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Screening of bioactives, anti-oxidant and anti-cancer potential of a herbal formulation

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Abstract
Medicinal plants have a role in cancer management. The purpose of this study was to develop a herbal adjunct for symptom management in cancer treatment and evaluate its *in vitro* antioxidant and anti-cancer activity. Locally available and traditionally used medicinal plants with known anti-cancer properties were selected for development of the formulation. Two variations of the formulation viz., Raw herbal formulation (RHF) and Heat treated formulation (HTF) were prepared. The activity of the variations was compared with Triphala (TRF). The 80% methanol extract of all the samples exhibited higher antioxidant activity and was chosen for screening the anti-cancer ability. The results of GC-MS showed that bioactives having potential anti-cancer effect were identified in HTF with lower probability. However, bioactive components with anti-oxidant, anti-cancer, anti-tumor and cyto-toxic activity were higher in RHF. The morphological studies confirmed apoptotic effect of RHF. Overall, RHF exhibited higher *in vitro* antioxidant, anti-cancer and cytotoxicity effect.

Keywords: Herbal formulation, Nutraceuticals, HT-29 cell line, cancer, antioxidants, GC-MS, morphology

1. Introduction
Cancer is a complex disease caused due to multiple genetic changes leading to uncontrolled proliferation of cells of metastatic ability [1]. Cancer occurs due to both external and internal factors. External factors include tobacco chewing/smoking, infectious organisms, unhealthy diet and lifestyle; whereas internal factors include inherited genetic mutations, hormones and immune conditions. These factors may act together or in sequence to cause cancer [2]. Studies by Ferlay *et al.* 2015 [3] reported that, the three most commonly diagnosed cancers in the developed countries were prostate, lungs and colorectum among males; and breast, colorectum and lungs among females. In the developing countries, commonly diagnosed cancers were lungs, liver and stomach cancer in males and breast, cervix, uterus and lung cancer in females. By 2030, the global burden of cancer is expected to grow to 21.7 million new cancer cases and 13 million cancer deaths due to growth and ageing of the population [2]. Apart from the conventional therapies such as chemotherapy and radiotherapy, researchers are now focusing on novel plant based products with anti-cancer properties [4]. Several epidemiological studies have indicated protective effect of vegetables and fruits against cancer. In recent years, isolation, identification, characterization, quantification of phytochemicals and evaluation of their potential benefits to humans has become an important area of pharmaceutical sciences. Approximately, 30 classes of phytochemicals have been isolated and listed for their anticancer potential [4].
The nutritional status of a cancer patient may be affected by the tumor and cancer treatment. Chemotherapy or radiation therapy directed against the tumor is not confined exclusively to malignant cells; thus, normal tissues may also be affected by therapy and contribute to specific nutritional problems like nausea, vomiting, diarrhea etc. When combined modality treatment is given, the nutritional consequences may be magnified [5]. Nutrition Impact Symptoms (NIS) such as taste and smell alterations, mucositis, nausea, constipation, pain and shortness of breath seem to occur frequently in patients undergoing cancer treatment [6]. Cancer patients burdened with drug induced toxicity are benefitting from the complementary and alternative medicines in a hope to find a better cancer management [7].
Alternatives of anticancer molecules of plant based origin have gained huge attention, which is cost effective and safe in use due to its natural origin. Aggarwal *et al.* 2006 reported the *in vitro* and *in vivo* anti-cancer ability of cumin and fennel seed via inhibitory effect on

Nuclear Factor-kB (NF-kB) [8]. Savita Dixit *et al.* 2010 reported that clove possesses cytotoxic effect on cancer cells, garlic pods have shown to reduce 50% colon cancer risk in post-menopausal women and neem leaves have shown immunomodulatory, anti-inflammatory, anti-carcinogenic and potential antioxidant activity which can be used in colon, stomach, lung, liver, skin, oral, prostate and breast cancer treatment [9]. Shukla S and Mehta A, 2015 reviewed and reported various plant based anti-cancer agents with a potential to treat and manage cancer than the conventional therapies which induce prolonged toxicity [10]. Among the various plants based formulations used commercially, Triphala (TRF), a herbal powder made from three primary fruits namely, Indian gooseberry (*Phyllanthus emblica*), dried fruit of *Terminalia chebula* tree and Vibhitaki (*Terminalia bellirica*) is reported to exhibit high antioxidant and colon cleansing effect. When the cytotoxic effects of aqueous extract of TRF was studied in human breast cancer cell lines (MCF-7), the treated cells were found to be decreasing with increasing concentrations of TRF [11]. TRF was used as reference standard in this study since HT-29 colon cancer cell line was chosen for *in vitro* anti-cancer studies. With this background, the study was conducted with an aim of developing a herbal drink to serve as an adjunct for cancer management and screening its potential as antioxidant and anticancer agent.

2. Materials and methods

2.1 Screening of ingredients

Considering the availability of ingredients for the development of the herbal drink, locally available and traditional used ingredients were selected and screened for various biological effects documented in literature. Ingredients such as *Garcinia indica* (Kokum), *Tinospora cordifolia* (Amritaballi), *Curcuma amada* (Mango Ginger), *Ocimum tenuiflorum* (Krishna Tulsi), *Mentha piperita* (Mint), *Zanthoxylum rhetsa* (Sichuan peppers), Cane jaggery, *Borassus flabellifer* (Palm jaggery) and Honey were selected for the study and TRF was used as standard.

2.2 Preparation of samples

Leaves of *Tinospora cordifolia*, *Ocimum tenuiflorum* and *Mentha piperita* were procured from the local market, sorted and washed. Excess water was removed using paper towels. Mango ginger was washed thoroughly, the outer skin was peeled and the root was cut into small pieces.

2.2.1 Preparation of Kokum extract: 400g of kokum rind was mixed with 2L of water, boiled for 1 hour and strained using a filter. One liter of the filtrate was obtained at the end of 1 hour and stored at refrigerated temperature until further use.

2.2.2 Preparation of Sichuan pepper extract: 13g of Sichuan pepper was weighed and boiled with 350ml of water for half an hour. The pepper pods were re-extracted in 300ml boiling water until the water reduced to half of its initial amount. Total of 250ml of the extract was obtained and stored at refrigeration temperature until further use.

2.3 Development and standardization of herbal formulation

Since the study aimed at developing a herbal adjunct for cancer therapy, a formulation was developed aiming to be used as a herbal drink for cancer patients, using the above mentioned ingredients at different concentrations. All the ingredients except kokum and sichaun pepper extracts were prepared on fresh basis with the necessary preliminary processing, ground to a paste without filtration and freeze dried (-40°C) to remove moisture and improve the storage stability. To study the effect of heat treatment on the phytochemical composition, the formulation was subjected to heat treatment (100°C) (HTF) before freeze drying. The composition of both the formulations remained the same except for the processing i.e., heat treatment. The freeze dried powder of the herbal formulations was used for further analyses.

2.4 Preparation of Extracts

The dehydrated powders of the standard (TRF) and herbal formulations (RHF and HTF) were extracted using four solvents viz., aqueous, ethanol, methanol and 80% methanol in the ratio 1:10 of sample and solvent respectively and extracted for 24h using a mechanical shaker. The aqueous extract was freeze dried and solvent extracts were prepared by flash evaporation. The dried extracts were stored in deep freezer until further use.

2.5 Chemicals: All the chemicals used were of analytical grade. Protease, amyloglucosidase, Glucose standards, ß-carotene standard, 2, 2,-diphenyl-1-picrylhydrazyl (DPPH), Dimethyl Sulfoxide (DMSO), Roswell Park Memorial Institute Medium (RPMI), Fetal Bovine Serum (FBS), Penicillin, Streptomycin, Trypsin, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), L-glutamine penicillin streptomycin solution, Triton X-100, Phosphate Buffered Saline (PBS), Colchicine were purchased from Sigma-Aldrich, USA. Propidium iodide (PI), 0.25% Trypsin-EDTA solution, RNase solution (20mg/ml), Dulbecco's Modified Eagle Medium (DMEM) and Fetal Bovine Serum (FBS) were purchased from Himedia chemicals, India.

2.6 Anti-oxidant Assay

Radical Scavenging Assay (DPPH)

The radical scavenging potential of the herbal formulation was analyzed using DPPH method described by E. J. Gracia *et al*, 2012 [12] and absorbance was read at 517nm. IC_{50} value was determined using Graph Pad Prism.

Reducing Power Assay (RPA)

Various concentrations of the plant extracts in corresponding solvents were mixed with phosphate buffer (2.5ml) and potassium ferricyanide (2.5ml). This mixture was kept at 50°C in water bath for 20 minutes. After cooling, 2.5ml of 10% trichloroacetic acid was added and centrifuged at 3000 rpm for 10 minutes. The upper layer of solution (2.5ml) was mixed with distilled water (2.5ml) and freshly prepared ferric chloride solution (0.5ml) was added. The absorbance was measured at 700nm against reagent blank. Increase in absorbance of the reaction mixture indicates increase in reducing power [13].

2.7 Screening of Bioactive Compounds using Gas Chromatography - Mass Spectrometry (GC-MS) analysis

In GC-MS analysis, chromatographic separation was carried out using the equipment, Thermo GC- Trace Ultra Version: 5.0, Thermo MS DSQ II with Db 35 – MS Capillary

Standard Non – Polar Column and with a dimension of 30 meters, ID: 0.25mm, film: 0.25µm. Helium was used as carrier gas with a flow rate of 1.0ml/minute. The oven temperature was programmed for 70°C and raised to 260°C at a rate of 6°C per minute. About 1µl of the extract was injected into the instrument. Interpretation on mass spectrum of GC-MS was conducted using the database of National Institute Standard and Technology (NIST). The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The name, molecular weight and molecular formula of the components of the test materials were ascertained. The compounds were identified using their retention time, molecular formula, molecular weight and identified using the library.

2.8 Cell lines and Culture

Colon cancer cells (HT-29) were obtained from National Centre for Cell Science, Pune, India. The cells were maintained in the Dulbecco's Modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 1 mM of sodium bicarbonate, L-glutamine (200 mM), streptomycin (10 mg/mL), Glucose (25 mM) and penicillin (10,000 units) (DMEM complete media) at 37 °C in a humidified 5% CO_2 atmosphere. The culture medium was replaced twice in a week. For the experiments, confluent cells were trypsinized and plated in 6-well, 12-well and 96-well plates. All cell culture operations were carried out in a model New Brunswick Galaxy 48 R CO_2 incubator from Eppendorf.

2.9 MTT Assay

The HT-29 cell lines were seeded at a density of 5×10^4 (100 μL/well) in 96-well plates and incubated at 37°C in a humidified 5% CO_2 atmosphere for 24h to form a cell monolayer. After 24h, the growth medium on the monolayer was aspirated and treated with 100 μL of 80% methanol extract of TRF, RHF, HTF at various concentrations (0, 500, 1000 and 2000 μg/mL) and Colchicine (320 μg/mL). After 24h treatment, cytotoxicity was tested by MTT [10 μL/well containing 100 μL of cell suspension; 5 mg/mL of stock in Phosphate Buffer Saline (PBS)] solution and the plates were incubated at 37°C for 4h in a 5% CO_2 atmosphere. The supernatants were aspirated from the wells and washed thrice with PBS. 100 μL of DMSO was added to each well and incubated for 15 min. After incubation, the plates were gently shaken to solubilize the formazan crystals and absorbance was measured at 590 nm using multimode plate reader (Varioskan Flash Top, Thermo Fisher Scientific, Finl and). The percentage of inhibition (%) was calculated using the formula below and IC_{50} values were calculated from log dose-response curves using Graph Pad Prism software version 6 for Windows (Graph Pad Software, USA).

Percentage of inhibition (%) =

$$100 - \frac{\text{Test Absorbance at 590nm}}{\text{Untreated Control Absorbance at 590nm}} \times 100$$

2.10 Cell Cycle Arrest

The HT-29 cell lines were seeded at a density of 5×10^5 (3 mL/well) in 6-well plates and incubated at 37°C in a humidified 5% CO_2 atmosphere for 24h to form a cell monolayer. After 24h, the growth medium was aspirated and treated with 80% methanol extract of RHF (1000 μg/mL) and Colchicine (320 μg/mL) for 24h. After treatment, the cells were washed, trypsinized and centrifuged at 1800 rpm for 8 min. After centrifugation, the supernatant was discarded and the cell pellet was washed twice with PBS. Further, the cells were re-suspended in PBS (300 μL) and fixed with 100% ethanol (700 μL) at -20°C for 1 hr. After fixing, the cells were washed with cold PBS and centrifuged at 4000 rpm for 10 min at 4°C. The cells were re-suspended in 1 mL of PBS containing PI (0.05 mg/mL), RNase A (0.05 mg/mL) and Triton X-100 (0.1%), and incubated for 30 min in the dark at room temperature. Finally, the cells were sorted in a flow cytometer (Cell Lab Quanta™, SC, Beckman Coulter, USA).

2.11 Morphological study by Phase contrast Microscopy

HT-29 cells were seeded in a T-25 flask at a density of 2×10^5 cells/flask and grown for 24hr. After seeding, the cells were treated with 80% methanol extract of RHF (1000 μg/mL) and Colchicine (320 μg/mL) for 24h, respectively. After 24h, cell morphology was evaluated using phase contrast inverted microscope with digital imaging (Axiovert A1, Zeiss, Germany).

3. Results

3.1 Composition of the herbal formulation

The final composition was standardized by conducting sensory analysis of the herbal formulation by semi-trained panel members. Table 1 depicts the composition of herbal formulation.

Table 1: Composition of herbal formulation

Ingredients	Amount
Kokum *(Garcinia indica*)	5ml
Amritaballi*(Tinospora cordifolia)*	2g
Mango Ginger (*Curcuma amada)*	3g
Krishna Tulsi (*Ocimum tenuiflorum)*	4g
Mint *(Mentha piperita)*	2g
Sichuan peppers (*Zanthoxylum rhetsa*)	2ml
Cane jiggery	2g
Palm jaggery (*Borassus flabellifer)*	3g
Honey	2g

The ingredients used in the herbal formulation have known antioxidant effect which is justified by literature reports. *Garcinia indica* rind has bioactive compounds garcinol and iso-garcinol which have shown anti-proliferative property *in vitro* [14]. *Tinospora cordifolia* plant extract supplementation in Swiss albino mouse provided protection against radiation induced alteration in internal mucosa [15]. The bioactive compound amadaldehyde present in *Curcuma amada* exhibited cytotoxicity against the A-549 cell lines [16]. Krishna Tulsi (*Ocimum tenuiflorum*) leaf extract suppressed chemically induced hepatomas in rats and tumors in the fore stomach of mice [17]. Mint *(Mentha piperita)* leaves provided protection against oxidative DNA damage associated with cancer [18]. Sichuan peppers (*Zanthoxylum rhetsa*) possess compounds that cause cell apoptosis i.e. by targeting phosphoinositide-3 kinase (PI-3kinase) pathway required for cell survival and was also effective in necrosis factor therapeutics as quoted in a study[19]. Palm Jaggery (*Borassus flabellifer)* seed coat inhibited the growth of He La cell lines (human cell line isolated from cervical cancer patient, Henrietta Lacks) [20].

3.2 Antioxidant activity of the herbal extracts

3.2.1. Radical Scavenging Assay (DPPH)

The DPPH assay was carried out in dose dependent manner. Among the samples, the radical scavenging activity of TRF (which served as standard), was the highest followed by RHF and HTF samples. Among the extracts of the RHF, 80% methanol showed the highest (67% at 320µg) antioxidant activity followed by ethanol (64%), aqueous (64%) and methanol (36%) extract. In the HTF extracts, 80% methanol showed the highest antioxidant activity (38% at 320µg) followed by ethanol (37%), methanol (29%) and aqueous (21%) extracts. All the extracts of TRF showed high antioxidant activity (>95% at 320µg) and followed the order ethanol > methanol > 80% methanol > Aqueous. The DPPH results of the RHF and HTF extracts as well as that of the TRF are depicted in table 2.

Table 2: Radical Scavenging Assay (DPPH) of RHF, HTF &TRF Exttracts

Variations	% Antioxidant activity						
	Concentration (µg)	10	20	40	80	160	320
RHF	Aqueous	34.76	24.74	28.11	38.24	29.55	63.8
	Ethanol	19.73	16.15	16.87	44.68	64.31	64.41
	Methanol	2.27	5.78	5.88	10.95	20.97	35.74
	80% Methanol	20.25	25.68	28.64	35.45	65.72	66.85
HTF	Aqueous	14.17	18.51	20.45	12.72	14.89	20.77
	Ethanol	11.75	14.09	15.37	17.06	26.97	37.43
	Methanol	12.39	15.61	14.33	15.78	19.96	29.3
	80% Methanol	15.85	18.25	20.51	22.98	28.85	38.22
TRF	Aqueous	45.52	66.64	94.16	95.28	94.8	95.52
	Ethanol	61.76	84.24	95.84	96.32	96.4	97.12
	Methanol	46.64	78.8	93.44	96.24	95.6	96.88
	80% Methanol	47.68	91.28	95.84	94.96	95.6	95.6

Note: RHF – Raw Herbal Formulation, HTF – Heat treated formulation, TRF - Triphala

3.2.2. Reducing Power Assay

Increase of absorbance in the Reducing Power Assay (RPA) indicates higher antioxidant activity[13]. The graphical representation of the extracts (RHF, HTF and TRF) subjected to reducing power assay is presented in figure 1. Among all extracts of the samples, 80% methanol extract showed maximum reducing power and the result correlated well with the results observed in the radical scavenging assay. The standard and variations showed an increasing trend in the antioxidant activity with increasing concentrations, however, the antioxidant activity of HTF was higher followed by RHF and TRF.

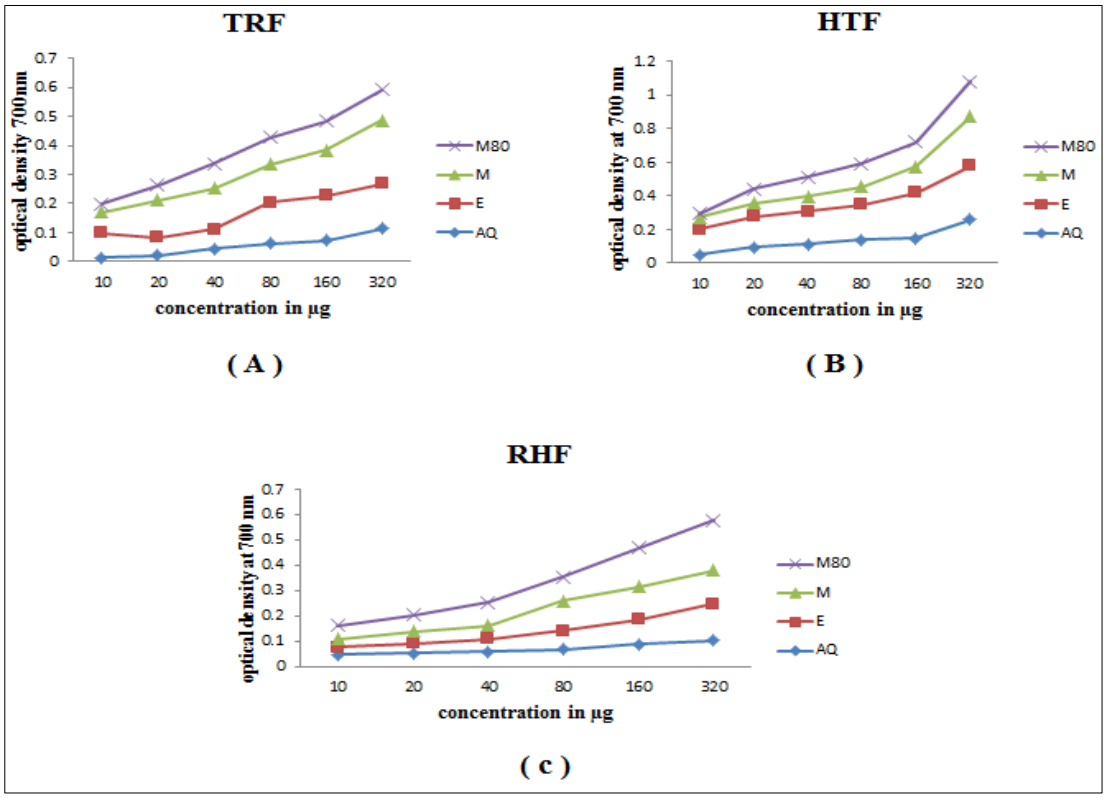

Fig 1: Reducing power assay of (A) Triphala; (B) Heat Treated formulation and (C) Raw herbal formulation

3.3 Gas Chromatography - Mass spectrometry (GC-MS) of herbal extract

Since 80% methanol extract exhibited higher antioxidant effect in all the samples, it was selected to screen potential bio-actives by GC-MS analysis and *in vitro* anti-cancer studies. The GC-MS results of 80% methanol extracts of RHF, HTF and TRF are depicted in figures 2, 3 and 4 and tables 3, 4 and 5 respectively.

3.3.1. Gas Chromatography and Mass Spectrum Analysis of the 80% Methanol Extract of Raw Herbal Formulation

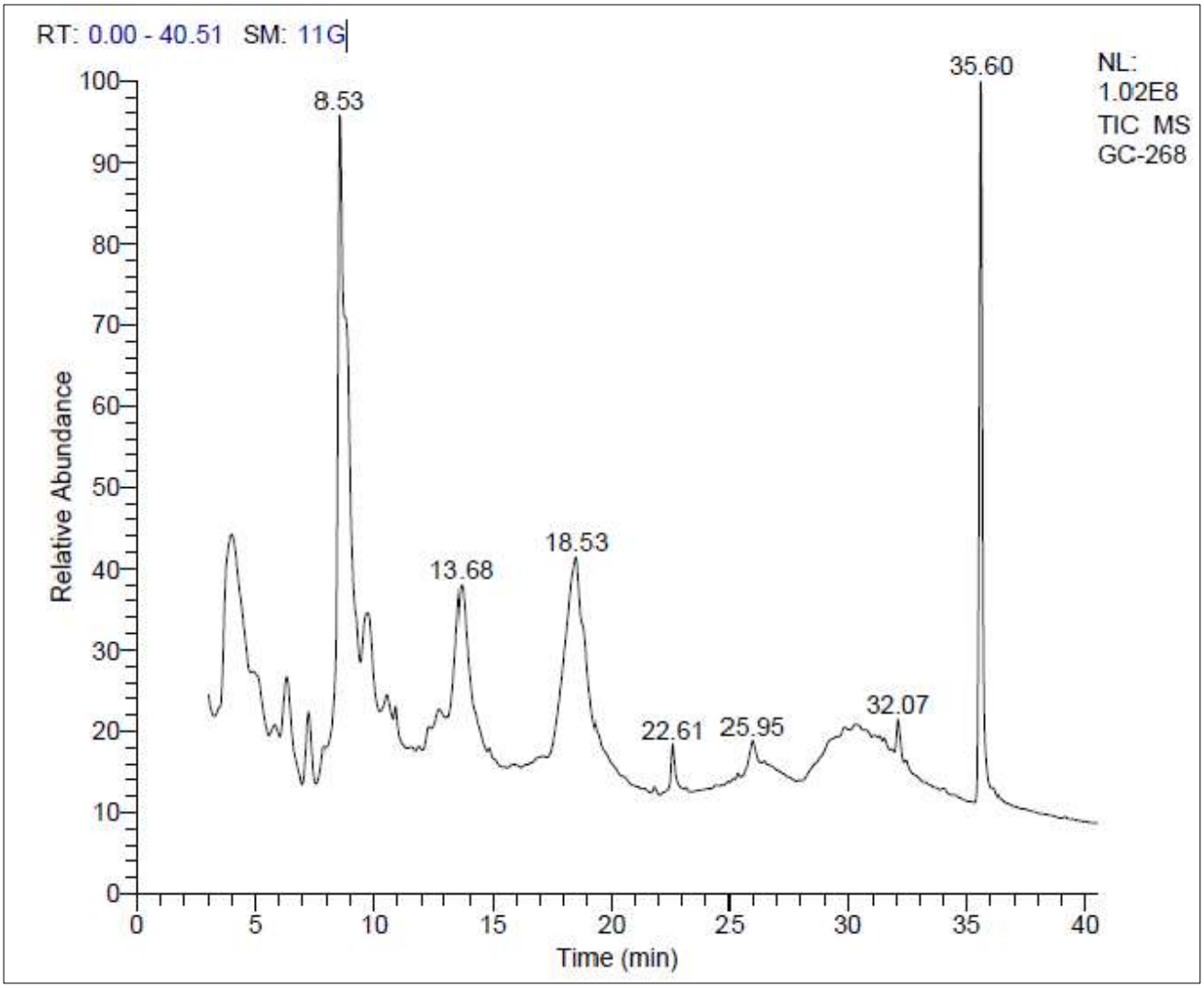

Fig 2: Gas Chromatography and Mass Spectrum Analysis of the 80% Methanol Extract of Raw Herbal Formulation

Table 3: Phyto-components detected in 80% Methanol Extract of RHF

Peak	Run Time	Compound Name	Probability
1	3.96	1,2Dihydroxyfurazan	47.24
5	7.26	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl- (CAS)	88.67
7	8.53	5-Hydroxymethylfurfural	60.74
8	9.24	4-Hydroxylamino-6-methylpyrimidin-2(1H)-one	5.05
19	21.83	Cyclopropanepentanoic acid, 2-undecyl-, methyl ester, trans- (CAS)	2.19
20	22.61	Hexadecanoic acid (CAS)	63.27
23	29.23	2-Hexadecanol (CAS)	2.44
27	32.07	trans-11-Icosenamide	1.57

The GC-MS results of 80% methanol extract of RHF is depicted in figure 2. From the GC-MS analysis of the RHF 80% methanol extract, compounds having potential anti-cancer, anti-oxidant, anti-tumorigenic, anti-mutagenic and cytotoxic activity were identified (table 3). Some of the compounds with these properties are 1,2Dihydroxyfurazan; 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl- (CAS); 5-Hydroxymethylfurfural;4-Hydroxylamino-6-methylpyrimidin-2(1H)-one; Cyclopropanepentanoic acid 2-undecyl-,methyl ester, trans- (CAS). Out of these compounds, 4H-Pyran-4-one, 2, 3-dihydro-3, 5-dihydroxy-6-methyl- (CAS) (88.67%) showed maximum probability compared to other compounds. It has been reported to exhibit both antioxidant and cytotoxic effect[21]. Apart from these Phyto-compounds 17 different peaks with run time varying from 5.12 – 31.50 minutes, with molecular weight from 73 to 207 were detected. However, these compounds could not be identified as they were not available in the NIST library.

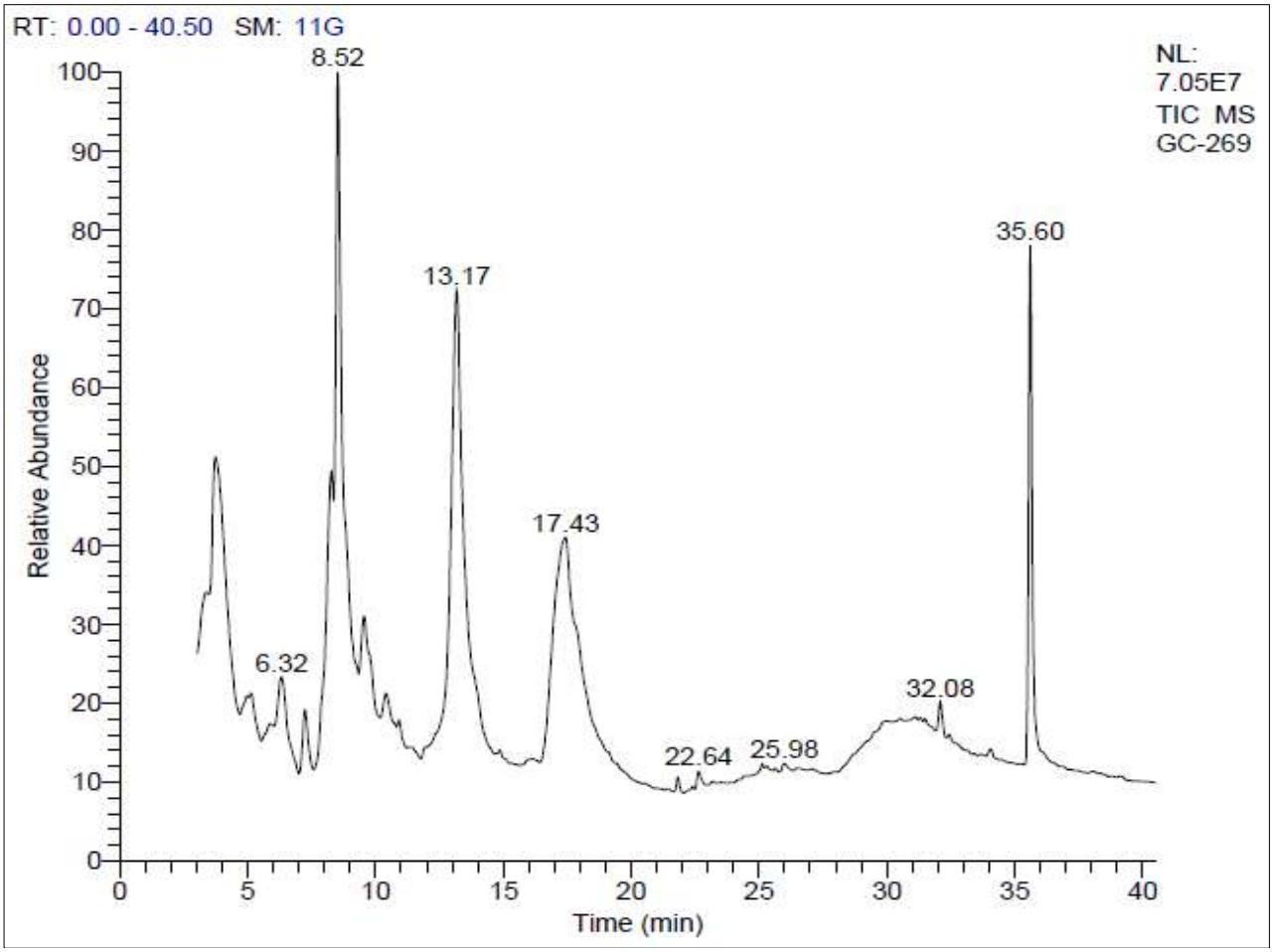

Fig 3: Gas Chromatography and Mass Spectrum Analysis of the 80% Methanol Extract of Heat Treated Herbal Formulation

3.3.2. Gas Chromatography and Mass Spectrum Analysis of the 80% Methanol Extract of Heat Treated Herbal Formulation

Table 4: Phyto-components detected in 80% Methanol Extract of HTF

Peak	Run Time	Compound Name	Probability
2	3.76	1,2Dihydroxyfurazan	29.18
5	6.32	Uracil, 1-N-Methyl-	0.78
6	7.24	2-Quinolinamine (CAS)	49.51
7	8.52	5-Hydroxymethylfurfural	75.45
10	10.93	Methyl-eugenol	50.09
17	17.30	3-Deoxy-d-mannoic lactone	74.67
19	21.83	Pentadecanoic acid, 14-methyl-, methyl ester (CAS)	6.14
20	22.62	n-Hexadecanoic acid	45.03
21	25.13	11-Octadecenoic acid, methyl ester	5.18
22	25.98	9-Octadecenoic acid (Z)- (CAS)	12.96
23	26.55	Pentadecanoic acid (CAS)	4.00
25	29.86	11-Octadecenal (CAS)	6.88
26	31.50	1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester	6.11
28	34.05	12-Methyl-E,E-2,13-octadecadien-1-ol	4.73

The GC-MS results of 80% methanol extract of HTF is depicted in figure 3. In HTF 80% methanol extract, the compounds with potential anti-cancer property that were identified are 1,2 Dihydroxyfurazan; Uracil, 1-N-Methyl-;5-Hydroxymethylfurfural; n-Hexadecanoic acid; 11-Octadecenoic acid; methyl ester, 9-Octadecenoic acid (Z)-(CAS); Pentadecanoic acid (CAS); 11-Octadecenal (CAS); 1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester. 5-Hydroxymethylfurfural (75.45%) which showed highest probability compared to other compounds and it has been reported to possess antioxidant as well as anti-cancer properties[22]. Apart from these phyto-compounds 14 different peaks with run time varying from 5.14 – 38.09 minutes, with molecular weight from 72 to 207 were detected. However these compounds could not be identified as they were not available in the NIST library.

3.3.3. Gas Chromatography and Mass Spectrum Analysis of the 80% Methanol Extract of Triphala

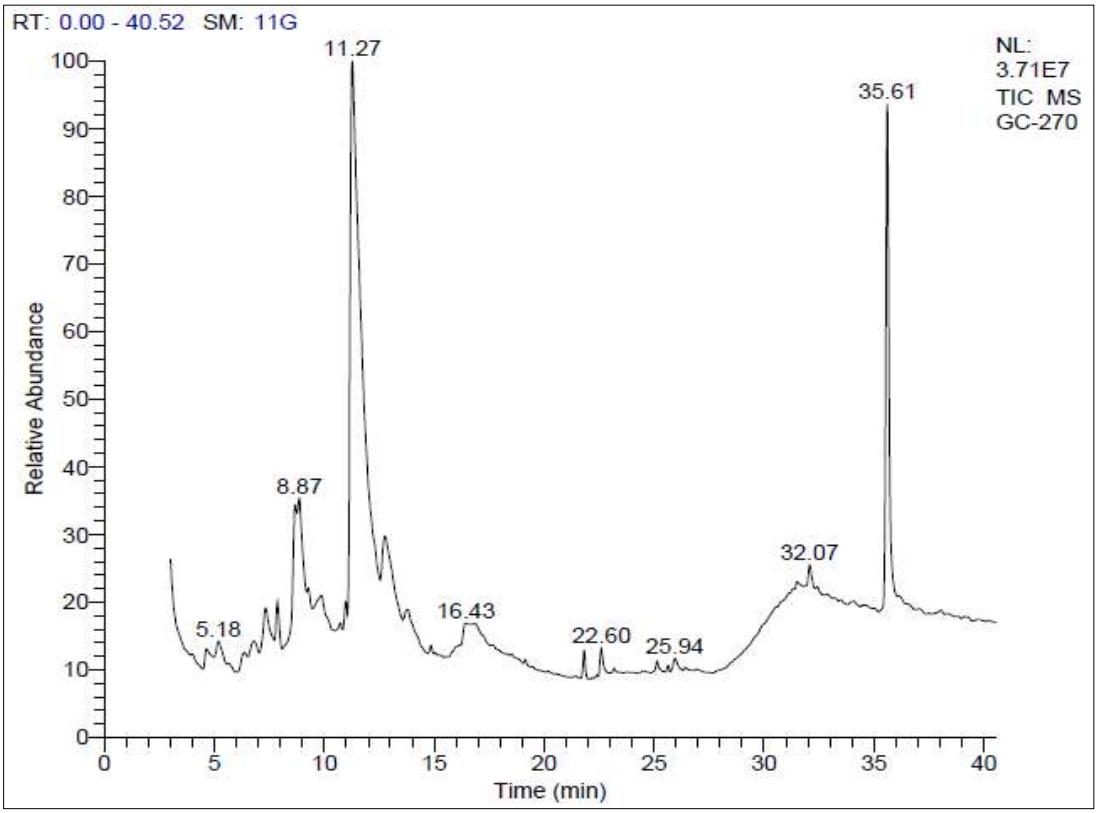

Fig 4: Gas Chromatography and Mass Spectrum Analysis of the 80% Methanol Extract of Triphala

Table 5: Phyto-components detected in 80% Methanol Extract of Triphala (TRF)

Peak	Run Time	Compound Name	Probability
5	7.32	2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	10.23
6	7.87	Dodecane (CAS)	21.51
7	8.67	5-Hydroxymethylfurfural	87.28
9	9.87	L-Arginine	11.68
12	11.27	1,2,3-Benzenetriol	90.27
16	15.98	1,2-Cyclopentanedicarboxylic acid, 4-(1,1-dimethylethyl)-, dimethyl ester, (1α,2α,4α)- (CAS)	5.51
18	21.83	Hexadecanoic acid, methyl ester (CAS)	35.66
19	22.6	n-Hexadecanoic acid	61.96
20	25.13	9-Octadecenoic acid (Z)-, methyl ester (CAS)	9.72
21	25.64	Octadecanoic acid, methyl ester (CAS)	33.45
24	32.07	9-Octadecenamide	38.13
28	38.07	Stigmasterol	4.84
30	39.26	4-Piperidineacetic acid, 5-ethylidene-2-[3-(2-hydroxyethyl)-1H-indo l-2-yl]-α-methylene-, methyl ester, [2S-(2α,4α,5E)]- (CAS)	12.11

The GC-MS results of 80% methanol extract of TRF extract is depicted in figure 4. TRF was used as reference standard. 80% methanol extract of TRF was screened for bioactives with anti-cancer properties by GC-MS analysis and the results showed the presence of compounds such as Dodecane (CAS); 5-Hydroxymethylfurfural; L-Arginine; 1,2,3-Benzenetriol; 1,2-Cyclopentanedicarboxylic acid,4-(1,1-dimethylethyl)-dimethyl ester, (1α,2α,4α)- (CAS); Hexadecanoic acid, methyl ester (CAS); n-Hexadecanoic acid; 9-Octadecenoic acid (Z)-, methyl ester (CAS); Octadecanoic acid, methyl ester (CAS); Stigmasterol which have potential anti-cancer properties[23]. Among these 1, 2, 3-Benzenetriol (90.27%) reported to exhibit antioxidant effect showed maximum probability[24,25]. Apart from these phyto-compounds 16 different peaks with run time varying from 4.63 – 35.61 minutes, with molecular weight from 72 to 207 were detected. However these compounds could not be identified as they were not available in the NIST library.

3.4Anti-cancer assay of the herbal extract

3.4.1. MTT Assay

MTT assay was carried out on HT-29 colorectal cancer cell line treated with 80% methanol extract of TRF, RHF and HTF in dose dependent manner and the percentage growth inhibition has been depicted in table 8.

Table 8: Summary of MTT of Test Samples

Extracts	Conc. µg/mL	OD at 590 nm	% Inhibition	IC_{50}
Control	0	0.62	0	
RHF	500	0.445	28.23	834.5
	1000	0.23	62.90	
	2000	0.16	74.19	
HTF	500	0.58	6.45	2335
	1000	0.43	30.65	
	2000	0.355	42.74	
TRF	500	0.5	19.35	1492
	1000	0.365	41.13	
	2000	0.265	57.26	

Note: RHF – Raw Herbal Formulation, HTF – Heat treated formulation, TRF - Triphala

Results of MTT assay suggested that RHF showed the lowest IC_{50} value of 834.5 µg/mL and hence among the three extracts, RHF was selected for cell cycle arrest and morphological studies.

3.4.2. Cell cycle arrest

Anti-cancer agents have the capacity to bring about cell cycle arrest and to induce apoptosis. The human colorectal adenocarcinoma cell line, (HT-29), was used to investigate the capability of the methanol 80% extract of RHF to induce apoptosis. Considering the IC_{50} from the MTT assay, 1000 μg/mL dosage was finalized for the cell cycle analysis. After 24h treatment, 1000 μg/mL dosage of RHF exhibited significant ($P < 0.001$) increase in the percentage of cells at S phase i.e. from 5.670±0.29% to 11.006±0.21% & 8.180±0.07% to 11.006±0.21% as compared to control & Colchicine 320 μg/mL respectively (Fig.5d). Significant decrease in the proportion of cells in G0/G1 Phase ($P < 0.001$) was observed.

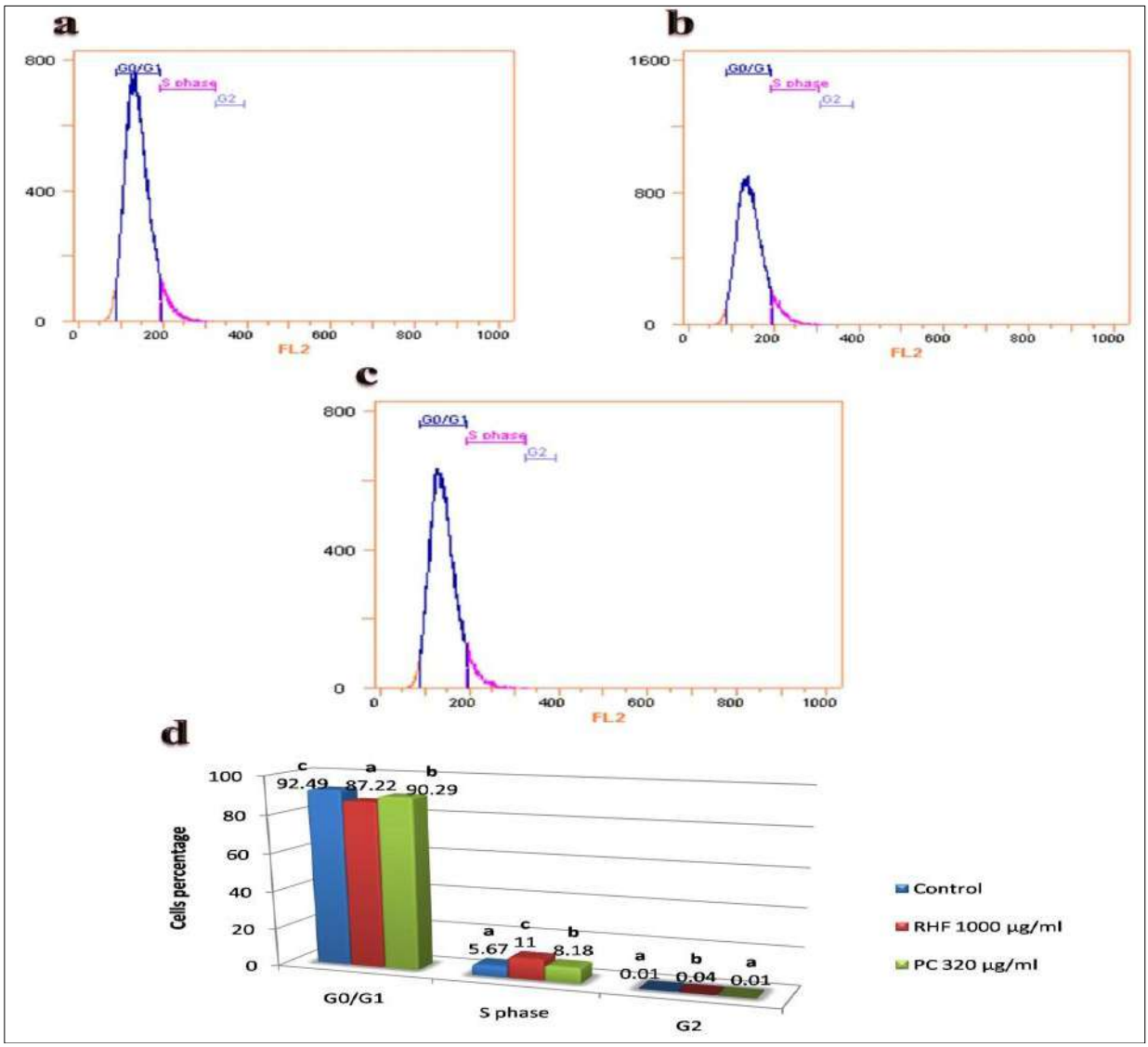

Fig 5: cell cycle analysis of RHF on HT-29 cells after 24 hours. a) Control; b)RHF 1000 µg/ml; c) Colchicine 320 µg/ml; d) Cell distribution of HT-29 cells after 24 hour treatment. All values are expressed as mean of triplicates (n=3). Mean values containing different superscript letters a, b, c…,d differ significantly ($P< 0.001, 0.05$)

3.4.3. Morphological Studies

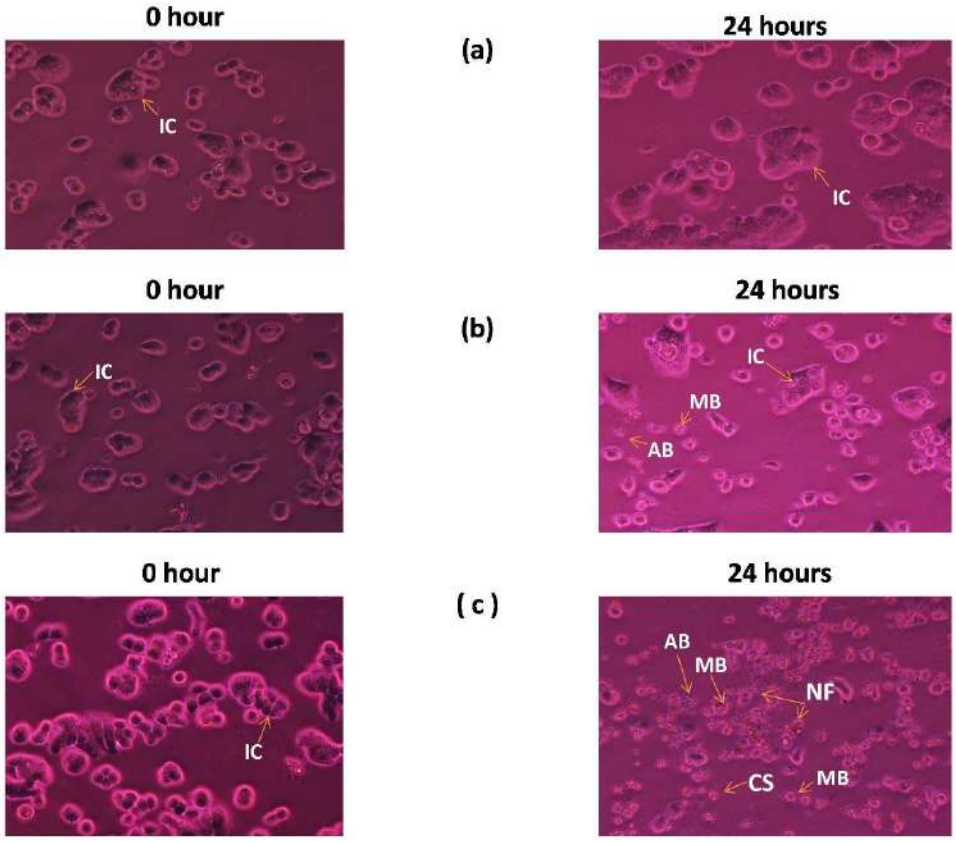

Fig 6: Morphological changes in HT-29 cell line as observed under phase contrast inverted microscope at 0 and 24 h. (a) Control; (b) Colchicine 320 µg/ml; and (c)RHF 1000 µg/ml., cellular shrinkage (CS), membrane blebbing (MB), nuclear fragmentation (NF), apoptotic bodies (AB); intact colonies (IC) (magnification 20X).

The HT-29 Cells were treated for 24 hours with the 1000 μg/mL of 80% methanol extract of RHF and Colchicine 320 μg/mL. After exposure for 24hrs the morphological characteristics of apoptosis, such as cellular shrinkage (CS), membrane blebbing (MB), nuclear fragmentation (NF), and apoptotic bodies (AB) were examined using phase contrast inverted microscope. Apoptosis was clearly observed in the RHF than in the Colchicine 320 µg/ml; which had few intact colonies along with apoptotic bodies. At higher dose, anti-cancer activity of RHF was comparable to colchicine.

4. Discussion

Medicinal plants are rich sources of secondary metabolites such as alkaloids, phenol, cardiac glycosides, flavonoids, tannins and terpenoids determined by gas chromatography and mass spectrum[21]. In a study it has been reported that the activities of some plant constituents with compound nature of flavonoids, palmitic acid (hexadecanoic acid, ethyl ester and n-hexadecaonoic acid), unsaturated fatty acid and linolenic acid (docosatetraenoic acid and octadecatrienoic acid) have antimicrobial, anti-inflammatory, antioxidant, hypo-cholesterolemic, cancer preventive, hepato-protective, anti-arthritic, anti-histimic, anti-eczemic and anti-coronary. Among the identified phyto-compounds Dodecanoic acid and n-Hexadecanoic acid have anti-oxidant and anti-microbial activities[21]. By GC-MS analysis 6 phyto-constituents in RHF 80% methanol extract, 9 phyto-constituents in HTF 80% methanol extract and 10 phyto-constituents in the 80% methanol extract of TRF were identified, which has potential anti-cancer activity as reported in literature. Both RHF and HTF extracts showed the presence of 1, 2 Dihydroxyfurazan and 5-Hydroxymethylfurfural which have anti-cancer effect and cytotoxic effect. The probability of 1, 2 Dihydroxyfurazan was higher in RHF, whereas the probability of 5-Hydroxymethylfurfural was higher in HTF. Comparison with the commercial herbal supplement TRF showed greater similarity with the HTF extract but the latter showed lower probability. Though the composition of both RHF and HTF sample were the same, a significant difference was observed in the types of phyto-constituents having anti-cancer effect. Phyto-constituents having potential anti-cancer effect were observed in HTF however with lower probability. The presence of various bioactive components with anti-oxidant, anti-cancer, anti-tumor and cyto-toxic activity suggests the possibility of using RHF in the management of cancer.

Dose dependent increase in the antioxidant activity was observed in most of the extracts of the samples with increase in concentration. This was not observed in aqueous extract of the formulations. A study showed that 80% methanol extract of barley seeds showed better antioxidant activity than 100% methanol extract[26]. Similar observation was made in both the RHF and HTF in antioxidant activity.

Plants have a long history of use in treatment of cancer. Plants are known to possess anticancer activities against different cancer cell lines. In a study it has been observed that use of plant extract on cell line (MCF-7) followed by *in vitro* anticancer assay with MTT standard, has shown up to 50% growth inhibition[27]. Similarly, 80% methanol extract of both RHF and HTF has shown cytotoxic effect via MTT assay in HT-29 cell line. It was observed that the percentage growth inhibition in RHF extract was better than the HTF

extract. This may be due to loss of essential volatile bioactive compounds, post heat treatment. TRF sample showed lower percentage growth inhibition than the RHF.

In cell cycle analysis of MDA-MB-231 cell lines after 24 hours treatment with the crude methanol extract of *E. platyloba*, it was observed that the percentage of cells in the S phase significantly increased from 4.58 ± 2.44% to 58.88 ± 3.11% as compared to the control[28]. Similar results were observed in our study where RHF showed cell cycle arrest at S phase. The possible mechanism may be due to disruption of mitochondrial membrane to arrest cells in S phase and inhibit cell proliferation mediated by RHF. This data is further supported by morphological studies. Clear apoptosis was seen after treatment with RHF indicating its apoptotic ability.

5. Conclusion

Several studies in the past have clearly demonstrated the beneficial effect of medicinal plants in the alleviation of human diseases. The therapeutic effects have been attributed to several compounds present in the plants either solely or in combination, which have been corroborated by extensive *in vitro* and *in vivo* studies. The present study has demonstrated the effectiveness of herbal formulation in curbing cancer cell growth *in vitro* by induction of apoptosis. Several bioactives with known anti-cancer effect have been identified in the formulation through GC-MS analysis. The effect of processing on the profile of bioactive compound has also been demonstrated. These *in vitro* observations pave ways for future clinical studies.

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7. Conflict of interests

Authors declare no conflict of interest.

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