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Anticancer Activity Of Herbal Syrup (Hibiscus Rosa Sinensis Flower)

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Abstract:

One defining feature of cancer is the rapid creation of abnormal cells that grow beyond their usual boundaries, and which can then invade adjoining parts of the body and spread to other organs, the latter process is referred to as metastasizing. The plant Hibiscus rosa-sinensis belongs to the family Malvaceae. With attractive and colour full flowers, plants of Hibiscus are widely planted as ornamentals and are used in traditional medicine. Methods: The nutrients were analyzed by AOAC method, Phytochemicals were identified by the GCMS method and the Anti-cancer activity was done by In-vitro analyzed by MTT assay in HELA cells. Results: Nutrients like Macro & Micro, 20 different varities phytochemicals and the herbal syrup had a anti- cancer activity. Conclusion: Some of the bioactive secondary metabolites identified may become commercially important phytopharmaceuticals. However, further studies are needed to ascertain their biological and pharmacological activity. Herbal syrup is a promising source of useful natural products and the new compound offers opportunities to develop novel anticancer drug.

Introduction:

Global burden of cancer:

Cancer is a general term for a group of diseases that can affect any part of the body. Cancer also called as malignant tumour and neoplasms. Nature of cancer is the rapid growth of abnormal immature cells that grow over their usual limit, and also occupy the adjoining parts of the body and spread to other organs of the body; this later stage is called as metastasizing. Cancer death mainly occurs due to the metastasis. (1)

Worldwide Cancer is the second leading cause of death. Cancer causes 9.6 million deaths in 2018 and globally 1 in 6 deaths occur due to cancer. Nearly 70% of cancer deaths occur in low- and middle-income countries. Around 1/3 of cancer death due to the 5 leading behavioral and dietary habits like tobacco use , alcoholism, low fruit and vegetable intake, high body mass index, lack of physical activity,(2)

The prevalence of cancer per year is expected around 29.5 million in 2040. The cancer-related deaths are also estimated around 16.4 million per year. (3) The economic impact of cancer is significant and is increasing. The total annual economic cost of cancer in 2010 was estimated at approximately US$ 1.16 trillion (4).

Burden of cancer in India:

According to the National Institute of Cancer Prevention and Research (NICPR), an estimated 2.25 million people in India live with cancer and more than 1,157,294 new cancer patients are registered every year. In 2018, 7, 84,821 people (4, 13,519 men and 3, 71,302 women) died of cancer. In fact, reports show that death rates due to cancer in the US have been decreasing gradually for the last 3 decades, typically 1.5% per year, while in India the death rate is rapidly increasing(5)

Finally, it is observed that a significant proportion of cancer patient households spend more than 10, 20 and 40 percent of their annual per capita household expenditure on inpatient treatment (Table 4).

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Overall, about 36.3 and 33.7 percent of households with cancer patients are spending more than 10 percent of their annual per capita household expenditure on public and private healthcare facilities, respectively

In 2017, the World Health Assembly passed the resolution *Cancer Prevention and Control through an Integrated Approach* (WHA70.12) urges governments and WHO to accelerate action to achieve the targets specified in the *Global Action Plan* and *2030 UN Agenda for Sustainable Development* to reduce premature mortality from cancer.

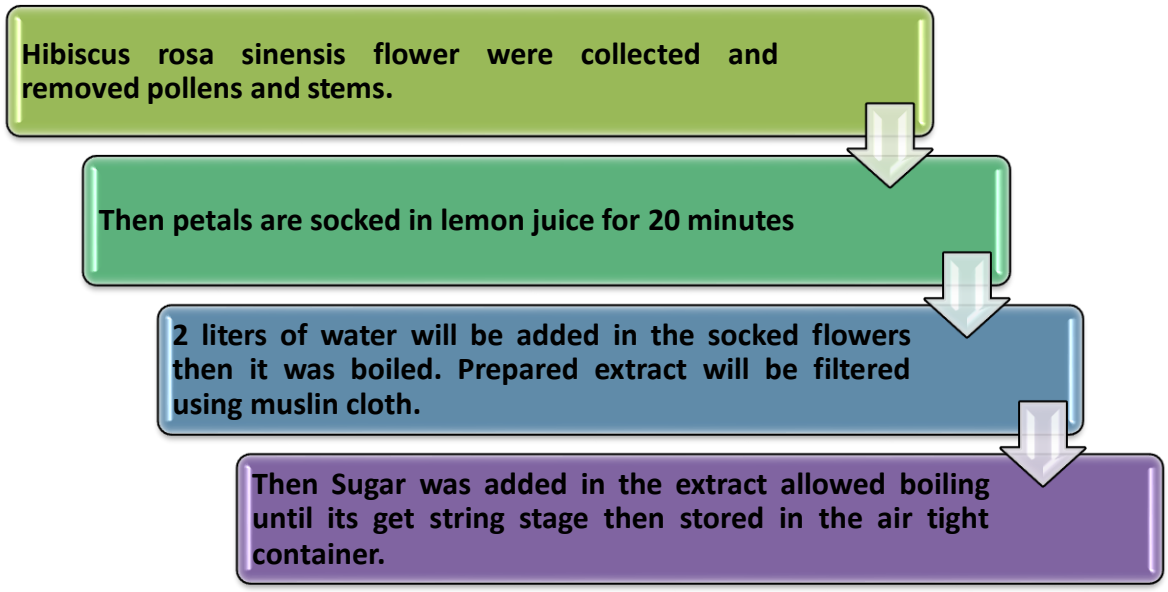
Therefore, there is a need for new strategies for the prevention and cure of cancer to control the death rate because of this disease. Herbal medicine has become a very safe, non-toxic, and easily available source of cancer-treating compounds. Herbs are believed to neutralize the effects of diseases in a body because of various characteristics they possess. (6)

Thus, medicinal plants have become a focal point to improve the present and future health needs. However, it is the need of the times to search new sources and compounds of specific antioxidants for determined objectives. The plant *Hibiscus rosa-sinensis* belongs to the family Malvaceae. With attractive and colour full flowers, plants of *Hibiscus* are widely planted as ornamentals and are used in traditional medicine. The plant species have been used as a folk remedy for the treatment of skin diseases, as an anti-fertility agent, antiseptic and carminative and the flower possesses anti-spermatogenic, androgenic, anti-tumor and anticonvulsant properties (7)

Materials and Methods

Plant Collection And Authentication: The plant *Hibiscus rosa sinensis* flowers was obtained from the southern part of India. The plant was identified and authenticated by Dr. G.V.S Murthy, Scientist 'G' & Head of Office, Botanical Survey Of India by TamilNadu Agriculture University, Southern Regional Centre, Coimbatore district, Tamil Nadu, India and a voucher specimen has been deposited at the herbarium for further reference (BSI/SRC/5/23/2016/TECH/1112).

Preparation of Herbal Syrup:



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Analysis of Micro and Macro Nutrients

Herbal syrup was subjected to nutrient analysis namely protein, fiber, moisture, ash, fat, carbohydrate, energy, vitamin C and iron was analyzed by using AOAC method.

Macro Nutrients

S.No	Parameter Analyzed	Method of Analysis
1	Protein	AOAC, 21st Edn, 2019, 984.13, Cha,4.2.09, Vol I, Pg: 31
2	Fat	AOAC, 21st Edn, 2019; 2003.05; Cha 4.5.05; Vol I; Pg:41.
3	Carbohydrate	Biochemical Methods by S. Sadasivam, et. al., Revised Second Edition; 2005; pg. 8-9
4	Fiber	AOAC, 21st Edn, 2019; 962.09; Cha 4.6.01; Vol I; Pg: 44

Table:1 Macro Nutrients

Micro Nutrients

1	Iron	AOAC, 21st Edn, 2019; 923.03; Cha 32.1.05; Vol II
2	Vitamin C	Sadasivam S and Manickam R, Biochemical Methods, 3rd Edition, New Age International Publications, Pg: 193-195

Table:2 Micro Nutrients

Non Nutrients

1	Moisture	AOAC, 21st Edn, 2019; 925.10; Cha 32.1.03; Vol II; Pg:1.
2	Ash	AOAC, 21st Edn, 2019; 923.03; Cha 32.1.05; Vol II; Pg:2
3	Energy (Kcal)	Food Labeling – Requirements for FDA Regulated products, by James L. Vetter, E. M. Melran, Ed., AIB International. Manhattan, K.S, 2007

Table:3 Non Nutrients

Phytochemicals

Phytochemicals are certain non-nutritive plant chemicals which have some disease preventive properties. It can act as an antioxidant and protect cells against free radical damage and to evaluate the nutraceutical value of the formulated products. Phytochemical compounds identification was done by GC-MS method.

Analysis of Samples

The samples were extracted with ethanol and analyzed through Gas Chromatography – Mass Spectrometry/ Mass Spectrometry for identification of different compounds.

GC Programme

- Column BR-5MS (5% Diphenyl / 95% Dimethyl poly siloxane), 30m x 0.25mm ID x 0.25µm df
- Equipment Scion 436-GC Bruker
- Carrier gas 1ml per min, Split 10:1
- Detector TQ Quadrupole Mass Spectrometer

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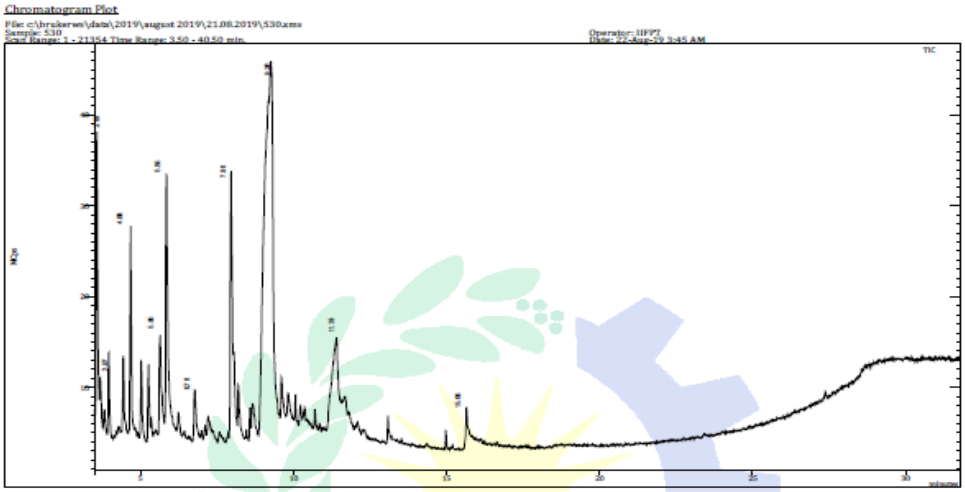
- Software MS Work Station 8
- Sample injected 2µl
- Oven temperature Programme -
- 110° C hold for 3.50 min
- Up to 200° C at the rate of 10 ° C/min-No hold
- Up to 280 ° C at the rate of 5° C / min- 12 min hold
- Injector temperature 280° C
- Total GC running time: 40.50 min

MS Programme

- Library used NIST Version-2011
- Inlet line temperature 290° C
- Source temperature 250 ° C
- Electron energy 70 eV
- Mass scan (m/z) 50-500 amu
- Solvent Delay 0 - 3.5 min
- Total MS running time: 40.50 min

GC- MS/MS Chromatogram



Invitro analysis

Test sample preparation (8,9,10)

For cytotoxicity studies, 32mg/ml stocks were prepared using DMSO. Serial two fold dilutions were prepared from 3.2mg/ml to 10mg/ml using DMEM plain media for treatment.

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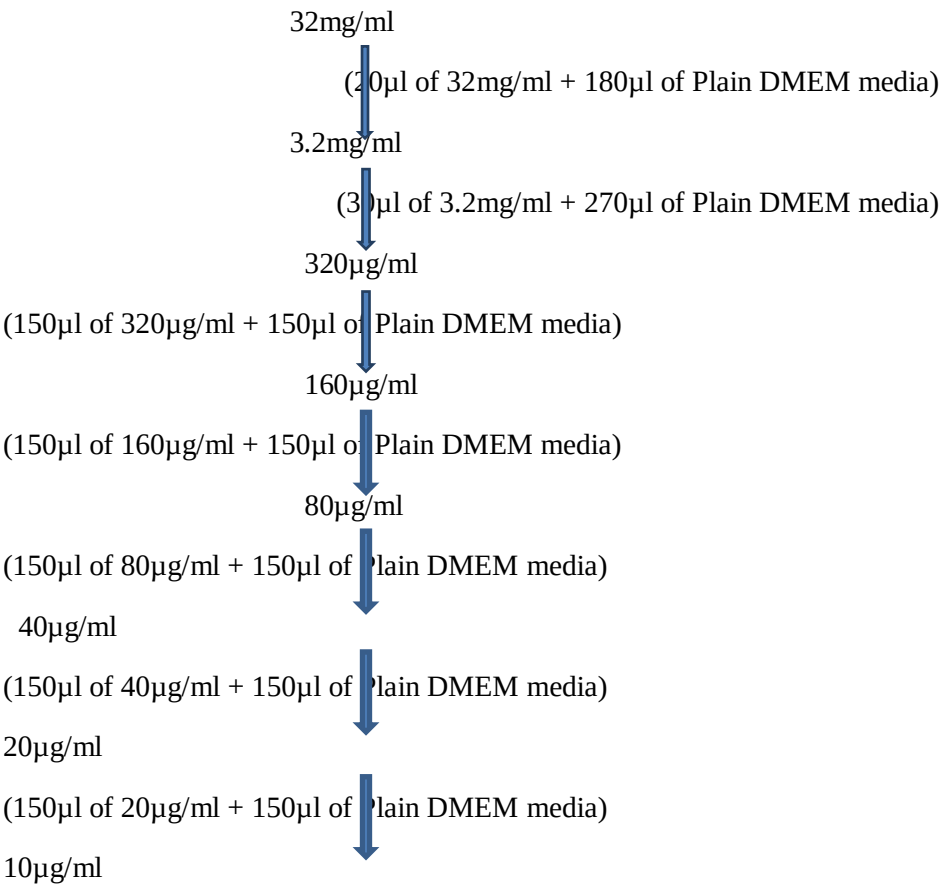

Cell lines and culture medium:

HeLa Cell line was procured from ATCC, stock cells was culturedin DMEM supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin(100IU/ml), streptomycin (100µg/ml) in a humidified atmosphere of 5% CO_2 at 37ºC until confluent. The cell was dissociated with cell dissociating solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The viability of the cells are checked and centrifuged. Further, 50,000 cells /well was seeded in a 96 well plate and incubated for 24 hrs at 37ºC, 5 % CO_2 incubator.

Source of reagents: DMEM, FBS, PenStrep, Trypsin-procured from Invitrogen.

Procedure:

The monolayer cell culture was trypsinized and the cell count was adjusted to $5x10^5$ cells/ml using respective media containing 10% FBS. To each well of the 96 well microtiter plate, 100µl of the diluted cell suspension (50,000cells/well) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100µl of different test concentrations of test drugs were added on to the partial monolayer in microtiter plates. The plates were then incubated at 37ºC for 24hrs in 5% CO_2 atmosphere. After incubation the test solutions in the wells were discarded and 100 µl of MTT (5mg/10ml of MTT in 1X PBS) was added to each well. The plates were incubated for 4 h at 37ºC in 5% CO_2 atmosphere. The supernatant was removed and 100 µl of DMSO was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 590 nm. The percentage growth inhibition

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was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% (IC_{50}) values is generated from the dose-response curves for each cell line.(11,12,13,14)

Calculating Inhibition:

% Inhibition = ((ODof Control – OD of sample)/OD of Control) x 100.

Statistical evaluation:

IC50 Value

The half maximal inhibitory concentration (IC50) is a measure of the effectiveness of a compound in inhibiting biological or biochemical function. This quantitative measure indicates how much of a particular drug or other substance (inhibitor) is needed to inhibit a given biological process (or component of a process, i.e. an enzyme, cell, cell receptor or microorganism) by half.

The IC50 of a drug can be determined by constructing a dose-response curve and examining the effect of different concentrations of antagonist on reversing agonist activity. IC50 values can be calculated for a given antagonist by determining the concentration needed to inhibit half of the maximum biological response of the agonist.

IC_{50} values for cytotoxicity tests were derived from a nonlinear regression analysis (curve fit) based on sigmoid dose response curve (variable) and computed using Graph Pad Prism 6 (Graph pad, SanDiego, CA, USA)

Results and Discussion:

Nutrient Content of Herbal Syrup

The nutrient contents of herbal syrup were found to be protein, carbohydrate, iron, vitamin C, fiber.

Macro Nutrients

S.No	Parameter Analyzed	Results (g/100g)
1	Protein	1.09g
2	Fat	Not detected
3	Carbohydrate	56.86g
4	Fiber	0.63g

Table:4 results of Macro Nutrients

Micro Nutrients

1	Iron	3.5mg/100ml
2	Vitamin C	5mg/100ml

Table: 5 results of Micro Nutrients

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Non Nutrients

1	Moisture	41.20
2	Ash	0.22
3	Energy (Kcal)	231.80 Kcal

Table: 6 results of Non Nutrients

Bio Active Compounds Identified in the Herbal Syrup

S.No	RT	Name of the compound	Molecular Formula	Molecular Weight	Peak Area %
1.	3.58	Thymine	$C_5H_6N_2O_2$	126	5.98
2.	3.70	Methyl6-oxoheptanoate	$C_8H_{14}O_3$	158	0.94
3.	3.97	Oxirane,[(2-propenyloxy)methyl]-	$C_6H_{10}O_2$	114	1.56
4.	4.44	Thiomorpholine-3-carboxylicacidamide	$C_5H_{10}N_2OS$	146	1.43
5.	4.68	4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl-	$C_6H_8O_4$	144	3.47
6.	5.03	Isoglutamine	$C_5H_{10}N_2O_3$	146	1.57
7.	5.27	2H-Pyran-2-methanol,tetrahydro-	$C_6H_{12}O_2$	116	1.46
8.	5.65	2(3H)-Furanone,5-heptyldihydro-	$C_{11}H_{20}O_2$	184	3.24
9.	5.85	5-Hydroxymethylfurfural	$C_6H_6O_3$	126	7.35
10.	6.78	Guanosine	$C_{10}H_{13}N_5O_5$	283	1.52
11.	7.21	l-Gala-l-ido-octose	$C_8H_{16}O_8$	240	0.54
12.	7.96	3-Propylglutaricacid	$C_8H_{14}O_4$	174	7.27
13.	8.68	d-Glycero-d-galacto-heptose	$C_7H_{14}O_7$	210	1.54
14.	9.26	Sucrose	$C_{12}H_{22}O_{11}$	342	49.79
15.	11.39	3-Deoxy-d-mannoiclactone	$C_6H_{10}O_5$	162	9.02
16.	11.69	Desulphosinigrin	$C_{10}H_{17}NO_6S$	279	1.61
17.	12.10	d-Mannitol,1-decylsulfonyl-	$C_{16}H_{34}O_7S$	370	0.36
18.	13.09	Tridecanoicacid	$C_{13}H_{26}O_2$	214	0.30
19.	14.98	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	$C_{17}H_{24}O_3$	276	0.26
20.	15.65	n-Hexadecanoicacid	$C_{16}H_{32}O_2$	256	0.80

Table:7 results of Phytochemical compounds

Gas chromatogram of the ethanol extract of Herbal syrup It confirmed the presence of various bioactive compounds with different retention times (RT). Table:7 The peaks of each component were obtained from the mass spectra. The compounds identified by their RT, molecular weight, and percentage peak area are illustrated along with their molecular formulas. Twenty compounds were detected in the ethanol extract of Herbal syrup Based on the RT and peak area of individual bioactive compounds, the predominant compounds were thymine, 5- Hydroxymethylfurfural, Methyl6-oxoheptanoate, 4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl-, l-Gala-l-ido-octose, n-Hexadecanoicacid.

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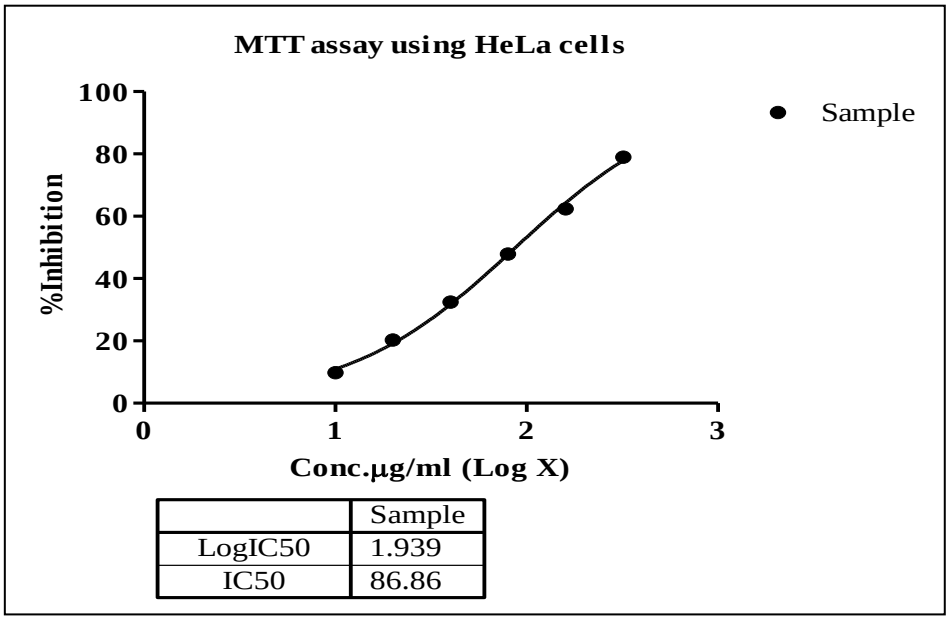
The use of medicinal plants in the treatment of various human ailments depends on their phytochemical constituents. This study revealed that the ethanol extract of Herbal syrup contained 20 compounds.

Invitro analysis

MTT assay is based on the reduction of MTT(3-(4,5- dimethyl thiazolyl)-2,5-diphenyl-tetrazolium bromide) by mitochondrial dehydrogenase to purple formazan product. The IC50 value of herbal syrup showed significant anticancer activity by MTT assay.(15)

Compound name	Conc. µg/ml	OD at 590nm	% Inhibition	IC50µg/ml
Control	0	0.784	0.00	
Herbal syrup	10	0.707	9.82	
	20	0.625	20.28	
	40	0.529	32.53	**86.86**
	80	0.408	47.96	
	160	0.295	62.36	
	320	0.165	78.98	

Table: 8 anticancer activity of herbal syrup



	Sample
LogIC50	1.939
IC50	86.86

Table:8 the Herbal syrup showed marked Anti-cancer activity, the concentration of Herbal syrup required for 50% death of the EAC cell lines (IC50) was found to be 86.86 µg/ml in HeLa Cells .

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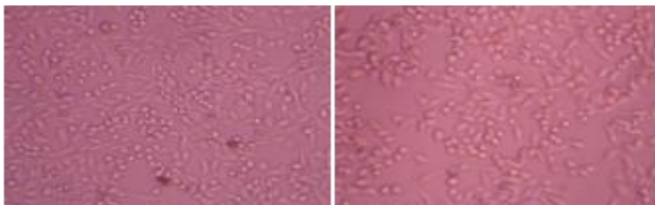
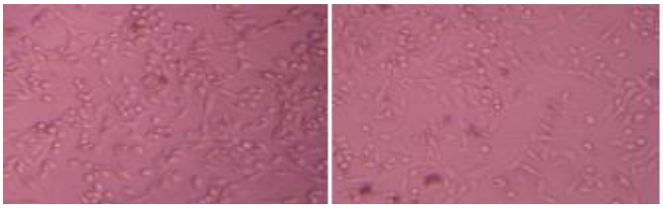
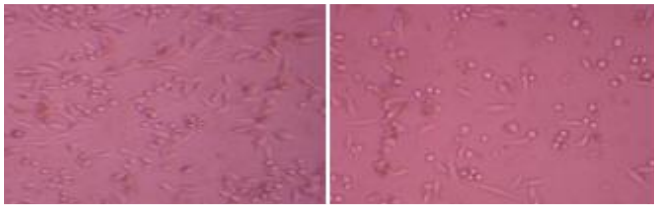


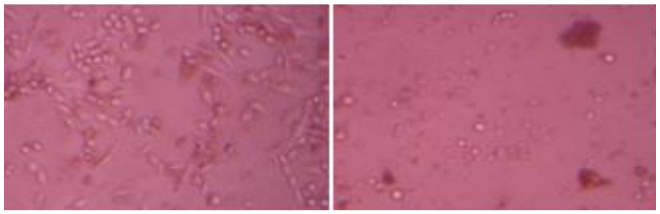
Herbal syrup 20µg/ml



Herbal syrup 40µg/ml



Herbal syrup 160µg/ml



Herbal syrup 320µg/ml

Conclusion

Hence the present study concluded that the Herbal syrup were rich in nutrients, phytochemicals and anti-cancer activity. Twenty compounds were identified from the ethanol extract of herbal syrup using GC–MS analysis. The presence of various bioactive compounds justifies the use of the whole plant for treating various ailments by practitioners of traditional Indian medicine and this study proved the herbal syrup having anti-cancer activity using MTT assay. Some of the bioactive secondary metabolites identified may become commercially important phytopharmaceuticals. However, further studies are needed to ascertain their biological and pharmacological activity. Herbal syrup is a promising source of useful natural products and the new compound offers opportunities to develop novel anticancer drug.

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Conflicts of Interest: No conflict of interest associated with this work.
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