doi.org/10.36721/PJPS.2021.34.2.SUP.755-760.1

# *In-vitro* evaluation of antiproliferative potential of various fractions of *Silybum marianum* using HeLa and HepG2 cell lines

**Aisha Nawaz[1], Tanzeela Riaz[1]* and Abrar Ahmad[2]**
[1]Department of Biochemistry, University of Central Punjab, Lahore, Pakistan
[2]University College of Pharmacy, University of Punjab, Lahore, Pakistan

**Abstract**: *Silybum marianum* (Milk thistle) has been proven to possess anticancer, lactogenic, neuroprotective, immunomodulatory, hepatoprotective and anti-inflammatory properties. The current study was designed to evaluate the antiproliferative potential of aqueous and various organic fractions (ethanolic, petroleum ether, ethyl acetate, chloroform, n-butanol) of *S. marianum* against cancerous [HeLa, HepG2] and noncancerous [BHK] cell lines. The MTT assay was performed to access the cytotoxicity of all these fractions and $IC_{50}$ values were calculated. The cytotoxicity of these fractions was also confirmed through crystal violet and Trypan blue assays. All the tested fractions of *S. marianum* possessed significant antiproliferative potential. Interestingly, ethyl acetate fraction of *S. marianum* exhibited the highest antiproliferative activity amongst all the other tested fractions with an $IC_{50}$ of 13.07 µg/ml, 18.92 µg/ml and 76.15µg/ml against HeLa, HepG2 and BHK cell lines respectively. So it is concluded that *S. marianum* possess strong anticancer activity against both cervical and liver cancer and low cytotoxicity against normal cell line so it could be used as a source of potent anticancer compounds having high efficacy and minimal side effects.

**Keywords**: *Silybum marianum*, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay, normal cells, anti-proliferative potential, cancerous cells.

## INTRODUCTION

Cancer is a deadly disease which is characterized by uncontrolled cellular multiplication and differentiation (Khan *et al*., 2019). It is basically a pathological condition that invades various organs resulting in impairment of their functions (Panth *et al*., 2017). It has become a major health issue in both under developed and developed countries (Wiseman, 2019). More than 100 different types of cancer are known but the most common are liver, breast, cervical, colorectal, breast, prostate and lung cancer (Andima *et al*., 2020). Numerous factors associated with the development of cancer include unhealthy diet, lack of physical exercise, tobacco use, alcohol consumption, UV radiation, viral or bacterial infections and environmental pollutants. The cancer treatment approaches which are currently available include hormonal therapy, chemotherapy, targeted therapy, immunotherapy, radiation therapy and surgery. All of these have side effects like anaemia, diarrhea, constipation, fatigue, vomiting, hair loss and loss of appetite (Biseko *et al*., 2019). A great hope is offered by the traditional alternative medicines as plants provide a potential, less toxic and cheap source of anticancer drugs (Kimani *et al*., 2018). Above 1000 species of plants having substantial anticancer activities are present in nature (Privitera *et al*., 2019). Numerous studies have proven the anticancer effect of plant extracts and their purified bioactive compounds that use different mechanisms to inhibit the growth of cancer cells including induction of apoptosis, inhibition of angiogenesis and enhancement of immune system (Almosnid *et al*., 2018). In underdeveloped countries like Pakistan, India and Bangladash plants derived medicines are reviving interest gradually due to high safety and fewer side effects as compared to synthetic drugs (Chanchal *et al*., 2018).

*Silybum marianum* also known as cardus marianus belongs to Asteraceae family. It was formerly native of Southern Europe and Asia, but now it is grown across the globe (Gioti *et al*., 2019). Silymarin is the extract of *S. marianum* which has multiple pharmacological activities including hepatoprotective, anticancer, antimicrobial, cardio protective, anti-diabetic, neuroprotective, anti-inflammatory and other activities. It also has potential to counter the toxic effects of metals, pesticides and antibiotics. Chemically silymarin is a blend of flavonolignans whose active constituents include silydianin, silychristin, silybin, dihydrosilybin and isosilybin (Wang *et al*., 2020). Anti-neoplastic characteristics of Silymarin have been established in various cancers like breast, skin, prostate, bladder, lung and ovaries (Choe *et al*., 2020). Various clinical trials have supported its therapeutic values in folkloric medicines (Valkova *et al*., 2020).

The current study was designed to evaluate the antiproliferative potential of *S. marianum* against cervical and liver cancers using HeLa and HepG2 cell lines respectively and normal cells using BHK cell line.

**Corresponding author:* e-mail: dr.tanzeela@ucp.edu.pk

## MATERIALS AND METHODS

The whole plant including seeds of *S. marianum* was collected from outskirts of Rawalpindi and Islamabad identified. It was shade dried and finally ground into powder.

***Extract preparation and fractionation***

Extract was prepared by soaking the powdered plant into 95% ethanol for 14 days with occasional shaking. The material was filtered using Whatman filter paper No.1 and the filtrate was dried under vacuum using a rotary evaporator at 45°C temperature. The ethanolic extract was fractioned by mixing it in distilled water and then shifting in a separating funnel. Petroleum ether was added into the funnel and after mixing thoroughly both layers were separated. Petroleum ether layer was dried using rotary evaporator. Then chloroform, ethyl acetate and n-butanol was added sequentially into the aqueous layer and separated subsequently. All separated fractions were dried in rotary evaporator while the aqueous layer was freeze dried using a lyopholizer. Different fractions of *S. marianum* such as ethanolic (ES), petroleum ether (PES), chloroform (CS), ethyl acetate (EAS), n-butanol (n-BS) and aqueous (AS) fractions were prepared (Liang *et al*., 2017).

***Culturing of cell lines and treatment with plant fractions***

Cell lines namely HeLa, BHK and HepG2 previously stored in cryogenic vials placed in the liquid nitrogen were acquired from the cell culture laboratory of University of Lahore and maintained in the culturing flasks containing DMEM-HG and 1% of antimycotic (0.25µg/ml amphotericin B, 100 U/ml penicillin G and 100µg/ml streptomycin) (Sigma, USA) and 10% of fetal bovine serum (FBS) (Gibco, USA). Cultured cells were incubated at 37°C, 95% humidified atmosphere and 5% $CO_2$. For splitting the cells into 96 well plates, 70-80% confluent cells were detached from the flask and treated with different concentrations ranging from 2.5 to 200 µg/ml of *S. marianum* fractions (ES, PES, CS, EAS, n-BS and AS). Seven groups of each cell line were made, out of which one group was untreated and the other 6 groups were treated with ES, PES, CS, EAS, n-BS and AS fractions. All fractions were dissolved into DMSO at a concentration of 3000µg/ml first and then different concentrations (2.5, 12.5, 25, 50, 100 and 200µg/ml) of all fractions were prepared in DMEM and applied to all groups of cell lines for 72 hours (Maqbool *et al*., 2019).

***Cell viability assays***

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetr-azolium bromide), crystal violet and trypan blue assays were performed to check the post treatment viability of cancerous [HepG2 and HeLa] and normal cell lines [BHK].

***MTT assay and $IC_{50}$ calculation***

After treatment with different concentrations of all fractions of *S. marianum* for 72 hrs, 25µl MTT reagent was added into each well followed by incubation for 2 hrs. Then the medium containing MTT reagent was removed from each well carefully and 100µl DMSO was added into each well. Overnight incubation was given and absorbance was taken at 570nm using ELISA reader. Percentage viability of cells was calculated using the following formula:

Percentage cell viability = [absorbance of treated cells/absorbance of untreated cells]×100

All the experiments were repeated three times and $IC_{50}$ was calculated. Crystal violet and trypan blue assays were executed only for $IC_{50}$ values of the above mentioned fractions (Maqbool *et al*., 2019).

***Crystal violet assay (Live cell detection)***

Viability of cells [HeLa, HepG2 and BHK] was assessed using crystal violet assay. After treating the cells in 96 well plates for 72hrs, the secretome was removed and cells were washed using PBS. The surface of each well was covered with crystal violet dye that was prepared by mixing 0.1% dye into 2% ethanol. Plate was incubated for 15 min at room temperature and dye was castoff carefully. All the wells were washed carefully so that the cells may not detach from the surface of plate. Then 100 µl SDS (1%) was added into each well and again 5-10 min incubation was given and absorbance was taken at 595nm using ELISA reader. Percentage viability of cells was calculated.

***Trypan blue assay (Dead cells detection)***

Percentage dead cells were calculated using trypan blue assay. After 72 hrs treatment, the cell lines [BHK, HepG2 and HeLa] were washed three times with phosphate buffer saline and trypan blue dye was added into each well. Then plate was incubated for 5 min followed by washing of cells with PBS. Dead cells absorbed trypan blue dye and were counted using an inverted microscope. The percentage dead cells were calculated.

## STATISTICAL ANALYSIS

All the experiments were performed in triplicate and the data was expressed as mean and SEM (standard error of mean) then statistical analysis was done using Graph pad prism version. 5. One way ANOVA and Bonferroni test was used and values having $p \leq 0.05$ were considered to be significant.

## RESULTS

***MTT assay***

All fractions of *S. marianum* showed cytotoxicity against both cancerous cell lines in a concentration dependent manner. The experiment was performed in triplicate and mean and SEM (standard error of mean) was used to

calculate the percentage viability of cancerous and non-cancerous cells. Percentage viability of HeLa, BHK and HepG2 cell lines after treatment with ES, PES, CS, EAS, n-BS and AS fraction of *S. marianum* is shown in fig. 1, 2, 3, 4, 5 and 6 respectively. table 1 shows the cytotoxic activity as inhibitory concentrations ($IC_{50}$) values of all the above-mentioned fractions of *S. marianum* on HeLa, BHK and HepG2 cell lines. Among these entire fractions ethyl acetate fraction showed the most promising cytotoxic effect on both HeLa and HepG2 cell lines with low $IC_{50}$ values of 13.07µg/ml and 18.93µg/ml respectively. This ethyl acetate fraction at these concentrations had least effect on normal cell line. Among organic fractions, the petroleum ether (PES) fraction is least effective against HeLa cell line while chloroform (CS) fraction of *S. marianum* was observed least effective against HepG2 cell line. The aqueous (AS) fraction possessed lowest antiproliferative activity against all tested cell lines. The $IC_{50}$ values of all organic and aqueous fractions of *S. marianum* are shown in the table 1.

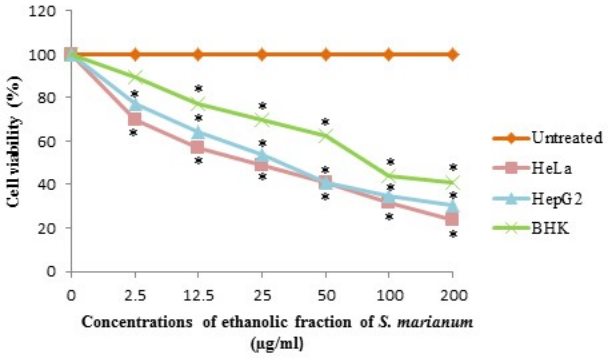


**Fig. 1**: Percentage viability of HeLa, BHK and HepG2 cell lines after treatment with ethanolic fraction of *S. marianum* for 72 hrs. The * indicates the significance in comparison to untreated group (P≤0.05).

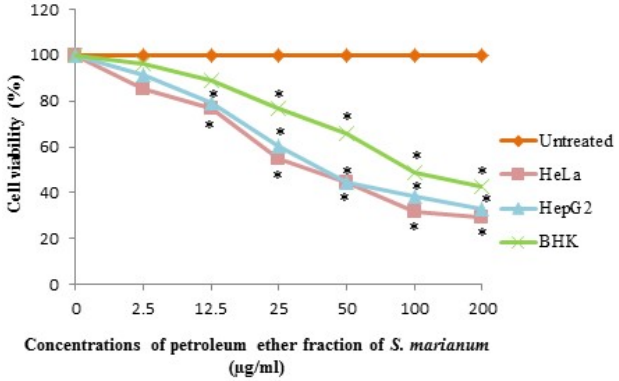


**Fig. 2**: Percentage viability of HeLa, BHK and HepG2 cell lines after treatment with petroleum ether fraction of *S. marianum* for 72 hrs. The * indicates the significance in comparison to untreated group (P≤0.05).

***Crystal violet and trypan blue assays***

The cytotoxic activity of all fractions of *S. marianum* was also assessed through crystal violet and trypan blue assays. Cell lines (HeLa, BHK and HepG2) were treated with $IC_{50}$ values of all fractions of *S. marianum* for 72 hrs and cell viability and cell death was calculated using crystal violet and trypan blue assay respectively. Results of both assays complemented each other. Fig. 7 shows percentage viability of all three cell lines after incubation with $IC_{50}$ values of different fractions of *S. marianum* and fig. 8 shows percentage dead cells at these concentrations.

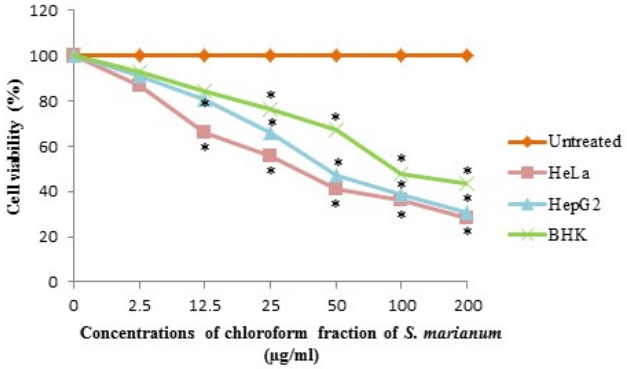


**Fig. 3**: Percentage viability of HeLa, BHK and HepG2 cell lines after treatment with chloroform fraction of *S. marianum* for 72 hrs. The * indicates the significance in comparison to untreated group (P≤0.05).

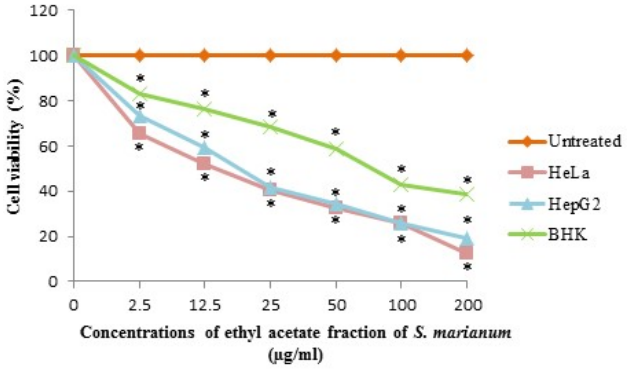


**Fig. 4**: Percentage viability of HeLa, BHK and HepG2 cell lines after treatment with ethyl acetate fraction of *S. marianum* for 72 hrs. The * indicates the significance in comparison to untreated group (P≤0.05).

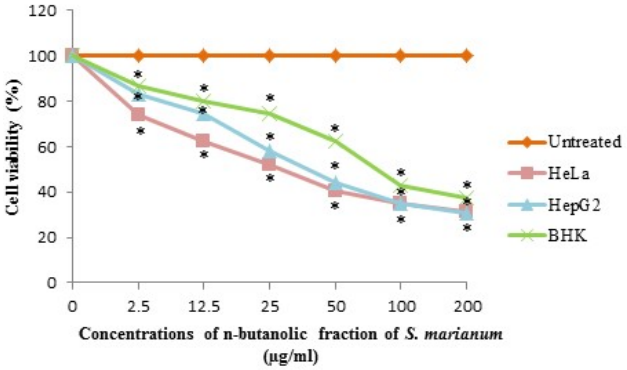


**Fig. 5**: Percentage viability of HeLa, BHK and HepG2 cell lines after treatment with n-butanolic fraction of *S. marianum* for 72 hrs. The * indicates the significance in comparison to untreated group (P≤0.05).

**Table 1**: $IC_{50}$ values of all fractions of *S. marianum* on both cancerous and non-cancerous cell lines

| Fractions of *S. marianum* | $IC_{50}$ values of all fractions of *S. marianum* (µg/ml) | | |
|---|---|---|---|
| | HeLa cell line | HepG2 cell line | BHK cell line |
| Ethanolic | 24.35 | 26.96 | 87.46 |
| Petroleum ether | 37.07 | 44.36 | 97.62 |
| Chloroform | 36.64 | 46.97 | 95.22 |
| Ethyl acetate | 13.07 | 18.93 | 76.15 |
| n-Butanol | 26.12 | 38.35 | 85.66 |
| Aqueous | 53.16 | 54.62 | 103.02 |

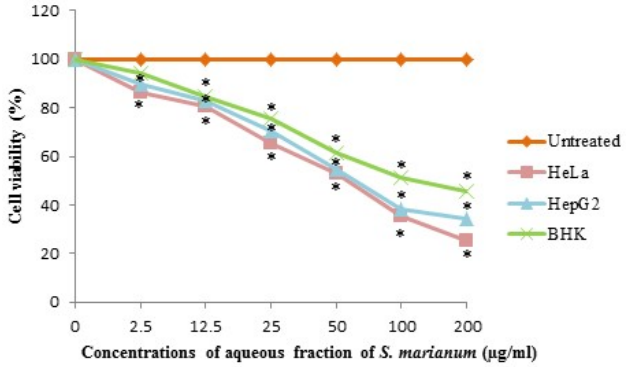


**Fig. 6**: Percentage viability of HeLa, BHK and HepG2 cell lines after treatment with aqueous fraction of *S. marianum* for 72 hrs. The * indicates the significance in comparison to untreated group (P≤0.05).

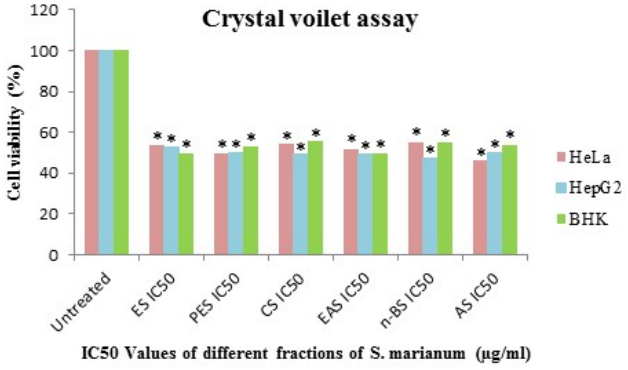


**Fig. 7**: Percentage viability of HeLa, BHK and HepG2 cell lines after treatment with $IC_{50}$ values of all fractions of *S. marianum* for 72 hrs.

## DISCUSSION

Globally a major health concern is oncological diseases and the high mortality rate associated with these. So the development of a novel anti-neoplastic drug is the need of the time (Feldman *et al*., 2018). Phytopharmaceutical products and phytotherapeutic agents being safer than synthetic drug have drawn the attention of human beings towards natural medicines (Agyare *et al*., 2018; Maqbool *et al*., 2019). Inconsistent composition and chemical complexity of the botanical extracts having anticancer activity are the two main challenges in crediting their biological significance (Chandrasekar *et al*., 2018). In recent years Silybum marianum have been renowned for its anti-tumor and anti-growth properties (Choe *et al*., 2020).

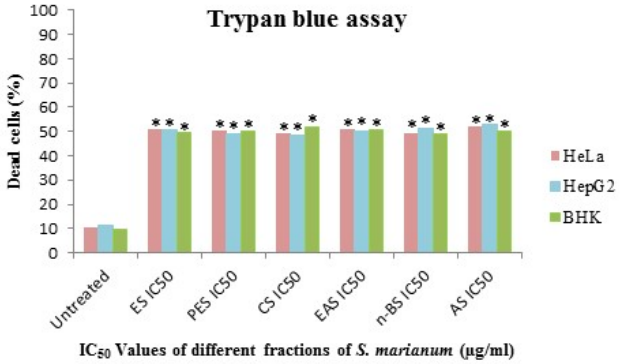


**Fig. 8**: Percentage dead cells of HeLa, BHK and HepG2 cell lines after treatment with $IC_{50}$ values of all fractions of *S. marianum* for 72 hrs.

Numerous studies have confirmed the anticancer effects of silymarin against human cancers (Won *et al*., 2018). In the present study the anticancer activity of various organic and aqueous fractions of S. marianum were investigated against HeLa (cervical cancer) and HepG2 (liver cancer) cell lines. In this study six fractions of *S. marianum* were obtained from the whole plant ethanolic extract. Different concentrations of *S. marianum* fractions including 2.5, 12.5, 25, 50, 100 and 200 µg were applied on three cell lines for 72 hrs and cytotoxicity was assessed using MTT assay. All fractions of *S. marianum* showed significant cytotoxic activity on both cell lines at different $IC_{50}$ values but the strongest cytotoxicity was shown by ethyl acetate fraction on both cancerous cell lines. Ethyl acetate fraction showed 50% inhibition of HeLa, HepG2 and BHK cell lines at 13.07µg/ml, 18.92µg/ml and 76.15µg/ml respectively so it had almost no cytotoxic effect on normal cells at its $IC_{50}$ value on cancerous cells. ES, PES, CS, n-BS and AS fractions showed 50% inhibition of HeLa cells at concentration of 24.35µg/ml, 37.07 µg/ml, 36.64µg/ml, 26.12µg/ml and 53.16µg/ml respectively. Similarly 50% inhibition of HepG2 cell line was shown by these fractions (Ethanolic, petroleum ether, chloroform, n-butanol and aqueous) at concentrations of 26.96µg/ml, 44.36µg/ml, 46.97µg/ml, 38.35µg/ml and 54.62µg/ml respectively.

The $IC_{50}$ values of these fractions were higher for normal cell line (BHK) that was 87.46µg/ml, 97.62µg/ml, 95.22 µg/ml, 85.66µg/ml and 103.02µg/ml for ethanol, petroleum ether, chloroform, n-butanol and aqueous fraction respectively. As these fractions have no cytotoxic activity on normal cells at their $IC_{50}$ values of cancerous cells so, it could be used as a potential drug for the treatment of cervical and liver cancers. Our findings are consistent with Singh *et al* (2013) who showed that *S. marianum* has anticancer activity against different cancers including liver, breast, colon, cervical and ovarian cancer. Davis-Searles *et al* (2014) reported the anticancer effect of *S. marianum* on human prostate carcinoma. Kim *et al* (2019) reported the apoptotic activity of silymarin (extract of *S. marianum*) against gastric cancer cells.

Cytotoxic activity of *S. marianum* was also checked by crystal violet and trypan blue assays. Results of these assays complemented each other. Cells (HeLa, BHK and HepG2) were plated and treated with $IC_{50}$ values of all fractions of *S. marianum* for 72 hrs and crystal violet assay was performed. The ES, PES, CS, EAS, n-BS, and AS fraction showed 53.97%, 49.67%, 54.08%, 51.52%, 55.34% and 46.28% respective viability of HeLa cells at their $IC_{50}$ values through crystal violet assay. HepG2 cell line showed percentage viability of 52.37%, 50.33%, 49.44%, 49.21%, 47.54% and 50.09% in the presence of $IC_{50}$ values of ES, PES, CS, EAS, n-BS and AS fractions of *S. marianum* for 72 hrs. BHK cell line showed percentage viability of 49.94%. 53.1%, 56.04%, 49.84%, 55.28% and 53.75% in the presence of $IC_{50}$ values of ES, PES, CS, EAS, n-BS and AS fractions of *S. marianum* for 72 hrs (Zahira *et al*., 2018).

Trypan blue assay was performed to calculate the percentage dead cells of HeLa, BHK and HepG2 cell lines after treatment with $IC_{50}$ values of all fractions of *S. marianum* for 72 hrs. Dead cells absorbed the dye and appeared bluish under the microscope. Dead cells were counted and percentage was calculated. HeLa cell line showed percentage dead cells of 50.67%, 50.33%, 49%, 51%, 49.33% and 52% after incubation with $IC_{50}$ values of ES, PES, CS, EAS, n-BS and AS fractions of *S. marianum* for 72 hrs respectively and 10.33% dead cells in untreated group of HeLa cells. Similarly dead cells percentage of HepG2 cell line was 50.67%, 49%, 48.33%, 50.33%, 51% and 52.67% after treatment with $IC_{50}$ values of ES, PES, CS, EAS, n-BS and AS fractions of *S. marianum* and 11.33% dead cells were observed in untreated group. The percentage dead cells of BHK cell line were 49.33%, 50%, 51.67%, 50.67%, 49% and 50.33% after incubation with $IC_{50}$ values of ES, PES, CS, EAS, n-BS and AS fractions of *S. marianum* for 72 hrs and 9.33% dead cells in case of no treatment. So *S. marianum* is a potential anticancer plant having strong growth inhibiting effects and could be used as a potential anticancer drug (Wang *et al*., 2020).

## CONCLUSION

In this study all organic and aqueous fractions of *S. marianum* exhibited considerable anticancer activity against both HeLa and HepG2 cell line with almost no cytotoxicity to normal cells at their $IC_{50}$ values, suggesting it a promising anticancer plant for the treatment of cervical and liver cancers. The bioactive compounds present in this plant which are responsible for its anticancer activity could be isolated and further studied.

## REFERENCES

Agyarea C, Spieglerc V, Asaseb A, Scholzc M, Hempeld G and Hense A (2018). An ethnopharmacological survey of medicinal plants traditionally used for cancer treatment in the Ashanti region, Ghana. *J. Ethnopharmacol*., **212**(2): 137-152.

Almosnida NM, Xiaolei Z, Lihe J, Amy R, Deborah K, Shuo W, Fan W, Jingquan Y, Elliot A, Ying G and Jianhua M (2018). Evaluation of extracts prepared from 16 plants used in Yao ethnomedicine as potential anticancer agents. *J. Ethnopharmacol*., **211**(1): 224-234.

Andima M, Coghi P, Yang LJ, Wong VKW, Ngule CM, Heydenreich M, Ndakala AJ, Yenesew A and Derese S (2020). Antiproliferative activity of secondary metabolites from *Zanthoxylum zanthoxyloides* Lam: *In vitro* and *in silico* Studies. *Pharmacogn. Commn*., **10**(1): 44-51.

Biseko EZ, Swai HS, Mbugua RW, Ndungu JW, Chepngetich J and Gathirwa JW (2019). *In vitro* antiproliferative potential of *Annona senegalensis* Pers. and *Allophylus africanus* P Beauv. plant extracts against selected cancer cell lines. *J. Med. Plants Res*., **13**(13): 304-311.

Chanchal DK, Alok S, Rashi S, Bijauliya RK, Yadav RD and Sabharwal M (2018). Various medicinal plants used in the treatment of anticancer activity. *Int. J. Pharm. Sci. Res*., **9**(4): 1424-1429.

Chandrasekar R, Sivagami B and Niranjan BM (2018). A pharmacoeconomic focus on medicinal plants with anticancer activity. *Res. J. Pharmacog. Phytochem*., **10**(1): 91-100.

Choe U, Whent M, Luo Y and Yu L (2020). Total phenolic content, free radical scavenging capacity, and anti-cancer activity of silymarin. *J. Food Bioact*., **10**(6): 53-63.

Davis-Searles PR, Nakanishi Y, Kim N, Graf TN, Oberlies NH, Wani MC, Wall ME, Agarwal R and Kroll DJ (2014). Milk thistle and prostate cancer: Differential effects of pure flavonolignans from *Silybum marianum* on antiproliferative end points in human prostate carcinoma cells. *Cancer Res*., **65**(10): 4448-4457.

Gioti K, Papachristodoulou A, Benaki D, Havaki S, Beloukas A, Vontzalidou A, Aligiannis N, Skaltsounis AL, Mikros E and Tenta R (2019). Silymarin enriched extract (*Silybum marianum*) additive effect on doxorubicin-mediated cytotoxicity in PC-3 prostate cancer cells. *Planta Med*., **85**(11/12): 997-1007.

Khan T, Amrita D, Hardik C and Krina P (2019). Polysaccharides as potential anticancer agents-A review of their progress. *Carbohydrate polymer*., **210**(4): 412-428.

Kim SH, Choo G, Yoo E, Woo J, Han S, Lee J and Jung J (2019). Silymarin induces inhibition of growth and apoptosis through modulation of the MAPK signaling pathway in AGS human gastric cancer cells. *Oncol. Rep*., **42**(5): 1904-1914.

Kimani PM, Mwitari PG, Njagi SM, Kirira PG and Kiboi DM (2018). *In vitro* anti-Proliferative activity of selected plant extracts against cervical and prostate cancer cell lines. *J. Cancer Sci. Ther*., **10**(9): 267-271.

Liang C, Pan H, Li H, Zhao Y and Feng Y (2017). *In vitro* anticancer activity and cytotoxicity screening of phytochemical extracts from selected traditional Chinese medicinal plants. *JBUON*, **22**(2): 543-551.

Maqbool T, Awan SJ, Malik S, Hadi F, Shehzadi S and Tariq K (2019). *In-vitro* anti-proliferative, apoptotic and antioxidative activities of medicinal herb Kalonji (*Nigella sativa*). *Current Pharmaceut. Biotech*., **20**(15): 1-21.

Panth N, Manandhar B and Paudel KR (2017). Anticancer activity of *Punica granatum* (Pomegranate): A review. *Phytother. Res*., **31**(4): 568-578.

Privitera G, Luca T, Castorina S, Passanisi R, Ruberto G and Napoli E (2019). Anticancer activity of *Salvia officinalis* essential oil and its principal constituents against hormone-dependent tumour cells. *Asian. Pac. J. Trop. Biomed*., **9**(1): 24-28.

Singh S, Mehta A, Baweja S, Ahirwal L and Mehta P (2013). Anticancer activity of *Andrographis paniculata* and *Silybum marianum* on five human cancer cell lines. *J. pharmacol. toxicol*., **8**(1): 42-48.

Valkova V, Duranova H, Bilcikova J and Haban M (2020). Milk thistle (*Silybum marianum*): A valuable medicinal plant with several therapeutic purposes. *J. Microbiol. Biotech. Food Sci*., **9**(4): 836-843.

Wang X, Zhang Z and Wu SC (2020). Health Benefits of Silybum marianum: Phytochemistry, pharmacology, and applications. *J. Agric. Food Chem*., **68**(42): 11644-11664.

Wiseman MJ (2019). Nutrition and cancer: Prevention and survival. *British J. Nutri*., **122**(5): 481-487.

Zahira NV, Nakhjavania M, Hajianb P, Shirazic FH and Mirzaeie H (2018). Evaluation of silibinin effects on the viability of HepG2 (human hepatocellular liver carcinoma) and HUVEC (human umbilical vein endothelial) cell lines. *Iranian J. Pharmaceutic Res*., **17**(1): 261-267.