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Combined Effect of *Cassia Auriculata* Linn. Leaf Extract with Cyclophosphamide and Methotrexate on their Individual Anti-Cancer Potencies using Cancer Cell Lines

Papanna K S[1], Dr. Bheemachari[1], Dr. Annegowda[2], Shruthi N[1], Srinivas[3]
[1]Department of Pharmacology, [2]Department of Pharmacognosy, [3]Department of Pharmaceutics,
Sri Adichunchanagiri College of Pharmacy, B. G. Nagara, Karnataka -571 448

Abstract:- Cancer can affect almost any part of the body and is a leading cause of death worldwide and even in India it is a major cause of morbidity and mortality. Few types of cancers can be cured, by surgery, radiotherapy or chemotherapy, especially if they are detected at early stage. Since the use of chemotherapeutic agents involves severe toxic effects hence, drugs derived from plant with least side effects are always exploited from natural resources. Among these one of the recently studied plants with anticancer activity was *Cassia Auriculata* Linn. (CA) which possess wide pharmacological activities along with anticancer activity. However, the effect of combined use of CA leaf hydro-alcoholic extract with synthetic anticancer drugs like cyclophosphamide and methotrexate on their individual anti-cancer potencies has not been properly studied and reported.

Keywords:- *Cassia Auriculata Leaf Extract, Cyclophosphamide, Methotrexate, MCF-7, HeLa Cell Line.*

I. INTRODUCTION

Cancer represents the uncontrolled growth of cells which often invade surrounding tissues and cause metastasis at distant sites. It can affect almost any part of the body and is a leading cause of death worldwide and even in India it is a major cause of morbidity and mortality. Few types of cancers can be cured, by surgery, radiotherapy or chemotherapy, especially if they are detected at early stage. Since the use of chemotherapeutic agents involves severe toxic effects hence, drugs derived from plant with least side effects are always exploited from natural resources. Among these one of the recently studied plants with anticancer activity was *Cassia Auriculata* Linn. (ca) which possess wide pharmacological activities along with anticancer activity. However, the effect of combined use of ca leaf hydro-alcoholic extract with synthetic anticancer drugs like cyclophosphamide and methotrexate on their individual anti-cancer potencies has not been properly studied and reported. Hence in the present project an attempt has been made to evaluate the combined effect of *Cassia Auriculata* Linn. Leaf extract with cyclophosphamide and methotrexate on their individual anti-cancer potencies using cancer cell lines.[1]

Health can be defined as homeostatic condition of physical, mental and social behaviour. Any change in the physiology of an individual without the involvement of infecting organism is known as disorder and with the involvement of infecting organism is known as a disease. Hence the panorama of a disorder/disease is as old as the origin of human beings and requires remedial measures in the form of medical treatment.

II. MATERIAL AND METHODS

A. Materials:

Cyclophosphamide, Methotrexate (Yarrow Chem), *Cassia Auriculata* Linn (Collected from local area of B.G. Nagar, Mandya)

➢ *For Anticancer Cell Line Activity:*

1.Cell lines:

a. MCF-7-Human Breast Carcinoma Cell Line (From NCCS, Pune)
b. HeLa- Human Cervix adenocarcinoma Cell Line (From NCCS, Pune)

2.Cell culture medium: DMEM (Dulbecco's Modified Eagle's Medium) High Glucose - (#AL111, Himedia) , Fetal Bovine Serum (#RM10432, Himedia) , MTT Reagent (5 mg/ml) (# 4060 Himedia) ,DMSO – Dimethyl Sulphoxide (#PHR1309, Sigma), Camptothecin (#C9911, Sigma) ,DPBS - Dulbecco's Phosphate Buffered Saline (#TL1006, Himedia)

B. Methodology:

➢ *Extraction*

The species for the study of leaves *Cassia Auriculata* Linn was carefully collected from surrounding area of B G Nagara, Mandya (District), Karnataka. The plant *Cassia Auriculata* Linn was identified and authenticated by renowned botanist Prof. L. B. Kulkarni, Head, Department of Botany, Sri Prabhu Arts and Science College, Surpur, Karnataka. After collection the leaves of *Cassia Auriculata* Linn were washed thoroughly with water to remove the dirt particles and any other foreign material adhered to leaves. Then the leaves were wiped off with soft cotton cloth and evenly spread over a newspaper to facilitate perfect drying.

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The *Cassia Auriculata* Linn leaves were subjected to shade drying,[02] until complete dryness. Then the dried leaves were powdered by using mixer grinder until to coarse powder is obtained, which was used for further extraction with solvent and photochemical studies. The extraction was performed using maceration technique. The coarse powder of *Cassia Auriculata* leaves (190 gm) was subjected to maceration for 72 hrs at room temperature using hydroalcoholic (water: methanol) in 30:70 ratio.[03] The extract was concentrated using rotary evaporator and stored in air tight container in a cool place until further use. The extract was used for phytochemical screening and to screen anticancer activities.[04]

➢ *Phytochemical Screening*

The leaves of *Cassia Auriculata* Linn. Are rich in carbohydrates, proteins, amino acids, flavonoids, alkaloids, tannins, phenolic compounds, and glycosides. The hydroalcoholic extract of Cassis auriculata Linn was subjected to different preliminary phytochemical tests to determine the chemical constituents present in the extract.[05]

➢ *Anticancer MTT Assay*

The yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay is a colorimetric assay used for the determination of cell proliferation and cytotoxicity, based on reduction of the yellow coloured water-soluble tetrazolium dye MTT to formazan crystals. Mitochondrial lactate dehydrogenase produced by live cells reduces MTT to insoluble formazan crystals, which upon dissolution into an appropriate solvent exhibits purple colour, the intensity of which is proportional to the number of viable cells and can be measured spectrophotometrically at 570 nm.

❖ *Steps Followed:*

1. Seed 200 µl cell suspension in a 96-well plate at required cell density (20,000 cells per well), without the test agent. Allowed the cells to grow for about 24 hours.
2. Add appropriate concentrations of the test agent mentioned from results.
3. Incubated the plates for 24 hrs at 37°C in a 5% CO2 atmosphere.
4. After the incubation period, takeout the plates from incubator, and remove spent media and add MTT reagent to a final concentration of 0.5 mg/mL of total volume.
5. Wrap the plates with aluminium foil to avoid exposure to light.
6. Return the plates to the incubator and incubate for 3 hours. (Note: Incubation time varies for different cell lines. Within one experiment, incubation time should be kept constant while making comparisons.)
7. Remove the MTT reagent and then add 100 µl of solubilisation solution (DMSO).
8. Gentle stirring in a gyratory shaker will enhance dissolution. Occasionally, pipetting up and down may be required to completely dissolve the MTT formazan crystals especially in dense cultures.
9. Read the absorbance on a spectrophotometer or an ELISA (Enzyme Linked Immuno adsorbent Assay) reader at 570nm and 630nm used as reference wavelength.
10. The IC50valuewas determined by using linear regression equation i.e. Y =Mx+C.

Here, Y = 50, M and C values were derived from the viability graph.[06-18]

III. CONCENTRATIONS USED FOR THE STUDY

In this study, three test compounds namely methotrexate, cyclophosphamide and *Cassia Auriculata* leaves extract were used to check the cytotoxicity study on the two cell lines namely, MCF-7 and HeLa. The concentrations of the compound used to treat the cells are as follows:

Sl. No	Test Compounds	Cell Line	Concentration treated to cells
1	Untreated	MCF-7 and HeLa	No treatment
2	Standard (Camptothecin)	MCF-7 and HeLa	25µM
3	Blank	-	Only Media without cells
4	Test -1(Methotrexate)	MCF-7 and HeLa	6.25,12.5,25,50,100µG/mL
5	Test - 2(Cyclophosphamide)	MCF-7 and HeLa	6.25,12.5,25,50,100µG/mL
6	Test -3(*Cassia Auriculata* leaves extract)	MCF-7 and HeLa	6.25,12.5,25,50,100µG/mL

Table 1:- Details of drug treatment to respective cell line

Combined anticancer study using synthetic drugs with CALE.

➢ *Concentrations used for the Study for Combination Effect*

In this study, the combination of methotrexate + *Cassia Auriculata* leaves extract (CALE) and cyclophosphamide + CALE are used to check the cytotoxicity study on the two cell lines namely, MCF-7 and HeLa. The used concentrations of the compounds to treat the cells as follows:

Sl No:	Test Compounds	Cell Line	Concentration treated to cells
01	Methotrexate + CALE	MCF-7 and HeLa	Three samples of IC_{50} of 50% meth+25%CALE, IC_{50} of 50% meth+50% CALE and IC_{50} of 50% meth+75% CALE
02	Cyclophosphamide + CALE	MCF-7 and HeLa	Three samples of IC_{50} of 50% cyclo+25%CALE, IC_{50} of 50% cyclo+50% CALE and IC_{50} of 50% cyclo+75% CALE

Table 2:- Details of combination drug treatment to respective cell line
(Meth- Methotrexate, Cyclo-Cyclophosphamide and CALE-*Cassia Auriculata* leaves extract)

IV. RESULTS

➢ *Phytochemical Analysis:*

The extract obtained was subjected to the qualitative chemical tests to detect the major primary and secondary phytoconstituents present in the leaf extract.

Sl No:	Phytoconstituents	Hydro-alcoholic extract
1.	Carbohydrates	+
2.	Proteins	+
3.	Alkaloids	+
4.	Tannins	+
5.	Phenolic compounds	+
6.	Flavonoids	+
7.	Glycosides	+
8.	Amino acids	+
9.	Terpenoids	-
10.	Steroids	-

Table 3:- Results of preliminary phytochemical investigations of *Cassisa auriculata* leaf extract.
(+ -"Present", - "Absent")

The Preliminary phytochemical investigations were carried out as per standard procedures given in reference book (Khandelwal and Kokate). Results reveals the presence of carbohydrates, proteins, amino acids, flavonoids, alkaloids, tannins, phenolic compounds, and glycosides are present whereas, terpenoids and steroids are absent in the hydro-alcoholic extract.

➢ *MTT Assay*

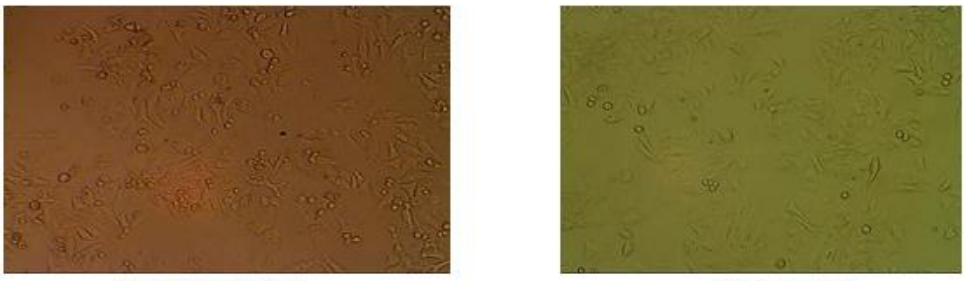

Fig 1:- HeLA Cell line:

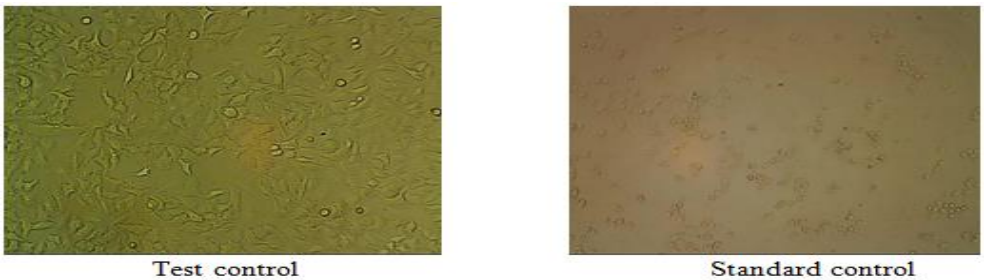

Fig 2:- MCF-7 Cell line

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Drugs	Viability at different conc. In µG/mL on HeLa cell lines					IC_{50} value
	6.25µG/mL	12.5µG/mL	25µG/mL	50µG/mL	100µG/mL	
Metho.	46.67	82.03	58.65	33.20	20.18	17.45µG/mL
Cyclo.	46.67	51.75	38.99	25.91	16.27	6.65µG/mL
CALE	91.5 2	83.82	75.35	61.51	39.84	77.32µG/mL

Table 4:- cell viability in the HeLa cell lines

Drugs	Viability at different conc. In µG/mL on MCF-7 cell lines					IC_{50} value
	6.25µG/mL	12.5µG/mL	25µG/mL	50µG/mL	100µG/mL	
Metho.	79.20	60.78	46.66	26.69	10.60	20.24µG/mL
Cyclo.	78.24	72.07	66.13	54.11	38.84	68. 12µG/mL
CALE	96.52	90.3 5	75.27	63.25	47.98	99.26µG/mL

Table 5:- cell viability in the MCF-7 cell lines

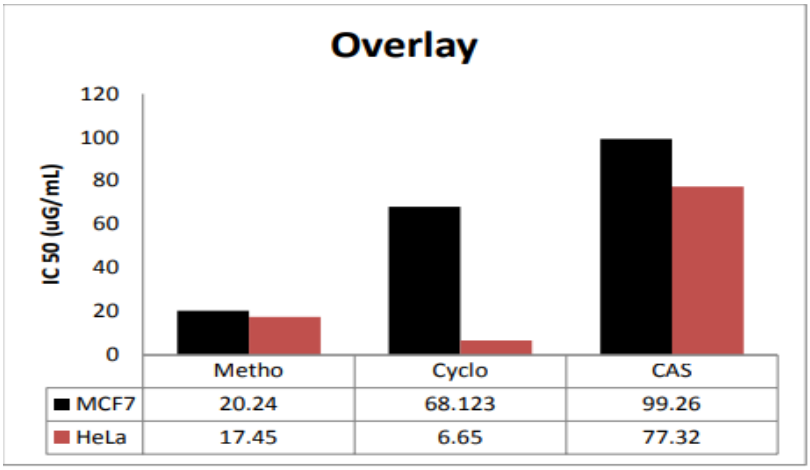
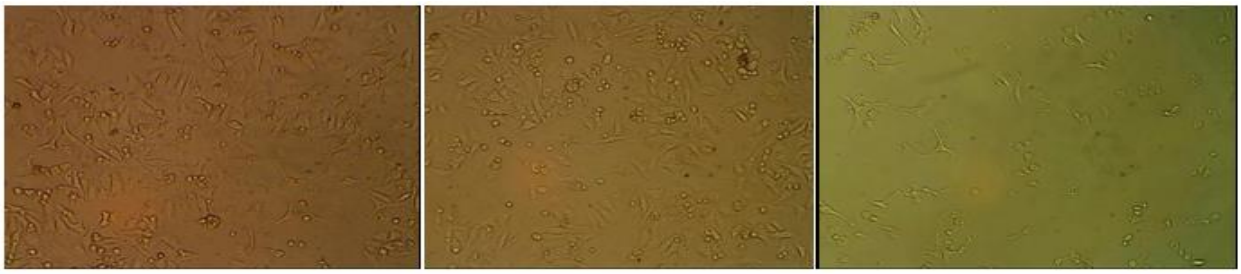


Fig 3:- Graphical representation of anticancer activity of tested samples against MCF-7 and HeLa cell lines.

V. COMBINED STUDY

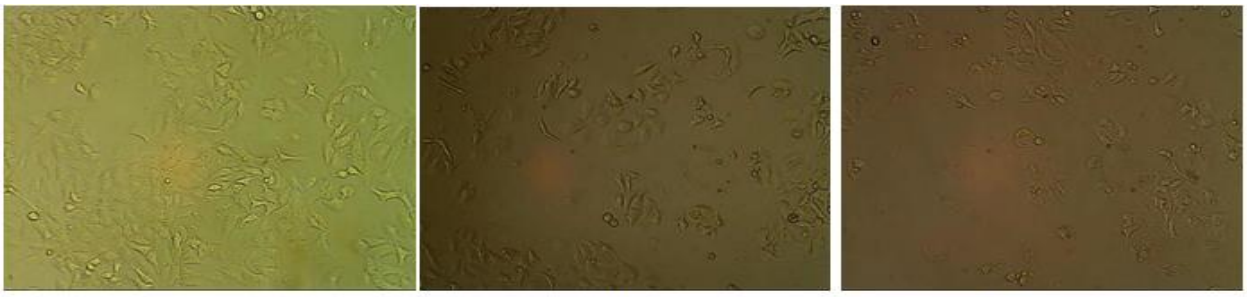
Sample of cell lines		Concentration with %viability on methotrexate		
HeLa cell line	Concentration	50% meth IC_{50} + 25% CALE IC_{50}	50% meth IC_{50} + 50% CALE IC_{50}	50% meth IC_{50} + 75% CALE IC_{50}
	%viability	95.70	73.17	49.58
MCF-7 cell line	Concentration	50% meth IC_{50} + 25% CALE IC_{50}	50% meth IC_{50} + 50% CALE IC_{50}	50% meth IC_{50} + 75% CALE IC_{50}
	%viability	80.38	49.39	32.67

Table 6:- combined study of methotrexate with CALE on HeLa and MCF-7 cell lines.



A B C

Fig 2:- on Hela cell lines=A-50% meth IC_{50} + 25% CALE IC_{50}.B-50% meth IC_{50} + 50% CALE IC_{50}.C-50%meth IC_{50} + 75% CALE IC_{50})

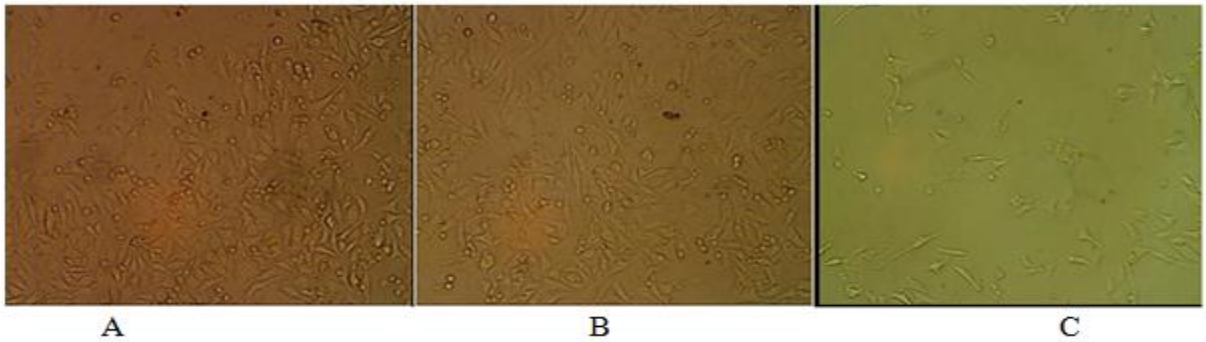


A B C

Fig 3:- on MCF-7 cell lines=A-50% meth IC_{50} + 25% CALE IC_{50}.B-50% meth IC_{50} + 50% CALE IC_{50}.C-50%meth IC_{50} + 75% CALE $IC_{50)}$

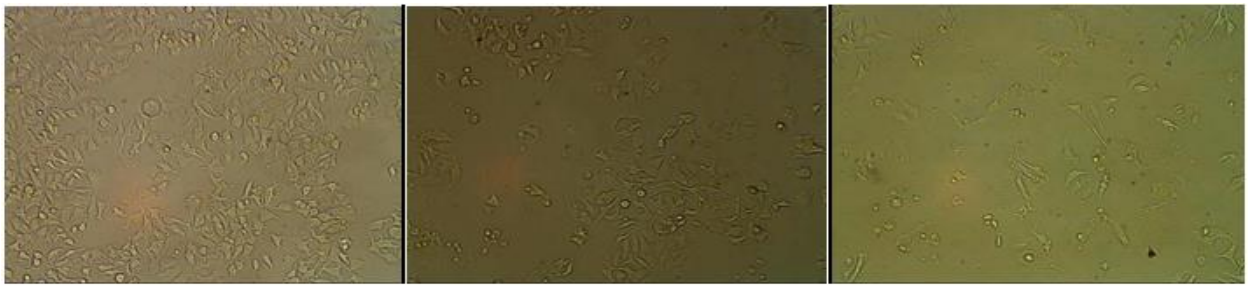
Sample of cell lines		Concentration with %viability on cyclophosphamide		
HeLa cell line	Concentration	50% cyclo IC_{50} + 25% CALE IC_{50}	50% cyclo IC_{50} + 50% CALE IC_{50}	50% cyclo IC_{50} + 75% CALE IC_{50}
	%viability	88.64	42.88	16.47
MCF-7 cell line	Concentration	50% cyclo IC_{50} + 25% CALE IC_{50}	50% cyclo IC_{50} + 50% CALE IC_{50}	50% cyclo IC_{50} + 75% CALE IC_{50}
	%viability	89.67	68.85	38.70

Table 7:- combined study of cyclophosphamide with CALE on HeLa and MCF-7 cell lines.



A B C

Fig 4:- on Hela cell lines=A-50% cyclo IC_{50} + 25% CALE IC_{50}.B-50% cyclo IC_{50} + 50% CALE IC_{50}.C-50% cyclo IC_{50} + 75% CALE $IC_{50)}$



A B C

Fig 5:- on MCF-7 cell lines=A-50% cyclo IC_{50} + 25% CALE IC_{50}.B-50% cyclo IC_{50} + 50% CALE IC_{50}.C-50% cyclo IC_{50} + 75% CALE $IC_{50)}$

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Combination	Percentage viability	
	MCF7 cell lines	HeLa cell lines
50% meth IC_{50} + 25% CALE IC_{50}	80.38	95.70
50% meth IC_{50} + 50% CALE IC_{50}	49.39	73.17
50% meth IC_{50} + 75% CALE IC_{50}	32.67	49.58
50% cyclo IC_{50} + 25% CALE IC_{50}	89.67	88.64
50% cyclo IC_{50} + 50% CALE IC_{50}	68.85	42.88
50% cyclo IC_{50} + 75% CALE IC_{50}	38.70	16.47

Table no.8. overall percentage cell viability in combination therapy

VI. DISCUSSION

In the present study, anticancer activity of methotrexate, cyclophosphamide and CALE was studied against MCF-7 and HeLa cell lines. The range of concentration of these samples used for the study was ranged between 6.25 µG/mL to 100 µG/mL. Results of the MCF-7 cell cytotoxic effect of methotrexate, cyclophosphamide and CALE is given in table no.4 on both the Hela and MCF-7 cell lines. It is apparent from the table no.4 that, methotrexate reduced cell viability in a dose dependent manner against MCF-7 cancer cell lines and the IC50 value was 20.24µG/mL. in case of cyclophosphamide but the IC50 value was found to be 68.12µG/mL against MCF-7 cell lines.

However, in case of *Cassia Auriculata* leaf extract, the IC50 value was found to be 99.26µG/mL and even at the higher dose of 100 µG/mL the MCF-7 cell viability was 47.98%. Results of the HeLa cell cytotoxic effect of methotrexate is given in table no.5. It is evident from the table no.5 that, methotrexate reduced cell viability in a dose dependent manner even against HeLa cancer cell lines and the IC50 value was 17.45µG/mL. In case of cyclophosphamide the IC50 value was found to be 6.65µG/mL against HeLa cell lines. *Cassia Auriculata* leaf extract possessed the IC50 value of 77.32µG/mL.

In the individual study of MTT assay, the viability of cells was seen in the test control and Standard control in the both cell lines, MCF-7 and HeLa cell lines. By progressive increasing the concentrations of CALE (6.25µG/ml, 12.5µG/ml, 25 µG/ml, 50 µG/ml, 100µG/ml) the viability of the percentage of the cells gradually decreases the both in MCF-7 and HeLa cell lines. The methotrexate on individual MTT assays of MCF-7 cell line at increasing concentrations gradually showed decreased percentage of cell viability by 10% at the concentration of 100µG/ml as compared to 6.25µG/ml. Similarly, against the HeLa cell lines, methotrexate showed decrease in the percentage of cell viability by 4% in the 100µG/ml concentration. In the HeLa cell lines the methotrexate shows maximum cytotoxic effect compared to human breast cancer cell lines.

In the combination study, various concentration of the plant extract based on the IC50 value (25%, 50% and 75% of IC50) was combined with the methotrexate and cyclophosphamide (50% of IC50 value) and was studied for anticancer activity in terms of cytotoxic effects against both MCF-7 and HeLa cell lines. In case of the combination study of CALE with cyclophosphamide, the combination of 50% of IC50 value of cyclophosphamide + 75% IC50 value of CALE against MCF-7 cell lines shows 38% of cell viability (Table no 6 and 7).

The found combination effect the *Cassia Auriculata* leaves extract with cyclophosphamide shown synergistic anticancer effect on the human cervical cancer cells (HeLa cells) compared to the combination of *Cassia Auriculata* leaves extract with methotrexate.
One of the finding of the present study is that, when methotrexate, cyclophosphamide and CALE used alone they have shown good anticancer activity against HeLa cell lines and less anticancer activity in terms of decreased number of viable cells against MCF-7 cell lines. However, cytotoxic effect of combination of methotrexate and CALE decreased cytotoxic effect against HeLa cell lines and increased cytotoxic effects against MCF-7 cell lines. Cytotoxic effect of the combination of cyclophosphamide and CALE was more prominent against the HeLa cell lines in comparison with the MCF-7 cell lines when used alone or in combination with CALE.

VII. CONCLUSION

Cassia Auriculata L leaves has been traditionally used to treat various disorders. The present study showed that *Cassia Auriculata* leaf extract possess significant anticancer effect in terms of individual cytotoxic assay and combined effect with both leaf extract and synthetic drugs.

Hence, it is possible to reduce the dose of above chemotherapeutic agents when used in combination with *Cassia Auriculata* leaves for the treatment/management of specific cancers, hence minimizing the dose related adverse effects of the chemotherapeutic agents which themselves are responsible for death of the subjects in some of the cancer cases. This is only a preliminary finding; however detailed assay studies are needed to be performed to determine the optimal concentration of the both drugs chemotherapeutic agents as well the CALE.

Our study has opened a new arena in the treatment/management of certain cancers **using herbo-synthetic combinations**, which would lengthen the life expectancy as well as quality of life of the cancer patients, which itself is a great contribution if outcomes are successful.

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