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Research Article

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In vitro cytotoxic potential of *Gymnema sylvestre* fractions on HT-29 cell line

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ABSTRACT

The HT-29 colon cancer cell line was used in the current work to investigate the anti-cancer efficacy of *Gymnema sylvestre* extracts. The viability, apoptosis, flow cytometry, and mode of action of the active fraction were also evaluated. The active fractions of *G. sylvestre* caused cell cycle arrest at the S phase and G2/M phase, and also caused programmed cell death. The alcoholic fractions showed anti-cancer effect in HT-29 cancer cells. By applying methanolic fraction of *Gymnema sylvestre to* the HT-29 cells, IC_{50} was observed at 140.39 µg/mL, whereas for chloroform fraction of Gymnema sylvestre showed IC_{50} at 154.44 µg/mL. The herbal extract for colon cancer treatment may be developed with the help of researchers due to *G. sylvestre*'s anti-cancer properties.

KEYWORDS: *Gymnema sylvestre*, Colon cancer, Cytotoxicity, Apoptosis, Cell cycle arrest

INTRODUCTION

The basis of traditional medicine is the use of medicinal plants (Ahvazi *et al.*, 2012). Humans relied on the therapeutic effects of medicinal plants before the development of modern drugs. Because of an antiquated belief that plants were made to give man food, medicine, and other benefits, people value these plants. Various plants utilised in herbalism, some of which have medical characteristics, are referred to as traditional herbs. These healing plants are recognised as a significant source of components for the creation and synthesis of medications (Hassan, 2012). Medicinal plants are currently gaining popularity due to their numerous benefits as a rich source of diagnostic phytochemicals that may facilitate the creation of new medicines.

Most plant-derived phytocompounds, including flavonoids, alkaloids, and phenolics, have been found to benefit health and prevent cancer (Kasagana & Karumuri, 2011; Azwanida, 2015). Oncogenesis expression is a critical event in the early stages of tumor formation (Hassanpour & Dehghani, 2017). The world's second leading cause of death is cancer (Imran *et al.*, 2017). One typical form of fatal malignant tumour is colon cancer. Around the world, there are 774,000 deaths from colon cancer each year and 1.36 million new cases (Liu *et al.*, 2021). Unbalanced diets, which mainly affect persons over the age of 50, are to blame for 70% of occurrences of colorectal cancer.

Because of side effects and limited differentiation between healthy and malignant cells, chemotherapy treatment for colon cancer is still challenging (Nelson *et al.*, 2019). For better colon cancer treatment, more research into organic products and their constituents with minimal toxicity and side effects is still necessary (Arun *et al.*, 2017). Numerous widely used anti-cancer medications are made from strong herbal phytochemicals. As a result, medicinal plants became a valuable source of anti-cancer agents. Herb-based drugs would be developed following a thorough evaluation and chemical modification (Zheng *et al.*, 2016). *G. sylvestre* is a perennial woody climber in the Asclepiadaceae family, also known as the "milk weed" family (Tiwari *et al.*, 2014).

Gymnema sylvestre is a large tropical plant native to the central and western regions of India as well as Australia and Africa. It is an Ayurveda and therapeutic herb that has been used to treat diabetes in India for over 2,000 years. *G. sylvestre* leaves are in high demand in the pharmaceutical industry these days because they are an important anti-diabetic medicinal plant (Packialakshmi & Sowndriya, 2019). Butyric acid, chlorophyll a, hentriacontane, phytin, chlorophyll b, Pentriacontane, inositol, formic acid, d-quercitol, tartaric acid, gymnemic acids, anthraquinone derivatives are all found in this plant. According to pharmacological investigations, G. sylvestre contains medicinal qualities such as anti-inflammatory, anti-microbial impact, anti-atherosclerotic effect, larvicidal effect, and anti-hepatoprotective action (Thirunavukkarasu *et al.*, 2016). The anticancer properties of *Gymnema sylvestre* on human colon cancer have not yet been investigated. This study was intended on investigating the anti-malignant effects of the methanol (GS-ME) and chloroform (GS-CH) fraction of *Gymnema sylvestre* on HT 29 cells (human colorectal cancer cells).

MATERIALS AND METHODS

Collection of Plant Samples, Extract Preparation and Fractionation

The leaves of *Gymnema sylvestre* were collected in Bharathidasan University campus, Trichy, Tamilnadu, India. The plant was authenticated by Botanical Survey of India, Coimbatore, Tamilnadu, India. Plant leaves were dried, powdered and extraction was done using Soxhlet apparatus with alcoholic solvent (Nowak *et al.*, 2021). 50g of plant sample was loaded into an extraction thimble and ran the Soxhlet for eight hours. Prior to drying in a freeze dryer, the retrieved solvents were collected and concentrated using a vacuum rotary evaporator set to -80°C. The extract was obtained until it had thickened and become viscous (Subramanian *et al.*, 2016). After the extraction, the concentrated extract was fractionated.

Cell Culture

HT-29 cells were obtained from the National Centre for Cell Sciences (NCCS) in Pune, India, and were cultured in Dulbecco's modified Eagles medium, DMEM. In a 25 cm^2 tissue culture flask, the cell line was cultured in DMEM supplemented with an antibiotic solution containing Penicillin (100 µg/mL), Streptomycin (100 µg/mL), 10% FBS (L-glutamine, sodium bicarbonate) and Amphotericin B (2.5 g/mL). Cell lines were cultured at 37°C in a humidified 5% CO_2 incubator. Cell viability was determined by observation of cells using an inverted phase contrast microscope (Venkatadri *et al.*, 2020).

Cytotoxic Potential of *G. Sylvestre* Against Colon Cancer Cell Line

In the tissue culture plate (96 wells), HT-29 cells were seeded and incubated for 24 hours consisting of DMEM. In yellow MTT (0.5 mg/mL) solution, cells were incubated and then treated with *G. sylvestre* alcoholic fraction in triplicates of aliquots of two fold dilution as mentioned above in 500 µl of DMEM. After incubation, the MTT solution was extracted from the medium and add 200µl DMSO to dissolve the purple formazan salt crystals. Then it was observed under the microscope (inverted phase contrast) (Olympus CKX41 with Optika Pro5 CCD camera) (Nelson *et al.*, 2020). At a wavelength of 540nm, the absorbance values were measured using a micro plate reader.

The growth of inhibition percentage was calculated by this formula:

$$\% \text{ of viability } = \frac{\text{Mean OD Samples}}{\text{Mean OD of control group}} \times 100$$

Flow Cytometry Assay

G. sylvestre alcoholic fraction treated with HT-29 cells. The sample was taken in a 12×75 mm polystyrene tube and then centrifuged at 3000 rpm for 5 mins. Discard supernatant and do not disturb the pellet, it will look like a white film at the bottom of the tube. PBS was added and gently vortex by pipetting. Centrifuged the cells at 3000 rpm for 5 mins. Remove the supernatant and resuspend the cell pellet with PBS by pipetting gently for several times. Drop by drop, the resuspended cells were added to a vial containing 1ml of ice cold 70% ethanol while vortexing at moderate speed. Close the tube and keep it in the freezer at -20°C (Nithya & Sumalatha, 2014).

Staining of Cell Cycle

The samples were centrifuged at 3000 rpm for 5 minutes at RT after the overnight incubation. After removing the supernatant, 250 µl PBS was added to the pellet. Repeat the centrifugation process at the same rpm and time. After removing the supernatant, the pellet was taken and 250 µl of cell cycle reagent propidium iodide (PI) was added. It was incubated in the dark for 30 minutes (it is sensitive to light). Following that, it was examined with a Flow Cytometer. To compare the untreated control cells and samples were analyzed by gating (Kim *et al.*, 2021).

Apoptosis

According to standard procedures HT-29 cells were cultured and treated for 24 hours with IC_{50} values of samples GS-ME (154.438 µg/mL) and GS-CH (140.387 µg/mL). The cells were stained with a mixture of acridine orange (AO) 100 µg/mL and ethidium bromide (EtBr) 100 µg/mL for 10mins at room temperature before the staining process washed with cold PBS (Zheng *et al.*, 2016). The stained cells were washed twice with 1X PBS before being examined under a fluorescence microscope with a blue filter (Olympus CKX41 with Optika Pro5 camera) (Manosroi *et al.*, 2015).

RESULTS

Extraction and Fractionation of Plant Sample

The air-dried alcoholic extract (5 g) was made into fractions such as petroleum ether, ethyl acetate, chloroform and methanol (100%) of gradient organic solvents in increased polarity in equal amounts as per sample. The supernatant was taken as a fraction of respective solvents by centrifuging at 3000 rpm for 10 mins. The methanolic and chloroform fractions (GS-ME and GS-CH) were taken for further studies based on high cytotoxic potentials on HT-29 cell line.

Cytotoxic Evaluation of *Gymenema Sylvestre* Fractions on HT- 29 Cell Line

2,5- diphenyltetrazolium bromide assay was done to check the anti-cancer effect of GS fraction in a human colon cancer cell line (HT-29). This is a qualitative measurement which results in the formation of purple formazan crystals and diluted with 200 µL of DMSO. The lethal concentration value of HT-29 was found to be 154.438 µg/mL & 140.387 µg/mL for GS-ME & GS-CH fractions which was calculated using Software (ED50 PLUS

V1.0). The max viability showed in the least concentration. Figures 1 & 3 shows the % viability of HT-29 and Figures 2 & 4 shows the photographic image of HT-29 treated with the methanol and chloroform fraction of *Gymenema sylvestre*.

Apoptosis

The AO/EtBr staining methods provide impact results of *G. sylvestre* fractions that cause apoptosis (a systematic death) in HT-29 cells. With the LC_{50} value obtained in the MTT assay, the experiment was carried out for the drug treatment and stained further. The HT-29 cells were classified as normal green nucleus (living cells), bright green nucleus with condensed or fragmented chromatin (early apoptotic), orange-stained nuclei with chromatin condensation or fragmentation (late apoptotic) and uniformly orange-stained cell 21 nuclei (necrotic). Cells were viewed at 20X magnification in a fluorescence microscope (Figure 5). HT-29 cells were retrieved as per the standard protocols and treated with LC_{50} values of samples GS-ME (154.438 μg/mL) and GS-CH (140.387 μg/mL).

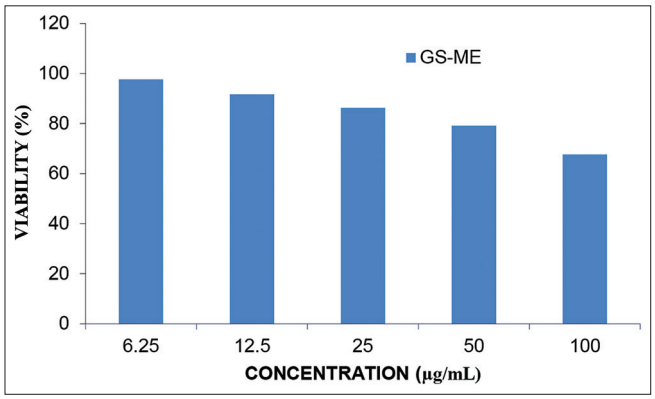

Figure 1: Cytotoxic effects of *Gymnema sylvestre* methanolic fraction against HT-29 cell lines: LC_{50} VALUE = 154.44 µg/mL

Flow Cytometry Analysis

Flow cytometry analysis confirms that the *G. sylvestre*-alcoholic fraction has high activity against HT-29 cell lines. To clarify whether *G. sylvestre* exerts anticancer activity effect only by inducing programmed cell death or they also induce cessation of cell cycle assessed its distribution by FACS analysis in HT-29 colon cancer cell line. The effect of the sample is represented in (Figures 6 & 8). The fraction arrests the cell cycle process at the G2/M and S phases as shown in the figures. Treatment with the isolated fractions at a low dose of 6 µg/mL, medium dose of 25 µg/mL and high IC_{50} dose shifted 20 the population of HT-29 cells into phase G1 from G2/M. At 6 µg/mL, 20% of cells were in the S phase while at 25 µg/mL cells are in the phase of G2/M were increased to 30% of the cell cycle Arrest at G1 phase as compared to the control. As a result of the analysis, we can conclude that the GS-ME fraction induces cell cycle arrest in the S phase, meanwhile, the GS-CH fraction provokes cell cycle arrest in the G2 phase (Figures 7 & 9).

DISCUSSION

In this study, GS-ME & GS-CH was incubated with HT-29 cell lines at various doses. *In vitro* research on the effects of GS on colon cancer cells revealed that it may trigger apoptosis. Cell cycle analysis revealed that the GS extracts arrest cellular proliferation in the S phase (Chang *et al.*, 2013). According to the efficiency of GS on colon cancer cells, the growth of cancer cells is greatly reduced. The findings imply that *G. sylvestre* therapy may reduce colon cancer cell growth. The use of *G. sylvestre* to treat colon cancer cells may not result in necrosis. The current data imply that after *G. sylvestre* treatment, carcinogen damages the colon cells which further decrease the growth. The extracts of *Gymnema sylvestre* is thought to prevent the development of colon cancer (Yadav *et al.*, 2021).

Gymnema sylvestre fractions ability to lower cancer cell viability is evaluated using the MTT assay. The cytotoxicity assay was

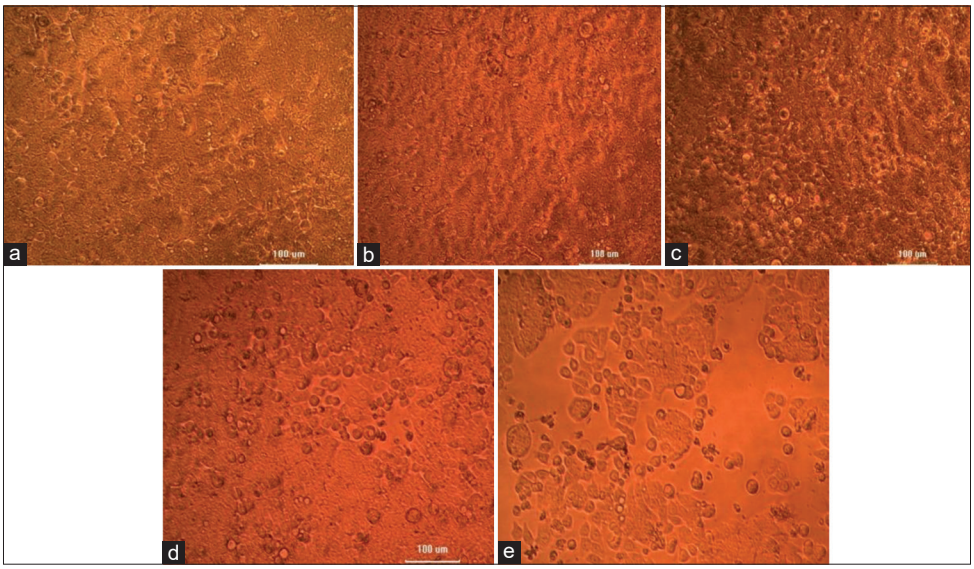

Figure 2: Phase contrast inverted microscopic image of treated HT-29 in MTT assay against *Gymnema sylvestre* methanolic fraction at different concentration. a) 6.25, b) 12.5, c) 25, d) 50 and e) 100 µg/mL

used to evaluate *G. sylvestre*'s effects on HT-29 cells after 72 hours, and the IC_{50} concentration that killed 50% of the cells was graphically determined. In screening results, *G. sylvestre* shown wide-ranging cytotoxicity with the most notable cytotoxic activity against colon cancer cells (Xu *et al*., 2014). *G. sylvestre* has demonstrated significant growth inhibition in the cancer cell line at low IC_{50} values. *G. sylvestre* therapy on HT-29 cell lines led to a significant decrease in growth rate when compared to the control (Revathi *et al*., 2019).

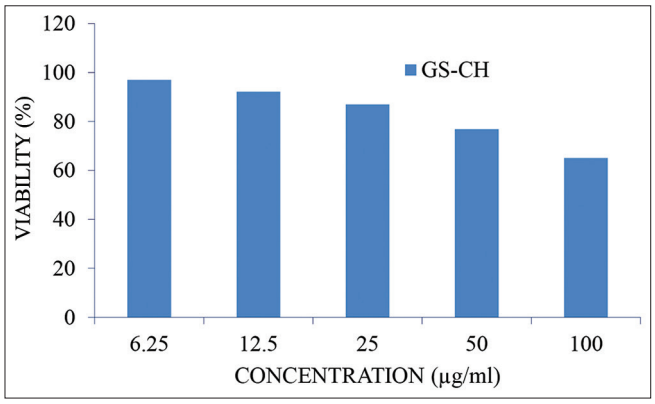

Figure 3: Cytotoxic effects of *Gymnema sylvestre* Chloroform Fraction against HT-29 Cell lines: LC_{50} Value = 140.39 µg/mL

The detrimental effects of *G. sylvestre* on MTT have been further confirmed by morphological investigations employing fluorescence microscopy, acridine orange, propidium iodide staining assay and flow cytometric cell cycle analysis on HT-29 cells. A minimum of 300 counted cells were studied under a fluorescent microscope to confirm the autophagy characteristics. The proportion of apoptotic cells was determined from the total cell populations, and the apoptotic cells were estimated as the fraction of apoptotic bodies. The graph and apoptotic cell percentage showed that *G. sylvestre* treatment increased the fraction of apoptotic cells got larger with time. Morphologic description was achieved by identifying and quantifying apoptosis using fluorescence and phase contrast microscopy (Yang *et al*., 2016).

Quantitative analysis of the cell cycle is crucial in the investigation of molecular mechanisms underlying cell death and cell cycle advancement. In order to measure apoptosis, DNA flow cytometry (FCM) was used on untreated and treated HT-29 cells to count the number of apoptotic cells (Alabsi *et al*., 2012).

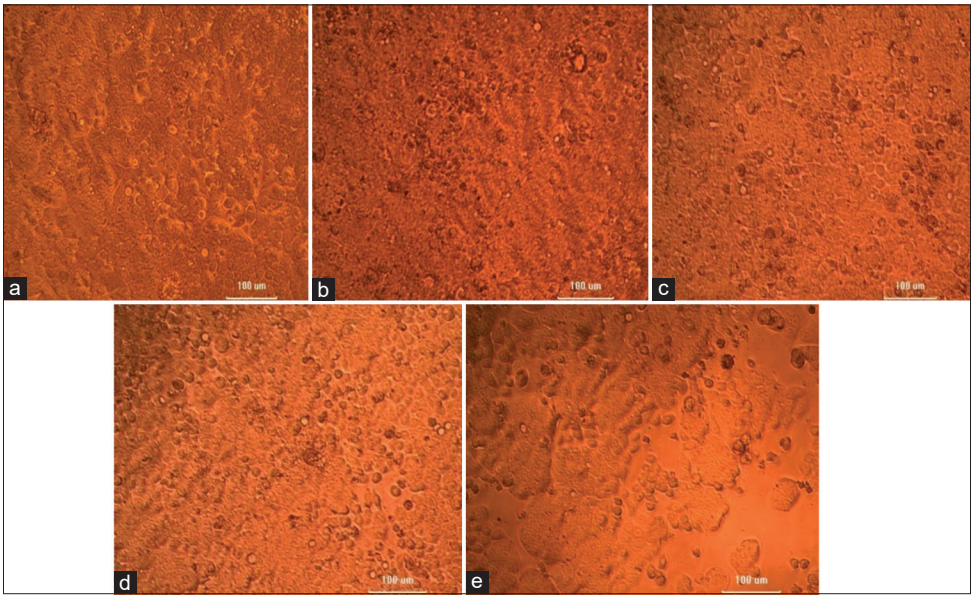

Figure 4: Phase contrast inverted microscopic image of treated HT-29 in MTT assay against *Gymnema sylvestre* Chloroform Fraction at different concentration. a) 6.25, b) 12.5, c) 25, d) 50 and e) 100 µg/mL

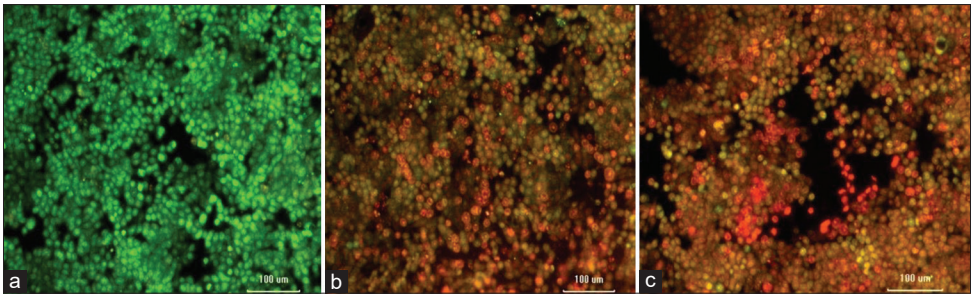

Figure 5: Fluorescence microscopic images of Apoptotic activity of *Gymnema sylvestre* Fraction against HT-29 cell line. a) Control, b) HT-29 Against GS-Methanolic fraction, c) HT-29 against GS-Chloroform fraction

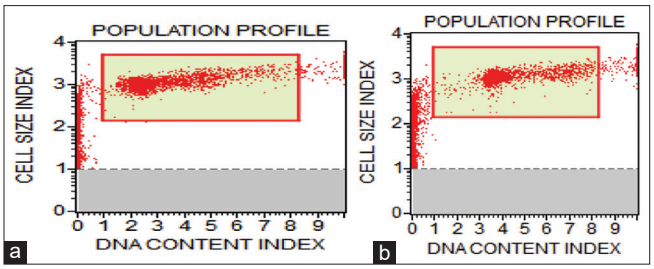

Figure 6: Flow Cytometry analysis cell population profile. a) DNA content index – control, b) DNA content index of *Gymnema sylvestre* methanolic fraction treated cells.

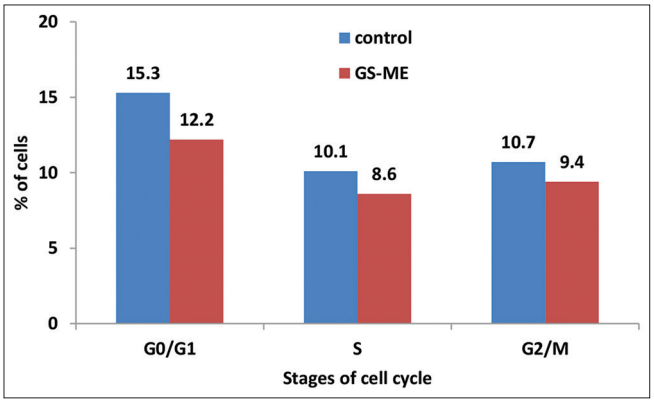

Figure 7: Flow cytometry analysis of *Gymnema sylvestre* methanolic fraction on HT-29 cell lines

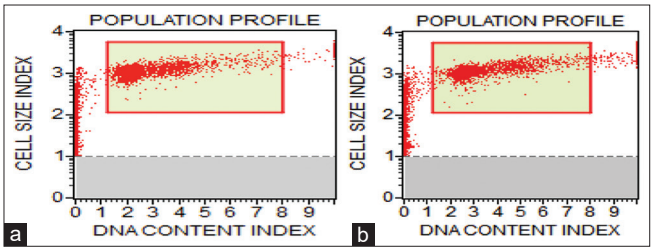

Figure 8: Flow Cytometry analysis cell population profile. a) DNA content index – control, b) DNA content index of *Gymnema sylvestre* chloroform fraction treated cells

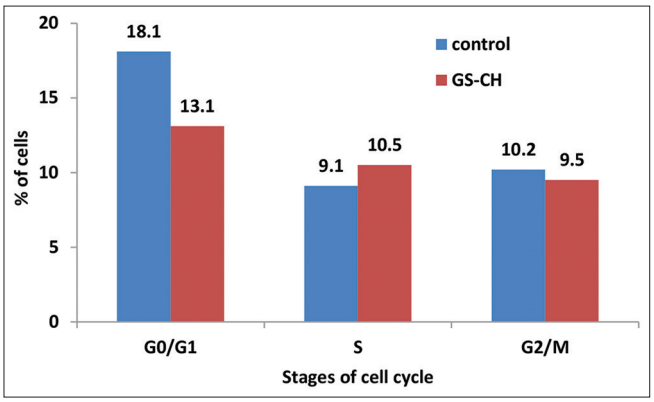

Figure 9: Flow cytometry analysis of *Gymnema sylvestre* chloroform fraction on HT-29 cell lines

Flow cytometry analysis of the cell cycle evaluates apoptotic changes in cells by colouring cells with DNA dyes. The crucial dye, PI, was taken up more readily by apoptotic cells than by live cells, most likely due to a decrease in membrane permeability. This technique works well for quantitatively determining the percentage of cells at each stage of the cell cycle (Kuppusamy *et al.*, 2016). In this work, treatment of GS-ME caused the S phase cell cycle to be arrested and the death of HT-29 cells. Cell cycle patterns revealed that treatment of GS-CH results in cell cycle arrest and death at the G2/M phase. According to our research, *G. sylvestre* inhibits HT-29 cell proliferation by inducing apoptosis, which results in cell cycle arrest at S phase by GS-ME and cell cycle arrest at G2/M by GS-CH.

CONCLUSION

The findings of the present study suggest that *G. sylvestre* has the potential to inhibit colon cancer cell lines, implying that more research is needed to isolate an active molecule and should be scientifically evaluated using animal models and clinical trials in the future. The study also supports the anticancer properties of medicinal plant species in Indian medicine systems, as well as further evaluation of the selected plants for an effective anticancer drug with minimal side effects. As a result, enhancing a healthy diet with *Gymnema sylvestre* leaves may help to treat colon cancer.

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REFERENCES

Ahvazi, M., Khalighi-Sigaroodi, F., Charkhchiyan, M. M., Mojab, F., Mozaffarian, V.-A., & Zakeri, H. (2012). Introduction of medicinal plants species with the most traditional usage in alamut region. *Iranian Journal of Pharmaceutical Research, 11(*1), 185-194.

Alabsi, A. M., Ali, R., Ali, A. M., Al-Dubai, S. A. R., Harun, H., Kasim, N. H. A., & Alsalahi, A. (2012). Apoptosis induction, cell cycle arrest and *in vitro* anticancer activity of gonothalamin in a cancer cell lines. *Asian Pacific Journal of Cancer Prevention, 13*(10), 5131-5136. https://doi.org/10.7314/apjcp.2012.13.10.5131

Arun, A., Patel, O. P. S., Saini, D., Yadav, P. P., & Konwar, R. (2017). Anti-colon cancer activity of *Murraya koenigii* leaves is due to constituent murrayazoline and O-methylmurrayamine A induced mTOR/AKT downregulation and mitochondrial apoptosis. *Biomedicine & Pharmacotherapy*, *93*, 510-521. https://doi.org/10.1016/j.biopha.2017.06.065

Azwanida, N. N. (2015). A review on the extraction methods use in medicinal plants, principle, strength and limitation. *Medicinal & Aromatic Plants*, *4*(3), 1000196.

Chang, C., Liu, S.-P., Fang, C.-H., He, R.-S., Wang, Z., Zhu, Y.-Q., & Jiang, S.-W. (2013). Effects of matrine on the proliferation of HT29 human colon cancer cells and its antitumor mechanism. *Oncology Letters*, *6*(3), 699-704. https://doi.org/10.3892/ol.2013.1449

Hassan, B. A. R. (2012). Medicinal plants (importance and uses). *Pharmaceutica Analytica Acta*, *3*(10), 1000e139. https://doi.org/10.4172/2153-2435.1000e139

Hassanpour, S. H., & Dehghani, M. (2017). Review of cancer from perspective of molecular. *Journal of Cancer Research and Practice*, *4*(4), 127-129. https://doi.org/10.1016/j.jcrpr.2017.07.001

Imran, A., Qamar, H. Y., Ali, Q., Naeem, H., Riaz, M., Amin, S., Kanwal, N., Ali, F., Sabar, M. F., & Nasir, I. A. (2017). Role of molecular biology in cancer treatment: A review article. *Iranian journal of public*

health, *46*(11), 1475-1485.

Kasagana, V. N., & Karumuri, S. S. (2011). Conservation of medicinal plants (past, present & future trends). *Journal of Pharmaceutical Sciences and Research*, *3*(8), 1378-1386.

Kim, S., Yang, H. Y., Lee, H. J., & Ju, J. (2021). *In Vitro* Antioxidant and Anti-Colon Cancer Activities of *Sesamumindicum* L. Leaf Extract and Its Major Component, Pedaliin. *Foods*, *10*(6), 1216. https://doi.org/10.3390/foods10061216

Kuppusamy, P., Ichwan, S. J. A., Al-Zikri, P. N. H., Suriyah, W. H., Soundharrajan, I., Govindan, N., Maniam, G. P., & Yusoff, M. M. (2016). *In vitro* anticancer activity of Au, Ag nanoparticles synthesized using Commelinanudiflora L. aqueous extract against HCT-116 colon cancer cells. *Biological Trace Element Research*, *173*, 297-305. https://doi.org/10.1007/s12011-016-0666-7

Liu, Z., Liu, X., Li, W., Luo, Q., Liu, J., & Wang, D. (2021). Anti-colon cancer activity tracking isolation of peptide from ginseng leaves and potential mechanisms evaluation *in vitro* and *in vivo*. *Journal of Peptide Science*, *27*(4), e3297. https://doi.org/10.1002/psc.3297

Manosroi, A., Akazawa, H., Akihisa, T., Jantrawut, P., Kitdamrongtham, W., Manosroi, W., & Manosroi, J. (2015). *In vitro* anti-proliferative activity on colon cancer cell line (HT-29) of Thai medicinal plants selected from Thai/Lanna medicinal plant recipe database "MANOSROI III". *Journal of Ethnopharmacology*, *161*, 11-17. https://doi.org/10.1016/j.jep.2014.11.038

Nelson, S. S., Yadav, S. A., & Surendren, L. K. (2019). Evaluation of *in vitro* anticancer potential in Punica granatum, Psidium guajava, and Vitis vinifera seed extracts. *International Journal of Research in Pharmaceutical Sciences, 10*(1), 165-169.

Nelson, V. K., Sahoo, N. K., Sahu, M., Sudhan, H. H., Pullaiah, C. P., & Muralikrishna, K. S. (2020). *In vitro* anticancer activity of Ecliptaalba whole plant extract on colon cancer cell HCT-116. *BMC Complementary Medicine and Therapies*, *20*(1), 1-8. https://doi.org/10.1186/s12906-020-03118-9

Nithya, T. G., & Sumalatha, D. (2014). Evaluation of *in vitro* anti-oxidant and anticancer activity of Coriandrum sativum against human colon cancer HT-29 cell lines. *International Journal of Pharmacy and Pharmaceutical Sciences*, *6*(2), 421-424.

Nowak, A., Florkowska, K., Zielonka-Brzezicka, J., Duchnik, W., Muzykiewicz, A., & Klimowicz, A. (2021). The effects of extraction techniques on the antioxidant potential of extracts of different parts of milk thistle (*Silybum marianum* L.). *Acta Scientiarum Polonorum. Technologia Alimentaria*, *20*(1), 37–46. https://doi.org/10.17306/J.AFS.2021.0817

Packialakshmi, B., & Sowndriya, S. R. (2019). Anti-cancer effect of *Gymnema sylvestre* Leaf Extract against MG63, Human Osteosarcoma cell line-An *in vitro* analysis. *International Journal of Current Research and Review*, *11*(11), 18-24. https://doi.org/10.31782/IJCRR.2019.11114

Revathi, S., Hakkim, F. L., Kumar, N. R., Bakshi, H. A., Sangilimuthu, A. Y., Tambuwala, M. M., Changez, M., Nasef, M. M., Krishnan, M., & Kayalvizhi, N. (2019). *In vivo* anti cancer potential of pyrogallol in murine model of colon cancer. *Asian Pacific Journal of Cancer Prevention*, *20*(9), 2645. https://doi.org/10.31557/APJCP.2019.20.9.2645

Subramanian, R., Subbramaniyan, P., Ameen, J. N., & Raj. V. (2016). Double bypasses soxhlet apparatus for extraction of piperine from *Piper nigrum*. *Arabian Journal of Chemistry, 9*(S1), S537-S540. https://doi.org/10.1016/j.arabjc.2011.06.022

Thirunavukkarasu, K., Rajkumar, P., Selvaraj, S., & Kumaresan, S. (2016). GC-MS analysis of *Gymnema sylvestre* leaves methanolic extract for antidiabetic and anticancer drug identification. *Journal of Chemical and Pharmaceutical Sciences, 9*(2), 1011-1013.

Tiwari, P., Mishra, B. N., & Sangwan, N. S. (2014). Phytochemical and pharmacological properties of *Gymnema sylvestre*: an important medicinal plant. *BioMed Research International*. https://doi.org/10.1155/2014/830285

Venkatadri, B., Shanparvish, E., Rameshkumar, M. R., Arasu, M. V., Al-Dhabi, N. A., Ponnusamy, V. K., & Agastian, P. (2020). Green synthesis of silver nanoparticles using aqueous rhizome extract of *Zingiber officinale* and *Curcuma longa*: *In-vitro* anti-cancer potential on human colon carcinoma HT-29 cells. *Saudi Journal of Biological Sciences*, *27*(11), 2980-2986. https://doi.org/10.1016/j.sjbs.2020.09.021

Xu, L., Qi, Y., Lv, L., Xu, Y., Zheng, L., Yin, L., Liu, K., Han, X., Zhao, Y., & Peng, J. (2014). *In vitro* anti-proliferative effects of Zuojinwan on eight kinds of human cancer cell lines. *Cytotechnology*, *66*, 37-50. https://doi.org/10.1007/s10616-013-9534-x

Yadav, S. A., Faruck, L. H., Subramanium, R., Surendren, L. K., & Bakshi, H. (2021). Screening and assessment of molecular mechanistic actions of 5-hydroxy-1-methylpiperidin-2-one against free radicals, lung cancer cell line (A549), and binding properties on bovine serum albumin. *Future Journal of Pharmaceutical Sciences*, *7*, 129. https://doi.org/10.1186/s43094-021-00277-5

Yang, L., Liu, Y., Wang, M., Qian, Y., Dong, X., Gu, H., Wang, H., Guo, S., & Hisamitsu, T. (2016). Quercetin-induced apoptosis of HT-29 colon cancer cells via inhibition of the Akt-CSN6-Myc signaling axis. *Molecular Medicine Reports*, *14*(5), 4559-4566. https://doi.org/10.3892/mmr.2016.5818

Zheng, Y. M., Shen, J. Z., Wang, Y., Lu, A. X., & Ho, W. S. (2016). Anti-oxidant and anti-cancer activities of Angelica dahurica extract via induction of apoptosis in colon cancer cells. *Phytomedicine, 23*(11), 1267-1274. https://doi.org/10.1016/j.phymed.2015.11.008