 1
Solanum nigrum fractions and their cytotoxic activity (IC_{50} values) against different cell lines.

***Solanum nigrum* IC_{50} values of cell lines (µg/ml)**			
Fractions	**HepG2**	**HeLa**	**BHK**
Ethyl acetate	7.89	19.20	90.01
Petroleum ether	22.46	38.25	81.71
Chloroform	14.06	26.11	61.94
n-Butanol	25.91	28.34	92.3
Ethanol	23.88	39.16	82.58
Aqueous	44.53	60.32	92.08

obtained data by means of graph pad prism software version 5. The p value < 0.05 were considered statistically significant.

3. Results

Different concentrations of *S. nigrum* fractions were applied to HepG2, HeLa and BHK cell line by MTT assay in this study (Fig. 1). Cells were treated with 2.5, 12.5, 25, 50, 100 and 200 µg/ml of doses for 72 hrs. Experiments were performed in triplicate and IC_{50} values for each fraction was calculated (Table. 1). Ethyl acetate bioactive factions were promisingly reported in our studies (Table. 2). Among all isolated fractions, ethyl acetate fraction of *S. nigrum* have shown maximum and significant antitumor potential against HepG2 cells with IC_{50} value of 7.89 µg/ml. Other fractions exhibited more potent anticancer activity against HepG2 cells followed by HeLa cells. These IC_{50} values were further evaluated by trypan blue and crystal violet assay to confirm the antiproliferative activities (Fig. 2). IC_{50} values of only ethyl acetate being as most promising among other fractions showed significant inhibitory cancerous activity in HepG2 and HeLa cell lines (Fig. 3). Ethyl acetate fraction disclosed highlighted antiproliferative activity with presence of various bioactive compounds (Fig. 4).

4. Discussion

It is evident from history that plant have great therapeutic potential against many human diseases. Both crude extracts and fractions of *S. nigrum* displayed significant antiproliferative activity against various cancer cell lines in the present study. Recent advances in technology have greatly emphasized on the bioactive components of plants and their role in therapeutic medicines as well (Muthuvel et al., 2020). Globally, plants are the choice of treatment due to their significant therapeutic index and low cost against several life threating diseases. Many practicing drugs are derivatives of natural plant sources and modified through addition of synthetic compounds to combat the diseases globally. *S. nigrum* is reported for number of therapeutic effects including antipyretic, anti-inflammatory, antiproliferative and antioxidant (Lelario et al., 2018).

The present study aimed to assess the antiproliferative activity of *S. nigrum* fractions (ethanol, n-butanol, chloroform, ethyl acetate aqueous and petroleum ether) in cervical cancer (HeLa), liver cancer (HepG2) and normal cells (BHK). Different biochemical constituents have been isolated from *S. nigrum* such as, quercetin, kaempferol, solanine, cuscutin, beta sterol, stigmasterol, dulcitol, coumarin and oleanolic acid (Zhu et al., 2020). Data generated from HPLC in the present study have provided the chemical basis for the extensive practice of this plant as therapeutic potent agent for treating different diseases. Different bioactive fractions like quercetin, caffeic acid, benzoic acid, syringic acid, p coumaric acid and cinnamic acid as *S. nigrum* metabolites were reported in our study. Quercetin (Rauf et al., 2018), caffeic acid (Kanimozhi and Prasad 2015), benzoic acid (Anantharaju et al., 2017), syringic acid (Abaza et al., 2013), p coumaric acid (Jaganathan et al., 2013), cinnamic acid (Su et al., 2015) posses anti-oxidant, antimicrobial, anti-inflammatory and anti-endotoxic activities and retard cancer cell growth by inducing apoptosis in cancer cell lines.

Earlier researches have shown the effective cytotoxic activity of many organic and aqueous extracts of *S. nigrum* against multiple cancer cell lines including −29, HT MCF PC −7 (human breast cancer), −12 (human lung cancer) and - HCT 116 (human colon cancer) and its ethanolic extract from berries exhibited poor cytotoxicity against all the cell lines (Ikeda et al., 2003; Lee and Lin, 2003; Heo et al., 2004; Butt et al., 2018; Campisi et al., 2019; Carvalho et al., 2019; Emam et al., 2019, Ling et al., 2019)

Aqueous extracts have generally been reported to be prepared with dried berries, other plant and whole plants can also be used to screen out their therapeutic potential. The antiproliferative activities of the crude organic solvent-based extracts of *S. nigrum* and isolated compounds were evaluated on tumor cell lines of liver (HepG2), (Fekry et al., 2019) colon (HT29 and HCT-116), breast (MCF-7), (Churiyah et al., 2020) and cervical (U14 and HeLa) (Chothiphirat et al., 2019; Moglad, 2019). Cytotoxic effects of a number of glycoalkaloids have also been considered on several cancer cell lines such as HepG2 (Ahmad et al., 2017; Ling et al., 2019; Li et al., 2021)

The IC_{50} values obtained for the different treatments in our study revealed that ethyl acetate fraction among all other fractions have high antiproliferative activity in HepG2 cells while BHK cells have shown no cytotoxicity against any fraction. HeLa cells have shown less activity than HepG2 cells indicating that isolated fractions from *S. nigrum* are more effective against hepatocellular carcinoma than the cervical cancer. All tested organic and aqueous fractions were more compromising for HepG2 cells as compared to HeLa cells with promising IC_{50} values. The potential of the anticancer activity of the plant fractions to categorize between ordinary and cancerous cells is an important model for developing strategy and innovation of chemotherapeutical compounds. Trypan blue dye approach and crystal violet assays authorize the antiproliferative activities of the fractions against cancer cell lines related to the normal BHK. Leakage of the cytoplasmic enzyme LDH into the extracellular medium is determined through LDH assay.

Table 2
Characterization of bioactive compounds of *Solanum nigrum*.

Retention time	Area (mV.s)	Area (%)	Amount (g/kg)	Amount (%)	Compound name
2.840	240.954	8.5	0.000	0.0	Quercetin
12.753	112.282	3.9	0.000	0.0	Caffeic acid
14.820	83.601	2.9	0.000	0.0	Benzoic acid
16.367	40.520	1.4	0.000	0.0	Syringic acid
17.540	120.775	4.2	0.000	0.0	p-Coumaric acid
20.907	442.851	15.6	0.000	0.0	Un characterized
25.160	435.163	15.3	0.000	0.0	Cinnamic acid
Total	2845.236	100.0	0.000	100.0	

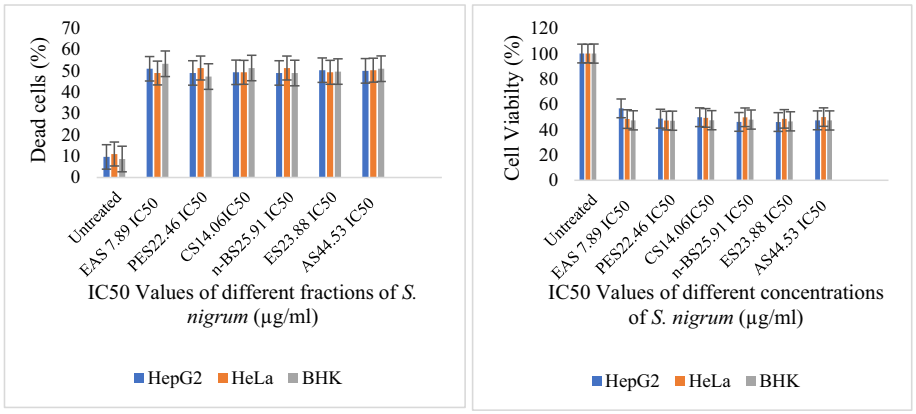

Fig. 2. HepG2 and HeLa and BHK cell lines were treated with obtained IC_{50} values of the *S. nigrum* fractions subjected trypan blue assay (a) crystal violet assay (b) for dead cells (%) and cell viability (%) respectively. The calculated mean values of replicates were expressed in percentage.

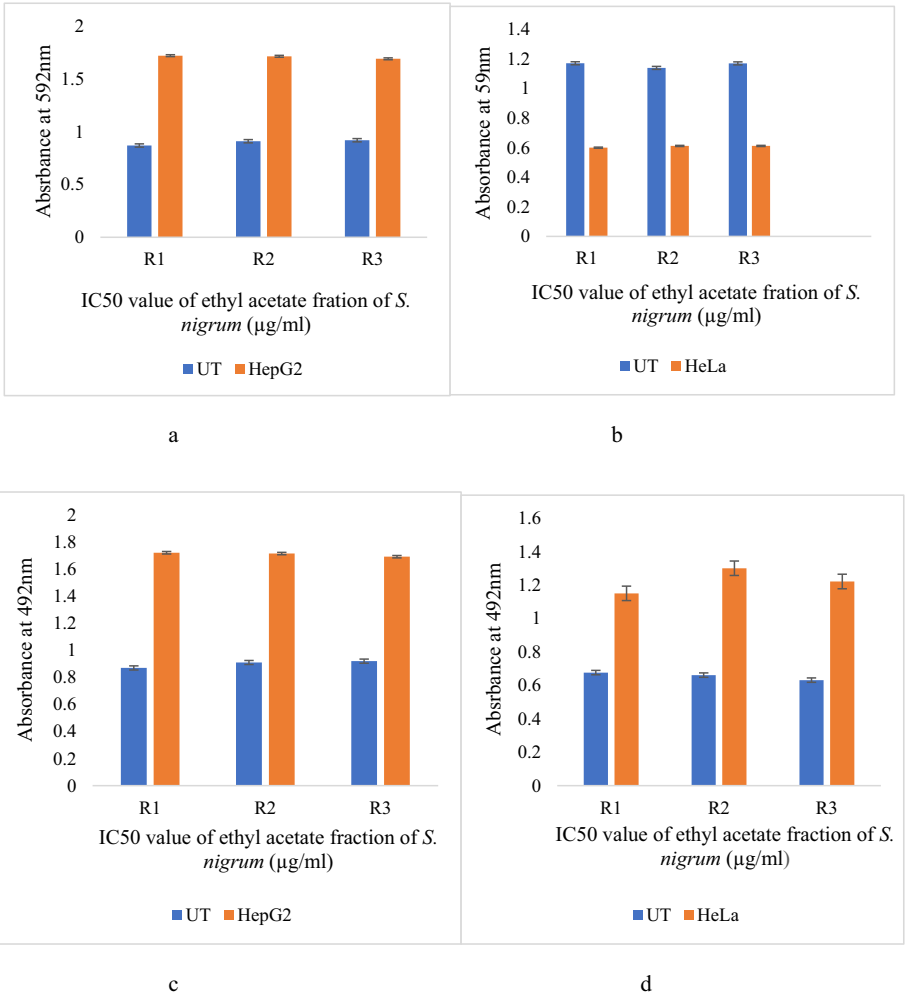

Fig. 3. Changes in VEGF protein in supernatant of HepG2 and HeLa cells (a, b) treated in triplicate (R1, R2, R3) with IC50 value of active ethyl acetate fraction of *S. nigrum*. Changes in lactate dehydrogenase (LDH) enzyme in supernatant of HepG2 and HeLa cells (c, d) treated in replicates (R1, R2, R3) with IC50 value of active ethyl acetate fraction of *S. nigrum*. The data was expressed as a mean value of replicates of each fraction.

The existence of the entirely cytosolic enzyme, LDH, in the cell culture medium displayed cell membrane damage (Chang and Geib, 2018)

Angiogenesis increases gradually during cell proliferations and secretion of VEGF by the cancer cells as shown in our study as well as reported in previously that is probably plays vital role in the

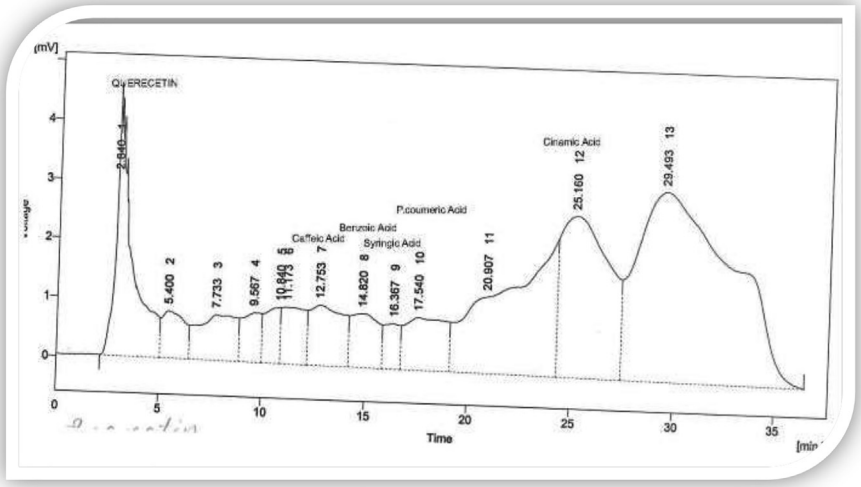

Fig. 4. HPLC chromatogram of most active fraction of *S. nigrum* and compounds correspond to peak 1–13 are marked.

initiation and maintenance of the abnormal angiogenesis. The proliferative vascular endothelium is responsive to VEGF stimulation and role of VEGF and its receptors in the biology of cancer cells as demonstrated by our study disclosed promising role of VEGF inhibitors and their receptors in cancer therapies (Jie et al., 2011; Han et al., 2018; Lee et al., 2018) The HPLC analysis of most active fraction was performed to screen out the nature of compounds accounts for that potential effect. Different compounds and fractions have been documented in literature for having significant anti-tumor and anticancerous activities. *S. nigrum* promisingly inhibit the proliferation of epithelial prostate cancer cells because of the presence of biologically compounds having anti cancerous property like phenols and flavonoids like caffeic acid, malic acid, chlorogenic acid and quercetin (Wang et al., 2011) which best support our findings. Our study findings showed the remarkable and promising results in HepG2 as compared to HeLa cell line. Ethyl acetate fraction screening by HPLC have shown many bioactive compounds as reported previously (Zhu et al., 2020). However, in future more biologically active compounds of medicinal values and cytotoxicity studies are required in different cell lines that could explore their elucidative role and key mechanism in cancer therapeutics.

5. Conclusion

The crude extract, as well as the aqueous and organic fractions exhibited noteworthy cytotoxicity against HepG2 and HeLa cells by inducing apoptosis. *S. nigrum* isolated fraction especially ethyl acetate showed promising results in HepG2 cells and might be established as a therapeutic agent in cancer treatment and therapeutics in future. Further investigations involving to its application as an additional antitumor agent is vital and can provide new therapeutic insights for further cancer studies.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Further reading

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