Anti-Cancer Effects of Plant-based herbal Formulation on Vero And Human Colorectal Cancer Cell Lines.

**Abstract**

The present study aims to search for excellent antineoplastic agents avertable to human colorectal malignancies. In vitro assay for cytotoxicity activity (3‑[4,5‑dimethylthiazol‑2‑yl] ‑2,5‑diphenyltetrazolium bromide assay) (MTT Assay), DNA fragmentation was analysed by single gel electrophoresis and the activities of certain tumor specific marker enzymes such as acid phosphatase, aspartate aminotransferase, lactate dehydrogenase, alanine aminotransferase, alkaline phosphatase are also analyzed. Our results envisage that the non-cytotoxic nature of polyherbal formulation on Vero and human colorectal carcinoma [HT – 29] cell lines and the poly herbal formulation possess the strong anticancer activities on human colorectal carcinoma cell lines.The evaluation and the discovery of new anticancer agents is a long-term process that encompasses many steps. The step broaches with the screening for anticancer properties, followed by the isolation and identification of bioactive compounds obliged to anticancer properties, toxicity estimation of the isolated compounds, and finally in vivo anticancer activity testing to verify the aptitude of the compounds.

**Key words:** Anticancer agent, Non-coding RNA, Colorectal tumours, Heterozygosity, Microsatellite instability, Polyherbal formulation.

**Introduction**

Plant-based compounds manifest many beneficial effects and can inhibit several stages of human malignancies. Despite there is significant progress in cancer therapeutics in the past decades, the need to discover and develop new synergistic plant-based anticancer agents are in the emerging stage. In the present study, we attempted to exploit the anticancer efficacy of a polyherbal formulation on colon cancer, and Vero cell lines were evaluated. Colorectal cancer has emerged as a major challenge for mankind. For the treatment of this cancer, some drugs are available in the market and many are under investigation. Colorectal tumors arise from one or a combination of chromosomal instability or instability of microsatellites. The instability of genetic material is usually caused by aneuploidy and dysfunctions or loss of heterozygosity. Mutations in the tumor suppressor proteins or cell cycle genes may also lead to cellular transformation. Similarly, epigenetic and or genetic alterations resulting in impaired cellular pathways, such as mechanisms of DNA repair may lead to the instability of microsatellite and mutator phenotype. Non-coding RNAs, more importantly, micro RNAs and long non-coding RNAs have also been implicated in colorectal tumorigenesis. Understanding the specific mechanisms of tumorigenesis and the underlying genetic and epigenetic traits is critical in comprehending the disease phenotype. Tremendous possibilities are reviewed and collected from the herbal source for the successful management of colorectal cancer. Intensive research has been done worldwide on the plant source that increases possibilities for providing great opportunities to improve the management of colorectal cancer. Natural therapies, such as the use of plant-derived products in malignant tumor treatment, may reduce the adverse side effects. Currently, a few plant products are being used to treat various cancers. However, a myriad of plant products exist that have shown very promising anti-cancer efficacies in vitro, but have yet to be evaluated in humans. Further study is required to determine the effects of medicinal plant-based products in treating malignancies in humans [1].

The winnowing of natural products particularly plant extracts effect the breakthrough of a few excellent anticancer agents [2]. Cancer remains a worldwide health problem, thus endeavouring the search for a new alternate approach. In defiance of astonishing advances in modern medicine, such as surgery, radiotherapy, chemotherapy, and hormone therapy [3].. The genus *Hugonia L.* of family *Linaceae* comprise about 40 species in the world; of which *Hugonia mystax L*. was reported from India. This plant *Hugonia mystax* is locally known as Modirakanni. Ethnobotanically, the fruits are used by the tribals of Kalakad- Mundanthurai for the treatment of Rheumatism [4]. These medicinal plant roots were used as anthelmintic, and astringent and also used for dysentery, snake bite, fever, rheumatism, and inflammations. Biological activities such as analgesic, anti-inflammatory, and ulcerogenic were reported [5]. *Wedelia* is an extensive genus of the family *Asteraceae*, comprising about 60 different species. *Wedelia trilobata* Linn. has long been used as traditional herbal medicine in South America, China, Japan, India and for the treatment of a variety of ailments. The aerial parts of this plant are used in traditional medicine in the Caribbean and Central America against bronchitis, colds, abdominal pains, dysmenorrhea, and even as a fertility enhancer [6]. In folk medicine, it is employed to treat backache, muscle cramps, rheumatism, stubborn wounds, sores and swellings, and arthritic painful joints [7]. The Miskito Indians of eastern Nicaragua use leaves for treatment of kidney dysfunction, colds, stingray wounds, snakebite, purge, and amenorrhea [8].The fruits, leaves, and stems are used in childbirth and in the treatment of bites and stings, fever, and infection [9]. In Trinidad and Tobago, used for reproductive problems, amenorrhea, and dysmenorrhea and it is used for the treatment of fever and malaria in Vietnam [10]. *Cassia alata Linn*. belongs to the family of *Caesalpiniaceae* and is a large shrub with thick downy branches, found wild almost throughout India. Leaflets are 8-12 pairs, lower leaflets oblong-elliptic; upper ones broadly obovate. It is known as Ringworm shrub and winged senna in English; Dadrughna and Dvipagsti in Sanskrit; semaiagathi and Vandugolli in Tamil [11]. Therefore, it is of interest to investigate the anti-cancer efficacy of chloroform extract of polyherbal formulation on Vero and HT-29 cell lines. So the present study aims to investigate the antitumor role of a polyherbal formulation on HT-29 cell lines induced human colorectal malignancy

**Materials and methods**

**Collection of samples**

The novel polyherbal formulation is used for this study. The herbal formulation was prepared by the available literature.

**Preparation of Herbal medicine**

The herbal formulation was prepared in the Department of Industrial Biotechnology, Bharath Institute of Higher Education and Research, Bharath University, Chennai, India. The equal volume of shade dried leaves of *Hugonia syntax*, W*edelia trilobata,* and *Cassia alata* were taken into mortar and pestle. The plant material was coarsely powdered, then filtered by muslin cloth, and the filtrate was used for further extraction.

**Preparation of extracts**

1000 grams of polyherbal powder was packed in the round bottom flask for sample extraction using solvent namely Chloroform. The extraction was conducted by 250 ml of the solvent mixture for a period of 24 hours. At the end of the extraction the respective solvents were concentrated under reduced pressure and kept in a water bath (at 50°c). Now the extracted experimental solutions were stored in the refrigerator.

**In vitro assay for cytotoxicity activity (3‑[4,5‑dimethylthiazol‑2‑yl] ‑2,5‑diphenyltetrazolium bromide assay) (MTT Assay).**

The anticancer activity of samples on HT- 29 and Vero cell lines was determined by the MTT assay. Cells (1 × 105/well) were plated in 0.2 ml of medium/well in 96‑well plates. Incubate at 5% CO2 incubator for 72 h. Then, add various concentrations of the samples in 0.1% DMSO for 24 h at 5% CO2 incubator. After removal of the sample solution and washing with phosphate‑buffered saline (pH 7.4), 20 μl/well (5 mg/ml) of 0.5%, MTT in phosphate‑buffered saline solution was added. After 4 h incubation, 1 ml of DMSO was added. Viable cells were determined by the absorbance at 540 nm. Measurements were performed, and the concentration required for a 50% inhibition of viability (IC50) was determined graphically. The effect of the samples on the proliferation of HT- 29 and Vero cells was expressed as the % cell viability, using the following formula: Calculation: % cell viability= A540 of treated cells / A540 of control cells × 100% [12].

**DNA fragmentation Analysis**

**Isolation of DNA**

Two millilitres of cells were taken and centrifuged at 3000 rpm for 5 min. The obtained pellet must be suspended in 200 μL of 1X Tris‑EDTA (TE) Buffer and 100 μL of 10% sodium dodecyl sulfate and mixed well. Then the tube is incubated at 50°C for 20 min. 300 μL of phenol: Chloroform: Isoamyl alcohol (25:24:1) w added in the incubated tube and centrifuge at 10,000 rpm for 10 min. The supernatant was transferred to a new 1.5 mL Eppendorf tube, and 1000 μL of isopropanol was added and mixed by inverting the tube (4–5 times), then centrifuged at 1000 rpm for 10 min. The supernatant was discarded, and 500 μL of 70% ethanol was added and centrifuged at 10,000 rpm for 10 min and the supernatant was discarded. Now, air‑dry the pellet till there are no traces of ethanol and resuspend the pellet in 20 μL of 1X TE Buffer [12].

**Agarose gel electrophoresis**

The extracted DNA was carried out with agarose gel electrophoresis in a horizontal submarine electrophoresis unit [13]. The casting was done with the 1.2% agarose gel and 0.72 g of Agarose in 60 mL of diluted 1X Tris‑borate‑EDTA (TBE) buffer (do not mix), which were dissolved by heating the content to get up to a clear solution. The solution was allowed to cool at room temperature, and then 5 μL of ethidium bromide added, mixed and poured the agarose into the casting system with combs. The gel was allowed to solidify, and then carefully disassemble the casting system without disturbing the wells. The gel is transferred to 1X TBE buffer filled electrophoresis tank. 5 μL of gel loading dye added to 20 μL of sample DNA, mixed well, and then the total 25 μl of sample loaded to gel. 10 μl of 1 kb DNA marker added near to the well. The power card terminals were connected at respective positions to run the gel. The unit was switched off after the gel loading dye migrated more than half of the length of gel the unit; the separated DNA bands visualized under ultraviolet transilluminator.

**Visualization of propidium iodide‑stained cells**

To identify those cells undergoing apoptosis, the cells were cytospined and mounted on the slides. Changes in cell morphology were examined under a differential microscope and a fluorescence microscope [14]. Cells were seeded in 12‑well plates at seeding densities of 5 × 105 cells/well and then treated with ginger extract at the specified concentration for 24 h. Then, the cells were washed with phosphate‑buffered saline (PBS); after washing once with PBS, the cells were stained with 100 μl of a propidium iodide (4 μg/ml). The cells were immediately washed with PBS, cytospined and mounted on the slides. Changes in cell morphology were examined to identify those cells undergoing apoptosis under Nikon‑inverted fluorescent microscope (TE‑Eclipse 300, Nikon, Tokyo, Japan) attached to the camera .

**Estimation of Tumour Specific Marker Enzymes**

**Lactate Dehydrogenase (LDH) (E.C.1.1.1.27)**

LDH enzyme activity was measured in the culture media using a diagnostic LDH-L kit according to the instructions provided by the manufacturer. In brief, the supernatant of each well was collected after treatment. A substrate provided by the kit was then incubated at 37°C for 5 min, followed by the addition of NAD to the mixture. Spectrophotometric absorbance at 340 nm was registered for 2 min. Finally, LDH concentration measured in units/liter (U/l) was obtained by the equation [LDH]=(ΔA/min) × factor, where ΔA indicates absorbance difference between second 120 and 1, and factor represents εNAD/NADH= 6230 M-1 cm-1. [15].

**Alkaline Phosphatase (EC: 3.1.3.1).**

A total of 1.0 or 1.5 × 106 cells were incubated with 10 ml RPMI-1640 medium with 25 mM HEPES and 5% fetal calf serum. The medium was changed after 24 h and unless stated otherwise the cells were incubated for 72 h before harvesting. The cells were washed with phosphate-buffered saline and extracted with 0.5% NP40, 0.25 M NaCl, 5 mM EDTA and 50 mM Tris pH 8.0. The protein concentration of the extract was determined using the BCA Protein Assay Reagent from Pierce, Rockford, IL, USA. Alkaline phosphatase was assayed at 37°C as previously described using para-nitrophenyl phosphate as substrate. Formation of the product was monitored by the change in absorbance at 410 nm [16].

**Alanine amino transferase (EC: 2.6.1.2), Aspartate amino transferase (EC:2.6.1.1) and Acid phosphatise (EC:3.1.3.2)**

The hepatic enzymes AST, ALT and ACP were used as the biochemical markers for human malignancies. The serum marker enzyme activities were determined by using a commercial diagnostic kit Biocompare, BioVision, Thermo scientific and Bio scientific Max discovery (USA).

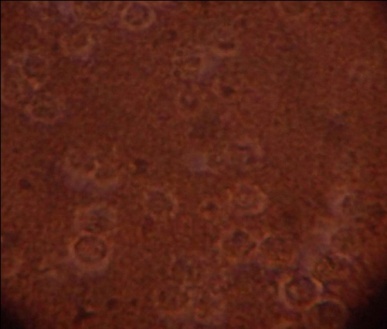
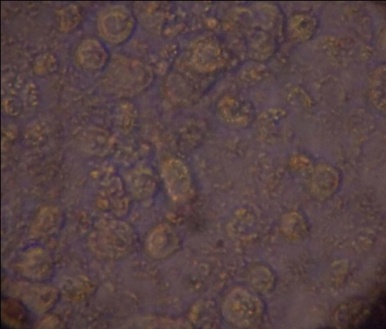
**Results and Discussion**

Figure 1: The Percentage of cell viability of a poly herbal formulation on *vero* cell lines.

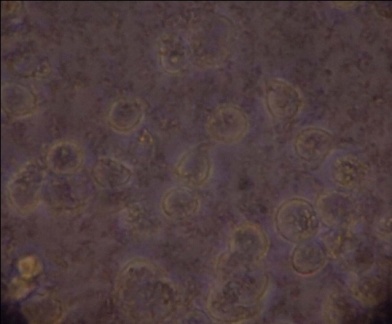
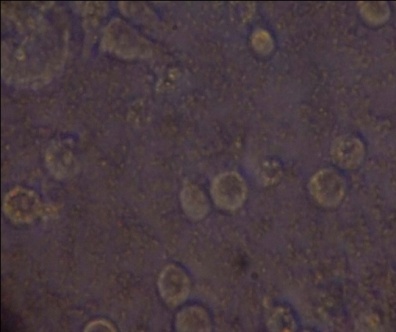
Figure 2: Percentage of cell viability of a polyherbal formulation on HT-29 cell lines.

Figure 3: The invitro cytotoxicity effects of a polyherbal formulation on vero cell lines.

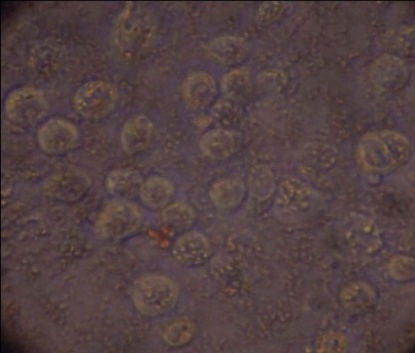
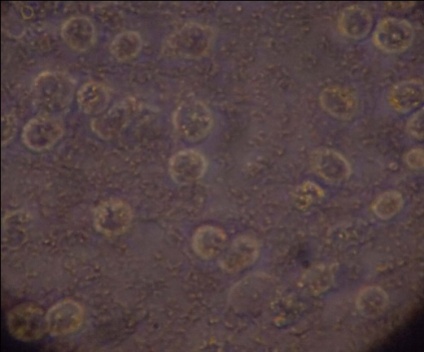
500 µg 250 µg

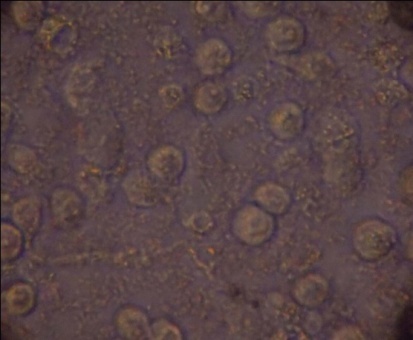
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125 µg 62.50 µg

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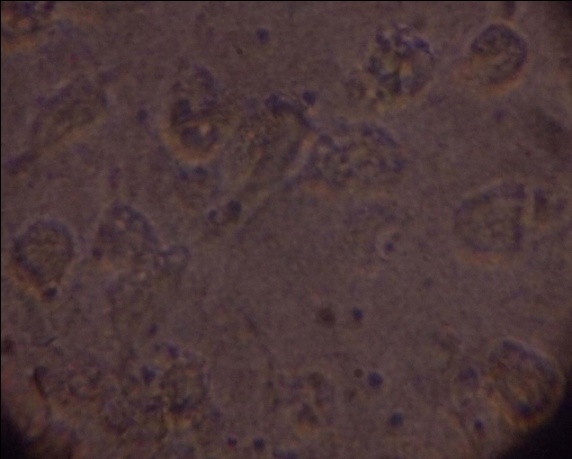
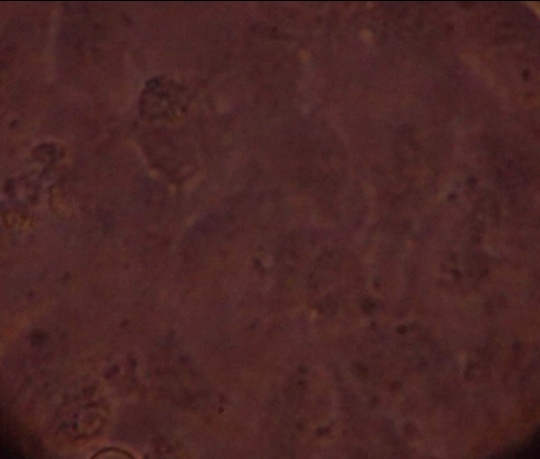
31.25µg 16.62µg

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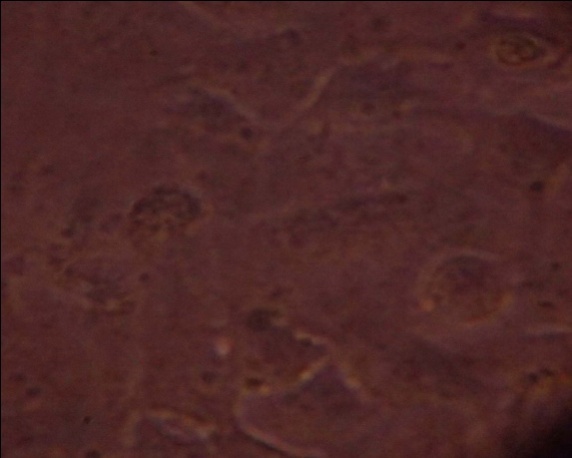
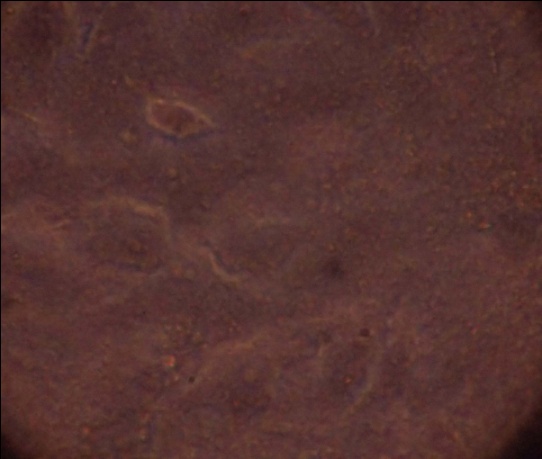
** HT-29 Control cell line**

**Figure 4: The invitro cytotoxicity effects of a polyherbal formulation on HT-29 cell lines.**

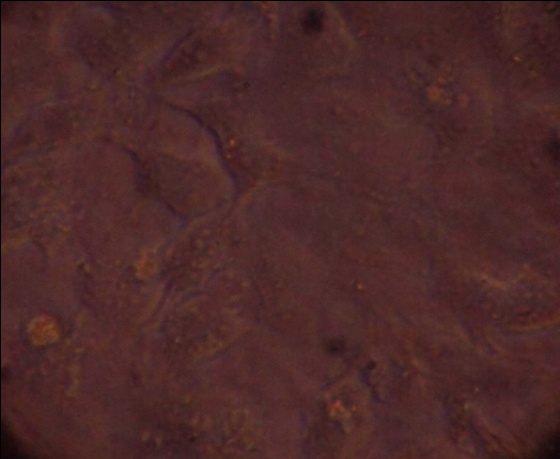
500 µg 250 µg

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125 µg 62.50 µg

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31.25 µg 16.62 µg

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**Vero control cells**

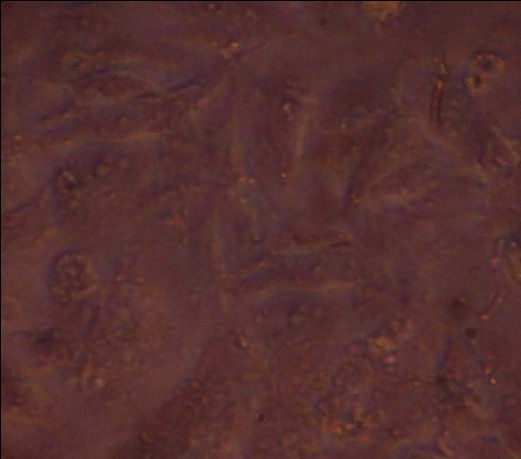
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Figure 5 : Alkaline phosphatase activity of poly herbal formulation on control cell lines.

Figure 6 : Alkaline phosphatase activity of poly herbal formulation on colon cancer cell lines.

Figure 7 : Lactate dehydrogenase activity of herbal formulation on control cell lines

Figure 8 : Lactate dehydrogenase activity of herbal formulation on colon cancer cell lines.

Figure 9: Acid phosphatase activity of a poly herbal formulation on *Vero* cell lines.

Figure 10: ACP Activity of poly herbal formulation on colon cancer cell lines.

Figure 11: ALT activity of herbal formulation on control cell lines.

figure 12: ALT activity of herbal formulation on colon cancer cell lines.

Figure 13: Aspartate amino transferase activity of herbal formulation on control cell lines.

Figure 14: AST activity of polyherbal formulation of colorectal cancer cell lines.

Figure 15: DNA Fragmentation analysis of herbal formulation on Vero cell lines

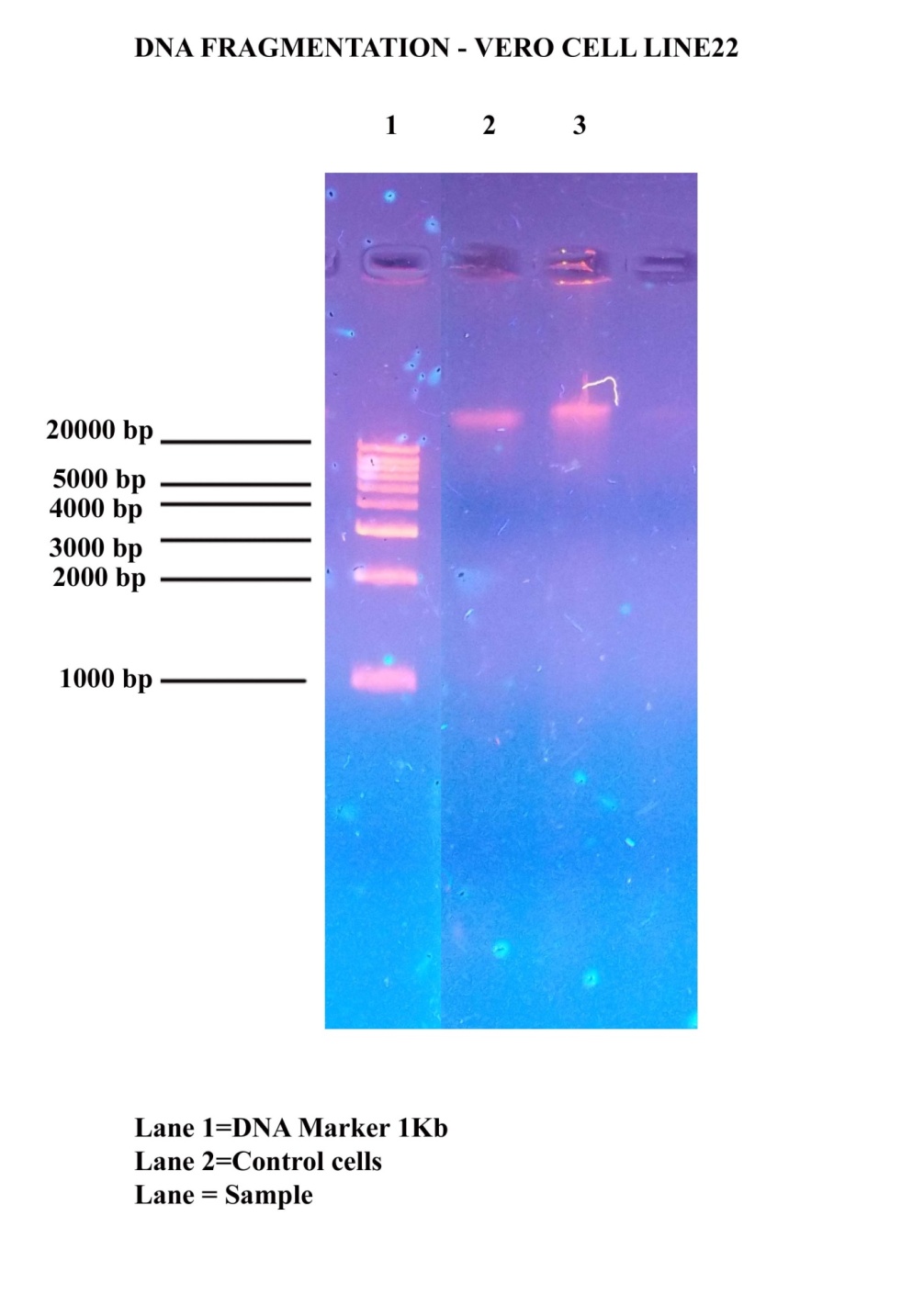
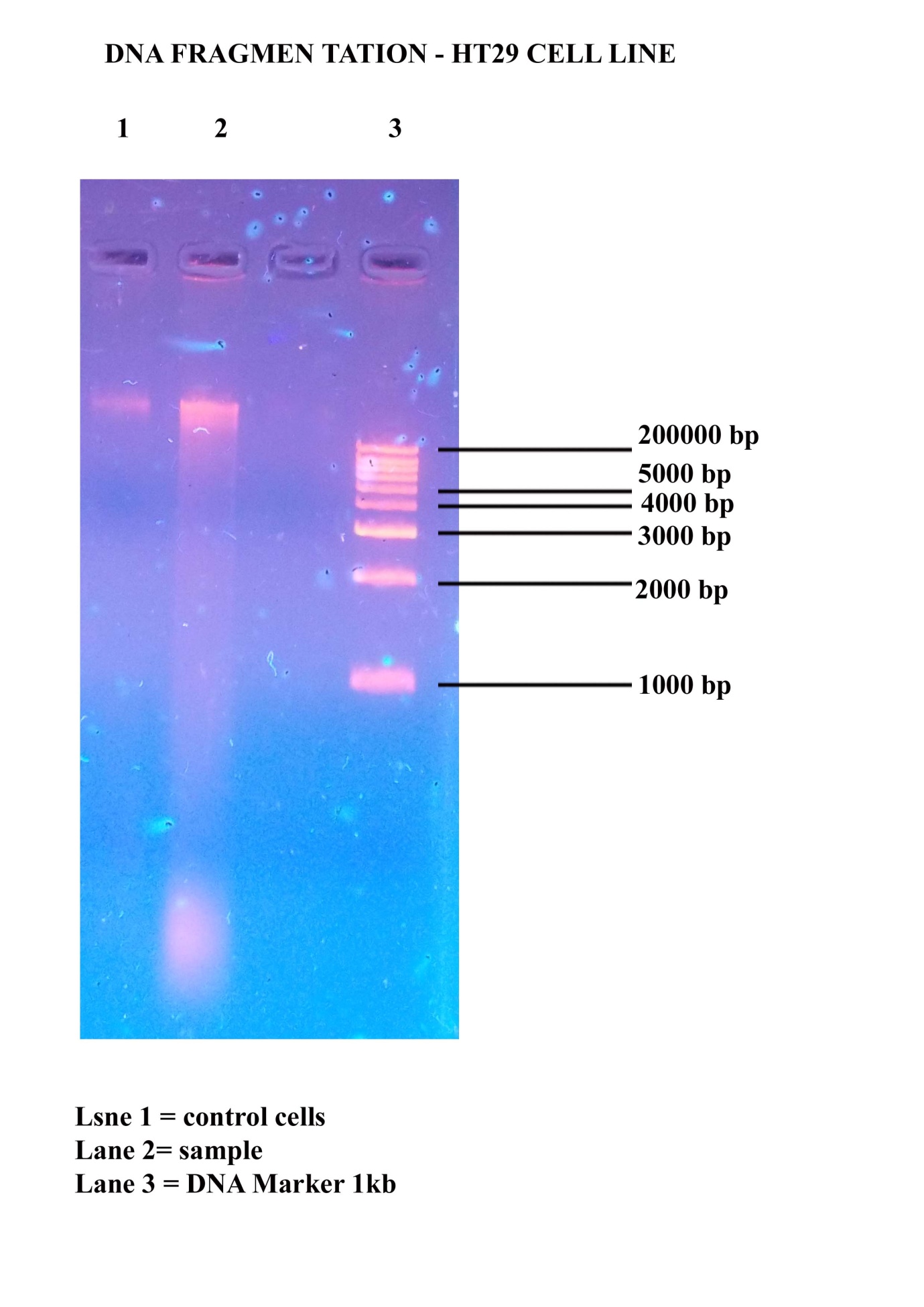


Figure 16: DNA Fragmentation analysis of herbal formulation on HT-29 Cell lines.



Medicinal plants continue to provide humanity with new remedies. It is therefore important to explore medicinal plants for their safety, quality, toxicity, appropriate amount of plant materials to use, and efficacy. Natural products of plants possess several biological activities including antioxidant and anti-inflammatory activity. Medicinal plants are rich in active phytochemical compounds with various biological activities. Researchers are highly interested in studying plants with the aim of isolating novel active drugs to replace synthetic drugs present in the market. The availability of these plants constituents provides a source of natural drugs for modern medicine. Hence, cytotoxic level of medicinal plants must also be evaluated against host cells. The safety of plants as a potential therapeutically agents must be ascertained and the side effects should be acceptable to the host. Bioactive compounds with no or less toxic effect to the host are the good candidates for formulation of drugs [17].

Figure 1 and 2 shows that the percentage viability of a polyherbal formulation on Vero and human colorectal carcinoma cell lines. Cytotoxicity analysis by MTT assay can be used for the measurement of cell proliferation in response to growth factors, cytokines, mitogens, and nutrients and analysis of cytotoxic and cytostatic compounds, such as anticancer drugs and other pharmaceutical compounds. A major concern about bioactive compounds from plants is that some of these compounds are toxic to our normal system; therefore, safety is critical in the development of novel drugs. In this study cytotoxic effect of chloroform extract of a poly herbal formulation at concentrations ranging from 100–500 μg/mL was investigated on Vero and human colon cancer cell lines were shown in figures 3 and 4. The effect of poly herbal formulation of chloroform extract on Vero and HT-29 cell line was evaluated after 24 and 48 hours of cell exposure to the extracts. Percentage cell viability was calculated by measuring the absorbance of pink color formation formed from the reduction of MTT solution by the presence of mitochondrial dehydrogenase in viable cells. The polyherbal extract was toxic to both cell lines at the higher concentration of 500 μg/mL. Morphology of cells was also altered from its normal shape of fibroblast to oval shape, because of the toxic effect of extract to the cells. However, at the lower concentrations (100 μg/mL), chloroform extract had more than 90% cell viability after 24 and 48 hours of exposure [18].

Identifying the mechanism of plant-derived anticancer agents provides helpful information in cancer therapy. Natural products have been used for the treatment of various diseases for centuries [19]. Evidence has shown that active principle compounds from plants may serve as potent chemotherapeutic agents with less toxicity to normal tissues and at low cost. They are associated with the induction of apoptosis, cell cycle arrest, and inhibition of various signal transducers, and signaling pathways. Thus, it is important to screen the crude extract of plants or isolated compounds for their apoptotic potentials. The use of alternative medicine is increasing and many pharmaceutical industries are interested in developing plant-derived medicinal compounds. The increasing cost of conventional treatments and the lack of effective drugs encouraged people to depend more on folk medicine [20].

Alkaline phosphatise activity of chloroform extract of a polyherbal formulation were shown in figure 5 and 6.ALP is an enzyme commonly expressed in almost all living organisms. In humans and other mammals, determinations of the expression and activity of alkaline phosphatase have frequently been used for cell determination in developmental studies and or within clinical trials. Alkaline phosphatase also seems to be one of the key markers in the identification of pluripotent embryonic stem as well as related cells. However, alkaline phosphatases exist in some isoenzymes and isoforms, which have tissue-specific expressions and functions [21].

Figure 7 and 8 shows that the effect of poly-herbal formulation on lactate dehydrogenase activity on Vero and HT-29 cell lines. LDH is a product regulates the anaerobic transformation of pyruvate to lactate for energy acquisition. The significance of serum elevation in LDH as a predictive indicator for the malignant potential and prognosis in colorectal cancer. Lactate dehydrogenase plays an important role in this process by mediating the conversion of pyruvate and lactate, and this enzyme is an emerging anticancer target. In addition, elevated lactate dehydrogenase levels are consistently reported as a prognostic factor for poor survival among several cancer groups [22]. Lactate dehydrogenase is a cytoplasmic enzyme with a wide distribution in tissue where it catalyzes the inter conversion of lactate to pyruvate. Functional LDH are homo- or hetero-tetramers composed of M and H protein subunits encoded by the LDHA and LDHB genes, respectively. Five isoenzymes are derived from the different monomeric compositions (LDH 1 to 5) and differ from each other in terms of their structural composition, biochemical properties and tissue distribution. LDH is involved in tumor initiation and metabolism. The LDHA gene is a transcriptional target of HIF1α and is induced in hypoxic conditions or when oncogenes activate HIF1α [23]. The figure 9 and 10 show that the activity of acid phosphatise on polyherbal formulation of vero and colorectal carcinoma cell lines. ACP is a ubiquitous lysosomal enzyme that hydrolyses organic phosphates at an acid pH. Although the post-pubertal prostatic epithelial cell contains a uniquely high concentration of acid phosphatase, cellular components of bone, spleen, kidney, liver, intestine, and blood also contain this enzyme. However, unequivocal documentation of the validity of these statements is not available. Newer immunologic techniques for measuring acid phosphatase may significantly alter our current concept of its role as a tumor marker [24].

Alanine aminotransferases are shown (figure 11 and 12) in the activity of polyherbal formulation on HT-29 cell lines as well as the control Vero cell lines. Figure 13 and 14 were shown the activity of chloroform extract of a polyherbal formulation on the Vero and colorectal tumor cell lines. These enzymes are the major critical enzymes in biological processes. The synthesized ALT and stored AST changes in serum levels have become diagnostic tools and markers for assessing liver function. Reports have suggested that their levels increase in different hepatic injuries, such as hepatitis and cirrhosis induced by alcohol, drugs, viruses, and also under oxidative stress. The liver could easily be exposed to internal stimuli which produce reactive oxygen species. The oxidative stress could damage the liver cells. The levels of ALT and AST in the serum have been generally accepted as a better predictor of liver injury. Oxidative stress and inflammation are related to the development of many human cancers at the same time, oxidative stress and inflammation could also lead to damaged liver cells. With the development of tumor biology, there is growing evidence that the presence of a systemic inflammatory response is linked to poor survival in patients with different types of malignancies [25, 26].

A DNA fragmentation assay was used to determine whether the action of chloroform extract of a poly herbal formulation was associated with apoptosis or not. As shown in (Figure15 and 16) Poly herbal formulation was strongly effective on control and experimental cell types. In this experiment, all tested cell lines were incubated with Poly herbal formulation at the IC50 concentration. DNA fragmentation was found on Vero cells and HT-29 cells at 2, 3, 4 and 7 days after exposure. In the HT-29 cells, the ladder pattern was observed for a maximum of 3 days because all cells died. Therefore, the effect of Polyherbal formulation was selective for colon cancer cell lines and mediated through the induction of apoptosis. Medicinal plant-based drug discovery continues to provide new and important leads against various pharmacological targets including cancer [27]. The DNA fragmentation forms a ladder pattern that can be used to distinguish between apoptosis and necrosis. This phenomenon can be generally detected by agarose gel electrophoresis. The DNA was isolated from the treated cells and subjected to agarose gel electrophoresis and examinations revealed a ladder formation, which is characteristic of apoptosis [28]. Here our results showed that the DNA ladders of HT-29 and Vero cells treated with Poly herbal formulation were observed within seven days. Therefore, DNA ladder formation indicated that the cytotoxic effect of Poly herbal formulation caused inhibition in the growth of colon cancer cells and normal Vero cells through apoptosis. Poly herbal formulation also inhibited growth in normal colon cells, but not to the point of death through apoptosis. In the Vero cells, Poly herbal formulation inhibited cell growth within six days and induced cell death on Day 7 after incubation. Although treated Vero cells showed DNA ladders at Day 7, the incubation time was longer and the dose was higher than in the colon cancer cell lines. Therefore, our Poly herbal formulation seemed to be safe for normal cells. However, further experiments are needed to evaluate the specific molecules in the apoptotic pathway [29].

**Conclusion**

The current mode of treatment for various diseases including human malignancies is based on synthetic drugs. These drugs are effective but they show serious adverse effects and also alter the genetic and metabolic activity of the patient. More some drugs prepared from medicinal plants and their constituents show more efficacy than the synthetic counterparts. Earlier reports have shown that the regular consumption of herbs, fruits, and vegetables is strongly related to reduced risk of various forms of diseases. Thus, it is of interest to investigate the anti-neoplastic role of chloroform extract in herbal formulation were undertaken. Our results indicate the efficacy of the plant-based drug on HT – 29 human colorectal carcinoma cell lines.

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