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In vitro antioxidant and anticancer activity of flavonoids from *Cassia Tora* linn. leaves against human breast carcinoma cell lines

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ABSTRACT

In previous work, three flavonoids viz Luteolin-7-O-β-glucopyranoside (I), Quercetin-3-O-β-d-glucuronide (II) and Formononetin-7-O-β-D-Glucoside (III) were isolated from Cassia tora leaves. As an extension of work, the present study aimed to evaluate the anti-oxidant and anticancer potential of these flavonoids using in-vitro models. The ethanol extract was standardized using HPLC by comparing with known markers. The total phenolic content and flavonoid content was accessed using Folin-Denis and $AlCl_3$ method respectively. The antioxidant potential of the samples was evaluated using inhibition of hydroxyl radical, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and nitric oxide scavenging methods. Breast cancer (MCF7) cell line was used as the in-vitro cancer model for MTT assay. In the HPLC analysis, 4 flavonoids were identified by comparison with retention time of standard marker viz., quercetin, kaempferol, formononetin and luteolin. The total phenolic content and flavonoid content was found to be 18.60 % w/w and 9.5% w/w respectively. The radical scavenging activity of the isolated flavonoids decreased in the following order: quercetin (IC_{50} values 15, 14, 18 µg/mL) > luteolin (IC_{50} values 19, 21, 14 µg/mL) > formononetin (IC_{50} values 20, 23, 18 µg/mL) respectively. The flavonoids luteolin and quercetin possess potent anticancer property against breast cancer cells (MCF7) with IC_{50} value of 14 and 19 µg/mL respectively. This study suggests potential antioxidant and anticancer activities of the flavonoids of Cassia tora.

Key words: Flavonoids, *Cassia tora*, Breast cancer, MCF7, MTT assay.

INTRODUCTION

Cancer is a multistep disease incorporating physical, environmental, metabolic, chemical, and genetic factors, which play a direct and/or indirect role in the induction and deterioration of cancers. For the past decades, much research has been developed in order to discover natural compounds with potential anticancer activity and several plant-derived agents (e.g., paclitaxel, docetaxel; vinblastine, vincristine; topotecan, irinotecan, etoposide, etc.) have been successfully used for cancer treatments [1-3]. Among the anticancer medications, 69% of drugs approved between 1940 and 2002 are either natural products or developed based on knowledge gained from natural products (4,5]. Application of plants in the treatment of cancer seems to be inevitable, constituting the basis for modern medical science and providing a great source for new drugs [6].

Flavonoids are naturally occurring polyphenolic metabolites distributed throughout the plant kingdom. As natural products, flavonoids are regarded as safe and easily obtainable, making them ideal candidates for cancer chemoprevention or associated agents in clinical treatment [7]. Almost all artificial agents currently being used in cancer therapy are highly toxic and produce severe damage to normal cells [8]. The ideal anticancer agent would exert minimal adverse effects on normal tissues with maximal capacity to kill tumor cells and/or inhibit tumor growth [9]. The lack of substantial toxic effects for long-term therapies and inherent biological activity of flavonoids make them ideal candidates for new therapeutics [10]. Indeed, flavonoids have been shown to reveal cytotoxic activity toward various human cancer cells with little or no effect on normal cells, and this fact has stimulated large interest in developing of potential flavonoid-based chemotherapeutics for anticancer treatment [11]. Due to the polyphenolic structure, flavonoids have been found to possess both anti- and prooxidant action [12]. While antioxidant effect and ability to scavenge reactive oxygen species (ROS) have been shown to account for most of the reported biological effects of phenolic compounds, several recent studies have revealed that anticancer activities of flavonoids may be mediated through prooxidant action. Cancer cells exhibit a higher and more persistent oxidative stress level compared to normal cells, rendering malignant cells more vulnerable to being killed by drugs that boost increased ROS levels, such as some flavonoids [13].

Cassia tora Linn. (Family: Caesalpiniaceae) is an annual herb, 30-39 cm high growing in India as wasteland rainy season weed. The leaves and seeds of *Cassia tora* are found to be used in leprosy, psoriasis, ring worm, flatulence, colic, dyspepsia, constipation, cough, bronchitis and cardiac disorders in the Ayurvedic system of medicine. It is a rich source of anthraquinone glycosides and flavonoids [14,15]. Hence the present study was designed to evaluate antioxidant and cytotoxic effect of flavonoids isolated from the ethanol extract of *Cassia tora* leaves.

MATERIALS AND METHODS

Plant Material

The plant specimen for the proposed study was collected in Chennai, Tamilnadu. It was identified and authenticated by Dr. P. Jayaraman, Director, Plant Anatomy Research Centre (PARC), Tambaram, Chennai. A voucher specimen No. PARC/2011/2141 has been deposited for further references.

HPLC analysis

The qualitative analysis of the sample was performed according to the method of Boligon et al., 2012 [16]. The Jasco HPLC system consists of a pump (model Jasco PU2080, intelligent HPLC pump) with an automatic injection system programmed at 20 µl capacities per injection. The detector consists of a UV/VIS spectrophotometer (Jasco UV 2075) at a wavelength of 270 nm. The software used was Jasco Borwin version 1.5, LC-Net II/ADC system. The column was Thermo ODS Hypersil C18 (250 ×4.6 mm, 5 µm) in isocratic mode. The separation was achieved using a mobile phase of methanol, water and phosphoric acid (100:100:1, v/v/v) at a flow-rate of 1.5 ml/min. The effluent was monitored using UV detection at a wavelength of 270 nm. The mobile phase was filtered through 0.45 µm nylon filter prior to use.

Sample preparation

Powdered sample of *C. tora* leaves was weighed and transferred to a 250 ml flask fitted with a reflux condenser. About 78 ml of extraction solvent (EtOH:H_2O:HCl, 50:20:8) was added, refluxed on a hot water bath for 135 min, cooled at room temperature and transferred to a 100 ml volumetric flask. About 20 ml of methanol was added to the 250 ml flask and sonicated for 30 min, afterwards the solution was filtered and the filtrate was transferred to the 100 ml volumetric flask, the residue was washed on the filter with methanol. The wash was collected in the same 100 ml volumetric flask and diluted to volume. Identification is based on retention times and on-line spectral data in comparison with authentic standards.

Estimation of Phytoconstituents

The phytoconstituents present in dried coarsely powdered *Cassia tora* leaves was estimated using standard procedures.

Total Phenolic Content

The content of total phenolics in the powdered drug was determined by using Folin-Denis reagent. About 1 gram of the powder was extracted in an ultrasonic wave bath with 80 ml of aqueous ethanol solution (70% v/v) for 2 hr. After cooling, the volume of the solution was adjusted to 100 ml. The final solution was centrifuged prior to the

colorimetric determination. Tannic acid standards (10 - 110 mg/ml) were dissolved in 100 ml of aqueous ethanol solution (70% v/v) respectively. About 10 ml of Folin-Denis reagent was added to 1 ml of the extract solution and 1 ml of standard solution. After reacting for 3 min, 10 ml of 35% sodium carbonate solution was added and the test solution was diluted to 100 ml with water and mixed. After 45 min, an aliquot was centrifuged for 5 min. The absorption coefficient for the supernatant was measured at 745 nm. The total phenolic content of the extract were calculated using the mean regression coefficient from the standards [17].

Determination of Total Flavonoids

Total flavonoid content in dried plant material was estimated by spectrometric method. (Perkin-Elmer UV-Vis spectrometer Lambda 16 (Germany)) [18]. Dried powdered plant material (10 gm) was extracted by continuous mixing in 100 ml of 70% ethanol, 24 hr at room temperature. After filtration, ethanol was evaporated untill only water remained. Water phase was subsequently extracted with ethyl acetate. The extract was dried over anhydrous sodium sulphate, filtered and concentrated under vacuum up to a concentration of 1 gm/ml of extract. They were further diluted with ethyl acetate to obtain 0.01 gm/ml solutions. About 10 ml of the solution was transferred into a 25 ml volumetric flask, 1 ml of 2% $AlCl_3$ was added and the solution was filled to volume with methanol-acetic acid and was kept aside for 30 min. The absorbance was measured at 390 nm against the same solution without $AlCl_3$ being blank. The total flavonoid content was determined using a standard curve with quercetin (100 - 1000 mg/L) as the standard. Total flavonoid content is expressed as mg of quercetin equivalents (Q) /g of extract.

In-vitro antioxidant

Several concentrations ranging from 50-400 µg/ml of the ethanolic extract and concentrations ranging from 5-40 µg/ml of flavonoids Luteolin-7-O-β-glucopyranoside (I), quercetin-3-O-β-d-glucuronide (II) and Formononetin-7-O-β-D-Glucoside (III) were tested for their antioxidant activity in different in-vitro models [19]. It was observed that free radicals scavenging property of the test was found to be in a concentration dependent manner in all the models.

Nitric oxide radical scavenging assay

Nitric oxide was generated from sodium nitroprusside and measured by Griess reaction. Sodium nitroprusside (5 mM) in standard phosphate buffer saline solution (0.025 M, pH: 7.4) was incubated with different concentrations of ethanolic extract (50-400 µg/ml) dissolved in phosphate buffer saline (0.025 M, pH: 7.4) and the tubes were incubated at 25^{o}C for 5 hr. Control experiments without the test compounds but equivalent amounts of buffer were conducted in an identical manner. After 5 hr, 0.5 ml of incubation solution was removed and diluted with 0.5 ml of Griess reagent (1% sulphanilamide, 2% O-phosphoric acid and 0.1% naphthyl ethylene diamine dihydrochloride). The procedure was repeated with isolated flavonoids (5-40 µg/ml). The absorbance of the chromophore formed during diazotization of nitrite with sulphanilamide and its subsequent coupling with naphthyl ethylene diamine was read at 546 nm.

DPPH radical scavenging activity

The free radical scavenging activity was measured in terms of hydrogen donating or radical scavenging ability, using the stable radical, DPPH. About 0.1 mM solution of DPPH in methanol was prepared and 1 ml of this solution was added to 3 ml of the different concentration of ethanolic extract (50-400 µg/ml), in different test tubes. The procedure was repeated with isolated flavonoids (5-40 µg/ml), and control (without the test compound, but with an equivalent amount of methanol). The mixture was shaken and allowed to stand at room temperature for 30 min and the absorbance was measured at 517 nm using a spectrophotometer. Decrease in absorbance of the reaction mixture indicates higher free radical scavenging activity.

Hydroxyl radical scavenging activity

The assay was performed by adding 0.1 ml EDTA, 0.01 ml of ferric chloride, 0.1 ml of hydrogen peroxide, 0.36 ml of deoxyribose, 1 ml of ethanol extract (50-400 µg/ml), 0.33 ml of phosphate buffer (50 mM. pH 7.4), 0.1 ml of ascorbic acid in sequence and incubated at 37^{o}C for 1 hr. The procedure was repeated with isolated flavonoids (5-40 µg/ml), and control (without the test compound, but with an equivalent amount of methanol). A 1.0 ml portion of the incubated mixture was mixed with 1.0 ml of 10% tri chloro acetic acid and 1.0 ml of 0.5% thio barbituric acid to develop the pink chromogen, which was measured at 532 nm. All determinations were performed in 6 replicates. Percentage inhibition was calculated by using the formula,

$$\text{Percentage inhibition (\%)} = \frac{(\text{Absorbance of control} - \text{Absorbance of test})}{\text{Absorbance of control}} \times 100$$

***In-Vitro* Anticancer Activity**

Cell Line and Culture

MCF7 cell line was obtained from National Centre for Cell Sciences, Pune (NCCS). The cells were maintained in minimal essential media supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 µg/mL) in a humidified atmosphere of 50 µg/mL CO_2 at 37°C.

Reagents

MEM was purchased from Hi Media Laboratories; fetal bovine serum (FBS) was purchased from Cistron laboratories; Trypsin, methylthiazolyl diphenyl-tetrazolium bromide (MTT), and Dimethyl sulfoxide (DMSO) were purchased from Sisco research laboratory chemicals, Mumbai. All of the other chemicals and reagents were obtained from Sigma-Aldrich, Mumbai.

Procedure

Cells (1×10^5/well) were plated in 5 mL of medium/well in 6-well plates (Costar Corning, Rochester, NY). After 48 hours incubation, the cell reaches the confluence. Then, cells were incubated in the presence samples for 24–48 h at 37°C. After removal of the sample solution and washing with phosphate-buffered saline (pH 7.4), 1 mL/well (5 mg/mL) of 0.5% 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide cells (MTT) phosphate-buffered saline solution was added. After 4 h incubation, 0.04 M HCl/isopropanol was added. Viable cells were determined by the absorbance at 570 nm. Measurements were performed, and the concentration required for a 50% inhibition of viability (IC_{50}) was determined graphically. The absorbance at 570 nm was measured with a UV-Spectrophotometer using wells without sample containing cells as blanks [20]. The effect of the samples on the proliferation of MCF-7 cells was expressed as the % cell viability, using the following formula:

$$\text{\% cell viability} = \frac{A_{570} \text{ of treated cells} \times 100}{A_{570} \text{ of control cells}}$$

Statistical analysis

Level of significance of all the parameters was expressed as the arithmetic mean ± SEM and was analyzed by one-way analysis of variance (ANOVA), followed by Dunnett's "t" test. P value less than 0.05 ($P< 0.05$) was the critical criterion for statistical significance.

RESULTS AND DISCUSSION

In an earlier work, three flavonoids were isolated viz., Luteolin-7-O-β-glucopyranoside (**I**), quercetin-3-O-β-d-glucuronide **(II)** and Formononetin-7-O-β-D-Glucoside **(III**) [21]. The therapeutic potential of flavonoids and the necessity for scientific validation in popular medicine have prompted increased interest in the field.

HPLC analysis

Qualitative analysis of the ethanol extract of the *Cassia tora* leaves demonstrates the presence of seven components. In the present investigation, three flavonoids were identified at 254 nm using peak area by comparison to a retention times of the standard marker, quercetin, Kaempferol, formononetin and luteolin (Figure 1a and 1b). The main difference was in peak, eluted at 12.07 min, 19.62 min and 31 min respectively. These results show therefore, that *Cassia tora* leaves are a rich source of the important biologically active flavonoids, quercetin, kaempferol, formononetin and luteolin.

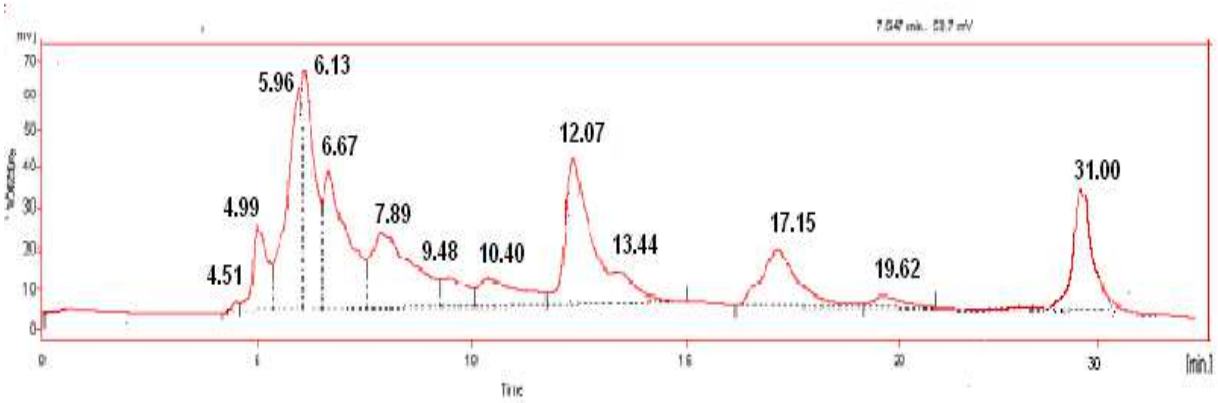

Figure 1a: HPLC fingerprint of ethanol extract of *Cassia tora* leaves

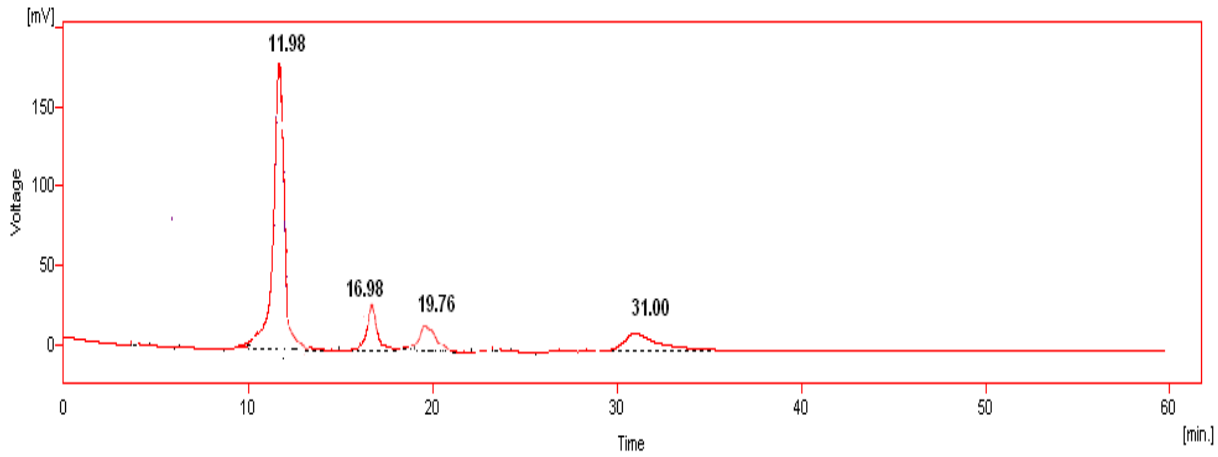

Figure 1b: HPLC fingerprint of reference standards
1: Quercetin 2: Kaempferol 3: Formononetin 4: Luteolin

Estimation of Phytoconstituents

Flavonoids are the most diverse and widespread group of natural compounds and are likely to be the most important natural phenolics. These compounds possess a broad spectrum of chemical and biological activities including radical scavenging activity. The contents of total phenols and flavonoids were estimated by the standard curves and expressed as gallic acid equivalents for total phenols and quercetin equivalents for flavonoids. The amount of total phenolic content and total flavonoid content in the ethanol extract showed 18.60 mg/g dry weight, expressed as Gallic acid equivalents and 9.5% mg/g expressed as quercetin equivalents respectively.

Anti-oxidant activity

Antioxidants are significant in the prevention of human illness and may function as free radical scavengers, complexes of pro-oxidant metals, reducing agents and quencher of singlet oxygen formation. Free radicals posses the ability to reduce the oxidative damage associated with many disease including neurodegenerative diseases, cancer, cardiovascular disease, cataracts and AIDS [22]. Antioxidants through their scavenging power are useful for the management of these diseases. The results were tabulated in Table 1.

Inhibition of Hydroxyl radical: The potentially reactive hydroxyl radicals can cause oxidative damage to DNA, lipids and proteins. The effect of ethanolic extract and isolated flavonoids on the inhibition of free radical-mediated deoxyribose damage was assessed by means of iron (II)-dependent DNA damage assay, which showed significant results. The IC_{50} value of ethanolic extract, standard and isolated flavonoids I, II, III being of 270 µg/mL, 18 µg/mL, 19 µg/mL, 15 µg/mL, and 20 µg/mL respectively.

Table 1: Free radical scavenging activity of ethanol extract and flavonoids from *Cassia tora* leaves

Drugs	Concentration (µg/ml)	Hydroxyl radical inhibition (%)	DPPH radical inhibition (%)	Nitric oxide inhibition (%)
Ethanol extract	50	11.24±1.14	13.36±1.36	14.14±1.29
	100	18.45±1.72	28.26±2.14	30.36±1.40
	200	39.13±1.32	56.25±1.46**	61.02±1.72**
	300	54.24±0.66**	80.14±2.52**	82.31±1.79**
	400	83.24±1.36**	97.17±2.40**	94.15±1.50**
	IC_{50}	**270 µg/ml**	**190 µg/ml**	**130 µg/ml**
Compound **I**	5	13.06±1.46	10.90±1.48	19.24±1.37
	10	24.41±2.10	21.69±2.13	34.42±2.15
	20	52.45±1.60**	48.83±1.56**	68.11±3.20**
	30	79.08±2.20**	72.56±3.22**	80.25±0.36**
	40	87.32±3.28**	90.54±1.38**	95.68±2.59**
	IC_{50}	**19 µg/ml**	**21 µg/ml**	**14 µg/ml**
Compound **II**	5	19.32±0.08	18.43±1.22	11.23±1.36
	10	33.48±1.64	34.21±1.09	28.32±2.20
	20	68.40±2.60**	69.68±0.83**	57.28±1.27**
	30	80.20±3.02**	85.35±1.60**	82.43±1.47**
	40	96.27±1.03**	98.08±0.94**	93.27±2.38**
	IC_{50}	**15 µg/ml**	**14 µg/ml**	**18 µg/ml**
Compound **III**	5	12.68±1.20	11.97±1.67	12.20±1.58
	10	26.46±1.89	22.24±0.05	27.21±1.69
	20	50.90±2.11**	44.90±1.12	54.35±1.50**
	30	74.43±1.45**	68.15±0.93**	69.12±1.40**
	40	88.16±2.36**	85.36±3.17**	90.14±0.75**
	IC_{50}	**20 µg/ml**	**23 µg/ml**	**18 µg/ml**
Ascorbic acid	5	14.31±1.20	12.38±1.80	10.81±1.72
	10	30.46±3.09	20.68±1.28	18.68±0.62
	20	59.68±1.38**	41.36±1.82	28.26±2.31
	30	88.25±1.49**	62.84±1.82**	44.36±1.28**
	40	94.60±2.30**	80.82±2.18**	70.10±2.16**
	IC_{50}	**18 µg/ml**	**24 µg/ml**	**35 µg/ml**

*Values are mean ±SEM of 6 parallel measurements. Statistical significant test for comparison was done by ANOVA, followed by Dunnet's 't' test (n=6). All the values are statistically significant at **P< 0.01.*

Inhibition of DPPH radical: DPPH assay is considered a valid and easy way to evaluate scavenging activity of antioxidants, since the radical compound is stable and does not have to generate as in other radical assays. DPPH radicals react with suitable reducing agents and then electrons become paired off and the solution loses colour with the number of electrons taken up [19]. The potential decrease in the concentration of DPPH radical due to the scavenging ability of ethanolic extract and isolated flavonoids I, II, III showed significant free radical scavenging activity of about 97%, 85%, 98%, and 90% respectively at higher doses with the IC_{50} value being 190 µg/ml, 21 µg/mL, 14 µg/mL and 23 µg/mL respectively.

Nitric oxide scavenging activity: Nitric oxide (NO) is a free radical produced in mammalian cells, involved in the regulation of various physiological processes. However, excess production of NO is associated with several diseases. Nitric oxide is a very unstable species under aerobic conditions. It reacts with O^2 to produce stable product nitrate and nitrite through intermediates NO_2, N_2O_4 and N_3O_4. It is estimated by using Griess reagent and in presence of test compound which is a scavenger that the amount of nitrous acid will decrease [19]. The scavenging of nitric oxide by ethanolic extract, and isolated flavonoids, was concentration dependent. The IC_{50} value of ethanolic extract, standard and isolated flavonoids I, II, III being of 130 µg/mL, 35 µg/mL, 14 µg/mL,18 µg/mL and 18 µg/mL respectively. On a comparative basis, the flavonoid formononetin showed better activity in quenching nitric oxide with an IC_{50} value of 14µg/ml.

***In Vitro* Anticancer Activity**

In order to understand the effect of ethanol extract and isolated flavonoids of *Cassia tora* on human breast cancer cells, experiments were conducted using cultured MCF7 cell lines. MTT assay is a well-established *in vitro* method for assessing cytotoxicity against cancer cell lines. Results of the cell viability were measured using MTT assay (Figure 2). The viability of MCF7 cells was remarkably decreased in a dose dependent manner after treatment with ethanol extract and isolated flavonoids at all concentrations (5–160 μg/mL) for 24 hour (Table 2). The IC_{50} values were determined from the graphs of the ethanol extract and flavonoids on MCF7 cell lines.

Table 2: Cytotoxicity effect of ethanol extract and isolated flavonoids on MCF7 cell line

S.No	Concentration (µg/ml)	% of cell viability			
		Ethanol extract	Luteolin	Quercetin	Formononetin
1	160	20.8	8.1	11.8	13.7
2	80	33.4	15.3	20.4	23.6
3	40	50.6	26.7	32.5	46.2
4	20	75.3	34.6	48.6	60.4
5	10	87.5	60.3	69.5	74.3
6	5	90.6	70.4	75.8	83.7
7	IC_{50}	40	14	19	34

The flavonoid luteoloin and quercetin showed potent cytotoxic effects with the IC_{50} values of 14 and 19 μg/mL in MCF7 cell line whereas ethanol extract and the flavonoid formononetin gave the IC_{50} values of 40 and 34 μg/mL respectively in MCF7 cell line. According to the National Cancer Institute (NCI), the criteria of cytotoxic activity for the crude extract are IC_{50} < 20 μg/mL [23]. The IC_{50} values indicated that the anticancer activity of isolated flavonoid fraction was higher than ethanol extract against MCF7 cell lines (Figures 2), and the IC_{50} of the isolated flavonoids luteoloin and quercetin fall within the NCI criteria, thus the flavonoids luteoloin and quercetin were considered as promising anticancer potential. Study shows relative activity being as luteolin> quercetin > formononetin.

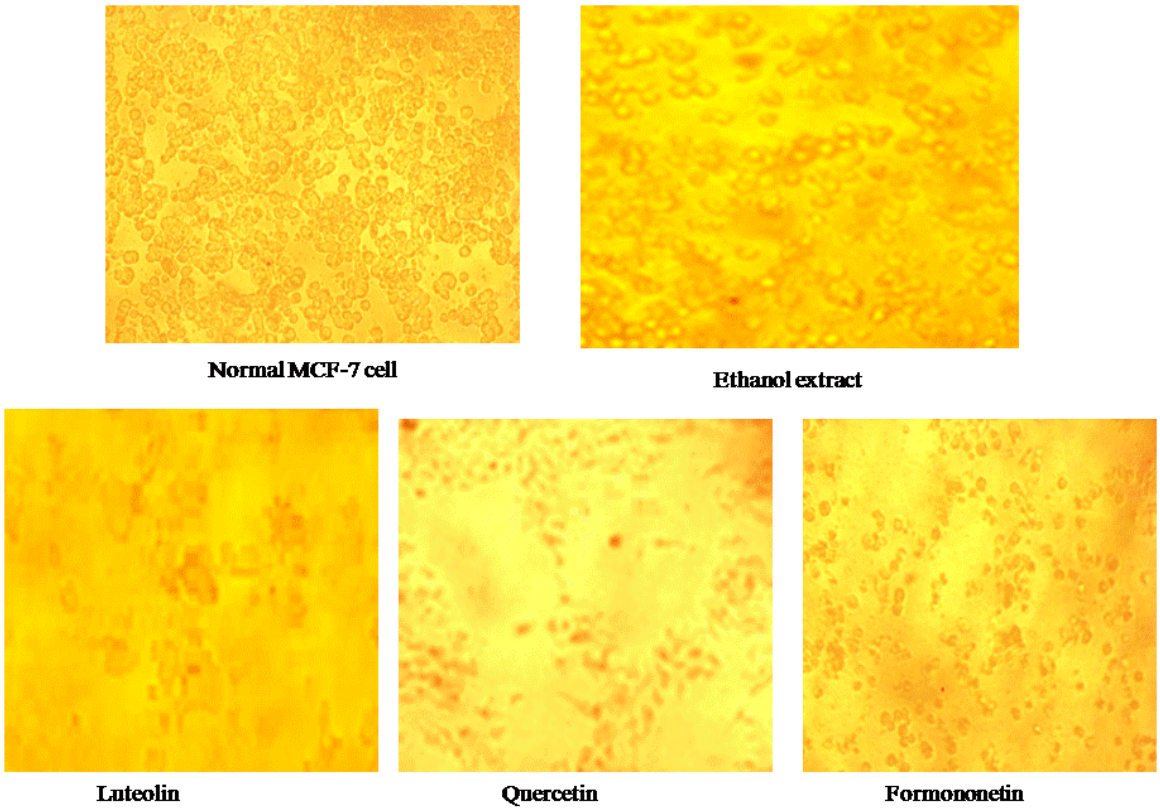

Figure 2: Morphological features in MCF-7 cells treated with ethanol extract and flavonoids

The anticancer efficacy of flavonoids is due, at least in part to their ability to induce apoptosis of tumor cells [24]. Most breast cancers are heterogeneous and consist of ER-positive and -negative cells. Therefore, agents that are able to inhibit the growth of both ER-positive and -negative tumors are of great interest [25]. Dietary flavonoids seem to display such dual activity, inhibiting both receptor-positive and -negative breast cancer cells.

CONCLUSION

In conclusion, considering that many chemotherapeutic agents against tumor cells without sparing normal cells remain a major obstacle and development of multidrug resistance further limits chemotherapy in cancer, the promising results will stimulate the development of flavonoids for cancer chemoprevention and chemotherapy. Beneficial effects have been described also by combining certain flavonoids with standard chemotherapeutic drugs leading to decrease in the dosage and associated toxicity while targeting specific resistance mechanisms. In this way, the genotoxic damage caused by standard chemotherapeutics to normal cells can be diminished, thereby reducing the chance of developing of secondary cancers [26]. Further work is certainly needed to develop and produce novel drugs from natural sources introducing structural variations into the backbone of flavonoids and modifying their structures to further improve biological activity and exhibit more potent anticancer effects [27].

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