



Contents lists available at ScienceDirect

## Asian Pacific Journal of Tropical Disease

journal homepage: www.elsevier.com/locate/apjtd



## Document heading

Anticancer activity of Rhein isolated from *Cassia fistula* L. flowerV Duraipandiyani<sup>1,2</sup>, A Albert Baskar<sup>3</sup>, S Ignacimuthu<sup>2</sup>, C Muthukumar<sup>1</sup>, N A Al-Harbi<sup>1</sup>.<sup>1</sup> Department of Botany and Microbiology, Addiriyah Chair for Environmental Studies, College of Science, King Saud University, Riyadh 11451, Saudi Arabia<sup>2</sup> Division of Ethnopharmacology, Entomology Research Institute, Loyola College, Chennai 600 034, India<sup>3</sup> College of Applied Medical Science, King Saud University, Riyadh 11451, Kingdom of Saudi Arabia

## ARTICLE INFO

## Article history:

Received 15 June 2012

Received in revised form 5 July 2012

Accepted 7 October 2012

Available online 28 October 2012

## Keywords:

Anticancer activity

*Cassia fistula*

Rhein

Colon cancer

## ABSTRACT

**Objective:** To evaluate anticancer activity of Rhein isolated from ethyl acetate extract of *Cassia fistula* (*C. fistula*) flowers against colon cancer celllines. **Methods:** Rhein was tested against human colon adenocarcinoma cell line COLO 320 DM and normal cell line VERO. Rhein exhibited minimal cytotoxic effect toward VERO cells. The compound rhein was identified by spectroscopical method. **Results:** Rhein was found to be cytotoxic toward COLO320 DM cells in a concentration and time dependant manner. Rhein exhibited 40.59%, 58.26%, 65.40%, 77.92% and 80.25% cytotoxicity at 200  $\mu$ g/mL concentration for 6, 12, 24, 48 and 72 h incubation time. The IC50 values of Rhein were 100, 25, 15, and 12.5  $\mu$ g/mL for 12, 24, 48 and 72 h incubation respectively. The COLO 320DM cells treated with Rhein showed the characters of apoptosis at 24 h period of treatment at 6.25 and 12.5  $\mu$ g/mL. Apoptosis in early stages was 2.29% at 6.25  $\mu$ g/mL and at late stages it was 1.94%. When the concentration was increased to 12.5  $\mu$ g/mL, apoptosis was 4.36% at early stages and 5.61% at late stages, respectively. **Conclusion:** The results indicated that Rhein could be utilized in the treatment of cancer.

## 1. Introduction

Medicinal plants are playing an important role as a source of effective anticancer agents and it is significant that 60% of currently used anticancer agents are derived from natural sources including plants<sup>[1]</sup>

*Cassia fistula* (*C. fistula*) L., (Leguminosae), a semi-wild Indian Labernum (also known as the Golden Shower), is distributed in various countries including Asia, South Africa, Mexico, China, West Indies, East Africa and Brazil. It is an ornamental tree with beautiful bunches of yellow flowers. *C. fistula* exhibited significant antimicrobial activity and showed properties that support folkloric use in the treatment of some diseases as broad-spectrum antimicrobial agent<sup>[2]</sup>. The whole plant is used to treat diarrhea; seeds, flowers and fruits are used to treat skin diseases, fever, abdominal pain and leprosy by traditional people<sup>[3]</sup>. The seeds are useful in jaundice<sup>[4]</sup>, biliousness, skin disorders and swollen throat. *C. fistula* has been reported to possess anti-inflammatory<sup>[5]</sup>.

The leaves are possessing hepatoprotective<sup>[6]</sup>, wound healing<sup>[7]</sup>, hypoglycaemic activity<sup>[8]</sup>.

This plant has a strong tendency to contain anthraquinone derivatives. Leaves and flowers contain anthraquinone, tannin, oxyanthraquinone, rhein and volatile oils<sup>[9]</sup>. The present study was undertaken to assess the anticancer effect of Rhein isolated from ethyl acetate extract of *C. fistula* flowers.

## 2. Materials and methods

## 2.1. Plant material

*C. fistula* flowers were collected from Loyola College Campus, Chennai, India. The plant was authenticated by Dr. S. Amerjathy, Department of Botany, Presidency College, Chennai, India. A voucher specimen (ERIC-D-73) was deposited at the herbarium of Entomology Research Institute, Loyola College, Chennai.

## 2.2. Preparation of crude extract

The extracts were taken using cold percolation method.

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Grant No.ERI/02/2007

Fresh flowers were collected (9 kg) and shade dried at room temperature and ground in a manual mill. The powder (1 kg) was extracted with 3 l (1:3 w/v) of hexane for 48 h. The extract was filtered through a Buchner funnel with Whatman number 1 filter paper. The filtrate was evaporated to dryness under reduced pressure using rotary evaporator at 40 °C. The remains of the plant material were extracted with chloroform (11 g), ethyl acetate (17 g), methanol (20) and water (13 g) sequentially in a similar manner. The crude extracts were stored at 4 °C until further use.

### 2.3. Isolation of active compound

The crude ethyl acetate extract (10 g) was subjected to column chromatography over silica gel (200 g–acme's 100–200 mesh) and eluted with hexane followed by the combination of hexane: ethyl acetate ranging from 95:5 to 100. 117 fractions were collected in 200 mL conical flasks. After checking TLC, the fractions were combined in to 24 fractions. Fraction 18 showed single spot on TLC ( $R_f = 0.36$ ) and yielded 210 mg; this fraction was eluted using hexane: ethyl acetate (10:9) as mobile phase solvent system (Figure 1). The spot turned pink on exposure to ammonia vapor; it indicated the presence of anthraquinone. The compound was subjected to spectroscopic analysis.

### 2.4. Spectroscopic analysis

IR, <sup>1</sup>H-NMR, <sup>13</sup>C NMR and MASS were taken with help of equipments available at Nicholas Primal Pvt. Ltd. Ennore, Chennai, India and used to identify the isolated compound.

### 2.5. Cell culture

Human colon cancer cell line COLO 320 DM was purchased from National Center for Cell Science (NCCS, Pune). The cells were cultured in 75 cm<sup>2</sup> flask containing Roswell park memorial institute 1640 (RPMI 1640; Sigma) medium, supplemented with 10% Fetal bovine Serum (FBS; invitrogen), 1.5 g/L sodium bicarbonate (Gibco), 100 U/mL of penicillin (Gibco), 100 μg/mL streptomycin (Gibco) and 0.25 μg/mL of Amphotericin B (Gibco). Cells were cultured as monolayers in culture flasks at 37 °C under a humidified atmosphere of 5% CO<sub>2</sub> in air. All experiments were performed using cells from passage 20 or less.

VERO cells were purchased from National Center for Cell Science (NCCS, Pune). The cells were cultured in 75 cm<sup>2</sup> flask containing Dulbecco's modified Eagle's medium (DMEM; Sigma), supplemented with 10% Fetal bovine Serum (FBS; invitrogen), 100 U/mL penicillin (Gibco) 100 μg/mL streptomycin (Gibco), and 0.25 μg/mL of Amphotericin B (Gibco). Cells were cultured as monolayers in culture flasks at 37 °C under a humidified atmosphere of 5% CO<sub>2</sub> in air. All experiments were performed using cells from passage 20 or less. During the experiment time, the serum medium was replaced by serum free medium containing 200–1.5 μg/mL of the compound, which was dissolved in DMSO. The stock was maintained at –20 °C. The final working concentration of DMSO was less than 1.0%.

### 2.6. Cytotoxicity assay

The survival of cells was determined by MTT assay as described by Mosmann<sup>[10]</sup>. Briefly, the tetrazolium salt MTT (3–(4,5–dimethylthiazol–2–yl)–2,5–diphenyl tetrazolium bromide) (Sigma) solution was prepared freshly as 0.5 mg/mL in phosphate–buffered saline (Gibco) just before use. Cells were seeded in 96– well plates (1×10<sup>5</sup> cells/well) and allowed for attachment for six hours and the cells were treated with various concentrations of compound (1.5–200 μg/mL serial dilution) for 6, 12, 24, 48 and 72 h; then 100 μL of MTT dye (50 μg) was added to each well. Control groups were maintained with the same amount of DMSO. The plates were incubated in a CO<sub>2</sub> incubator for 4 h. After 4 h incubation period, the inhibition of cell growth induced by the tested compound was detected by eluting the dye with DMSO and optical density (OD) was reordereed using a 96 well micro plate reader (BIO–RAD, model 680, USA) at 570 nm.

### 2.7. Annexin–V FITC assay

The ability of the compound to induce apoptosis was studied by staining the treated cells with annexin V FITC and propidium iodide. COLO320DM cells were plated in a 25 cm<sup>2</sup> flasks at a density of 1×10<sup>7</sup> cells and allowed to form confluence and the cells were treated with 6.25 and 12.5 μg/mL concentrations in 1% serum containing medium, control cells were also treated with the same as the treatment group without compound and the final concentration of DMSO was kept below 1%. At the end of the treatment cells were washed with Phosphate buffered saline (PBS) and resuspended in binding buffer (10 Mm HEPES/NaOH Ph 7.4, 140 Mm NaCl, 2.5 Mm CaCl<sub>2</sub>). Aliquots of cells (100 μL) were incubated with 5 μL of Annexin FITC (fluorescein isothiocyanate–conjugated) and incubated in dark for 15 m at room temperature and stained with propidium iodide (5 μg/mL). The cells were then gently vortexed and 10 000 events were acquired and analysed using Becton Dickinson FACS caliber. In brief, early apoptotic cells were defined as those cells exhibiting a fluorescein isothiocyanate–conjugated annexin V–positive and propidium iodide–negative staining pattern. Necrotic cells exhibited popidium iodide– positive and Annexin–V FITC negative staining pattern.

## 3. Results

### 3.1. Identification of active compound

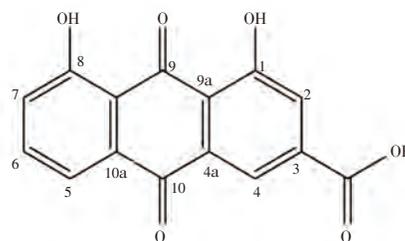
The present study deals with the separation of an active compound Rhein from ethyl acetate extract of *C. fistula* flower which was tested against cancer cell line and normal cell line. The active compound was identified as Rhein (1,8–dihydroxyanthraquinone–2–carboxylic acid) (Figure 2). The structural identification of compound was carried out by IR, MS, <sup>1</sup>H NMR and <sup>13</sup>C NMR spectra as follows: the EI–MS: m/z 284, 267, 256, 239, 228, 211, 183, 155, 142, 126. It showed the molecular ion at m/z 284, which is corresponding

to the molecular formula  $C_{15}H_8O_6$  of Rhein<sup>[11]</sup>. The  $^1H$  NMR spectrum revealed five aromatic protons of which two were broad singlets due to meta coupling.  $^1H$  NMR (300 MHz, DMSO): 11.9 (1H, brs, C1-OH), 11.5 (1H, brs, C8-OH), 8.13 (1H, brs, C2-H), 7.40 (1H, d,  $J = 8.5$  Hz, C5-H), 7.73 (1H,

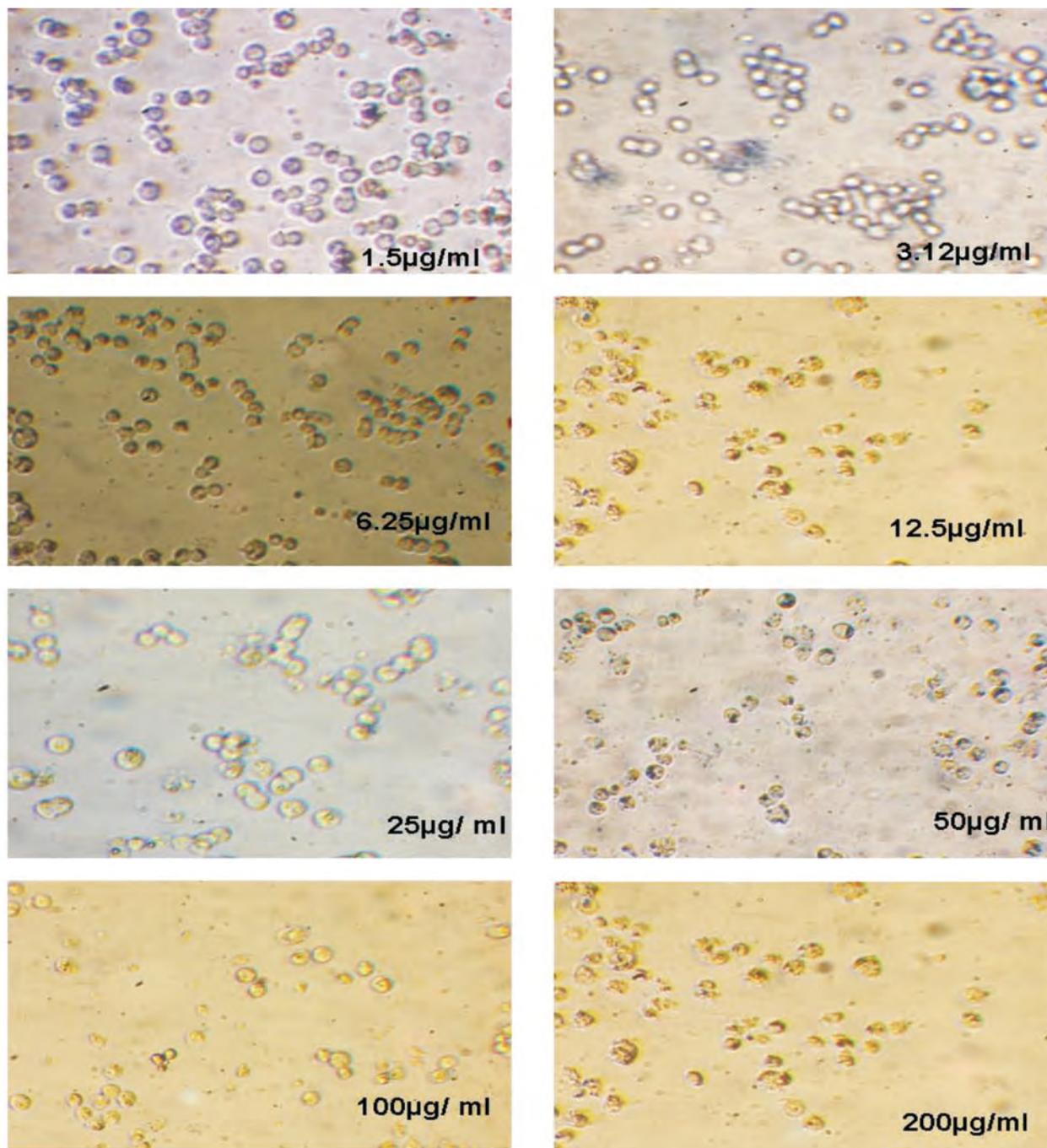


**Figure 1.** The compound rhein was eluted with hexane; ethylacetate(10:9)(Under UV light-254-360nm)

m, C6-H), 7.75 (1H, brs, C4-H), 7.81(1H, d,  $J = 8.0$  Hz, C7-H) corresponded to rhein<sup>[12]</sup>.  $^{13}C$  NMR [(300 MHz, dimethyl sulfoxide (DMSO))  $\delta$  ppm: 161.2 (C-1), 124.2 (C-2), 165.6 (C-3), 119.0 (C-4), 124.7 (C-5), 138.5 (C-6), 124.7 (C-7), 161.5 (C-8), 181.2 (C-9), 181.2 (C-10), 130.0 (C-4a), 118.9 (C-8a), 118.7



**Figure 2.** Rhein(1,8-dihydroxyanthraquinone-3-carboxylic acid) isolated from ethyl acetate extract of *C.fistula* flower.



**Figure 3.** Cytotoxic effect of Rhein against COLO 320DM

(C-9a), 133.3 (C-10a), 191.4 (3-COOH) which corresponded to those reported<sup>[11]</sup>.

**Table 1.**

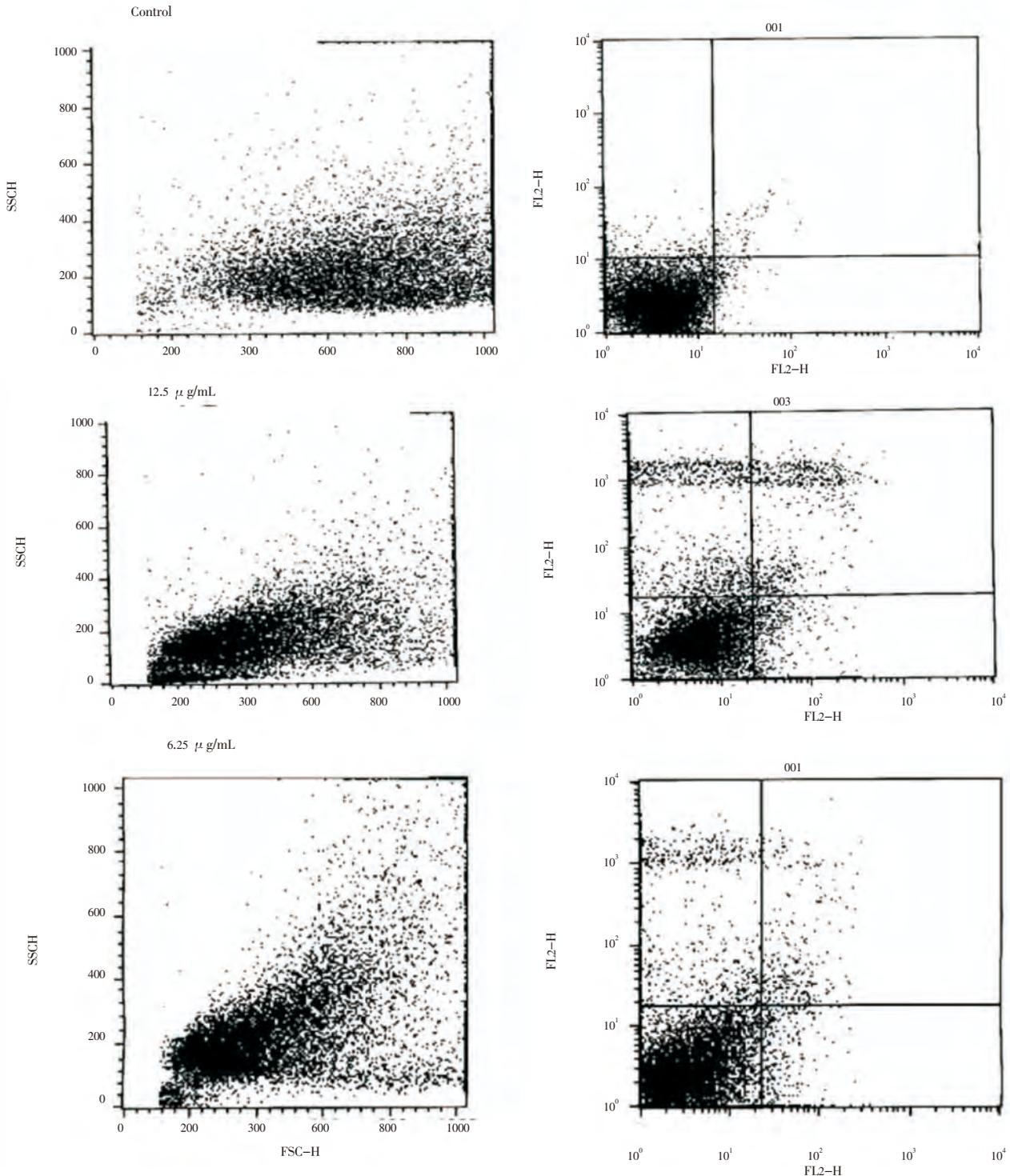
IC50 concentrations of rhein based on cytotoxic studies in COLO320 DM cells.

Cell lines	IC50 values in $\mu\text{g/mL}$			
	12 h	24 h	48 h	72 h
Colon cancer cell	100	25	15	12.5
VERO	-	-	>200	>200

-; no toxicity

**3.2. Effects of Rhein on colon cancer cell line and VERO cell line (cytotoxicity assay)**

Cancer specific cytotoxic effects of the isolated compound Rhein (1,8-dihydroxyanthraquinone-3-carboxylic acid) was studied using a carcinoma (human colon adenocarcinoma cell-COLO 320 DM) and a non carcinoma cell line (VERO). The degree of toxicity of Rhein towards the two cell lines was determined using MTT assay. The assay is a calorimetric assay based on the ability of the viable cells to reduce a soluble yellow tetrazolium salt (MTT) to blue formazan



**Figure 4.** Induction of apoptosis by Rhein.

**Table 2.**

Percentage of apoptosis induced by rhein (1,8–dihydroxyanthraquinone–3–carboxylic acid) using flow cytometer.

	Viable cell (%)	Necrotic cells (%)	Late apoptotic (%)	Early apoptotic (%)
Control	100.00	–	–	–
6.25 $\mu$ g/mL	86.40	9.37	1.94	2.29
12.5 $\mu$ g/mL	75.31	14.72	5.61	4.36

Control: negative control.

crystals.

The compound Rhein exhibited minimal cytotoxic effect toward VERO cells. Among the tested cell lines, a differential behavior was observed in colon cancer cell in a dose and time dependant manner. The viability of colon cancer cell was significantly decreased at 200  $\mu$ g/mL concentration. Maximum growth inhibitory effects (80.25%) were observed at 200  $\mu$ g/mL concentration after 72 h.

Rhein was first tested for cytotoxicity for COLO 320 DM cells and then the effects of the compound on VERO cells were detected. Rhein was found to be cytotoxic in COLO320 DM cells in a concentration and time dependant manner. Rhein exhibited 40.59%, 58.26%, 65.40%, 77.92% and 80.25% cytotoxicity at 200  $\mu$ g/mL concentration for 6, 12, 24, 48 and 72 h incubation time. The IC50 values Rhein were 100, 25, 15, and 12.5  $\mu$ g/mL for 12, 24, 48 and 72 h incubation respectively. Cytotoxicity of Rhein was tested against COLO 320 DM at various concentrations for 72 h (Table 1 & Figure 3) Based on MTT results it is clear that Rhein possessed minimal cytotoxicity towards VERO cells till the maximum time of incubation (72 h). Rhein did not show any toxicity towards VERO cells upto 200  $\mu$ g/mL concentration for 6, 12, and 24 h incubation time, but the same showed 31.14% and 45.99% toxicity towards VERO cells at 200  $\mu$ g/mL concentration for 48 and 72 h incubation. The IC50 values of Rhein in VERO cells were not detectable up to the tested concentration for various incubation periods.

### 3.3. Annexine V FITC assay

The cytotoxic activity of compound Rhein was much higher in colon cancer cell line. It showed the highest activity with IC50 of 12.5  $\mu$ g/mL for 72 h incubation period. The compound was able to induce apoptosis in colon cancer cells, 2.29% of cells were at early apoptosis and 1.94% of cells were at late apoptosis at 6.25  $\mu$ g/mL concentration at 24h incubation period. At 12.5  $\mu$ g/mL concentration 4.36% of cells were found to be at early apoptotic stage and 5.61% of cells were at late apoptotic stage (Table 2 & Figure 4).

## 4. Discussion

Current studies involved in developing effective cancer-preventive approaches are focused on the utilization of natural bioactive agents that are able to induce selective apoptosis in cancer cells<sup>[13]</sup>. Anthraquinones are biologically active substances that are useful to treat different ailments. We isolated Rhein as active substance from *C. fistula* flowers. The same compound was previously reported

from some other plants. Sun *et al*<sup>[14]</sup> chromatographed and purified emodin, chrysophanol and Rhein from Rheum officinale extract. Wang *et al*<sup>[15]</sup> separated and determined active anthraquinone components physcion, chrysophanol, aloe–emodin, emodin, and Rhein from the Chinese herb Polygonum multiflorum.

In the present study cytotoxicity was performed to determine the toxicity of isolated compound from *C. fistula* flowers. Two types of cell lines namely, VERO and COLO 320 DM were used for the study. From the results of cytotoxicity (against COLO 320 DM cell), Rhein showed notable toxicity towards cancer cell line and minimal toxicity towards VERO cells. And also at 12.5 and 6.25  $\mu$ g/mL concentration, Rhein exhibited 42.22% and 30.31% toxicity at 24 h incubation with no toxicity on VERO cells. Hence the above concentrations were fixed for apoptosis assay.

The compound Rhein showed 80.25% toxicity against COLO320DM cells at 200  $\mu$ g/mL (72 h) and also induced apoptosis in COLO 320 DM cell line with an IC50 at 12.5  $\mu$ g/mL for 24 h treatment. The COLO 320DM cells treated with Rhein showed the characters of apoptosis at 24 h period of treatment at 6.25 and 12.5  $\mu$ g/mL. Percentage of apoptosis in early stages was 2.29% at 6.25  $\mu$ g/mL and 1.94% at late stages. When the concentration was increased to 12.5  $\mu$ g/mL, the apoptosis was 4.36% at early stages and 5.61% at late stages, respectively. In our experiments, cell growth was also suppressed after higher concentrations of Rhein without any side effect. Isolated compound is an anthraquinone. Anthraquinones act as anticancer agents<sup>[16]</sup>. Siddhuraju *et al*<sup>[17]</sup> reported that alcoholic extract of *C. fistula* leaves and stem bark showed significant antioxidant properties. Naturally occurring dietary antioxidants found in medicinal plants could in theory serve as alternatives to chemically designed anticancer agents<sup>[18]</sup>.

Moreover, rhein has reported activation of p53/p21 signaling pathway with in rhein–induced apoptosis of human lung cancer cells, cervical cancer cells, and hepatoblastoma cells<sup>[19,20]</sup>; it affects oxidative phosphorylation by inhibiting electron transport and ATP–driven proton uptake<sup>[21]</sup>; and it was observed to inhibit the oxidation of NADH by ferricyanide in submitochondrial particles from the heart. The effect on mitochondrial oxidative functions and the finding that Rhein influences the aerobic and anaerobic glycolysis led to the hypothesis that Rhein could exhibit anti–neoplastic activity<sup>[22,23]</sup>. Furthermore, Rhein has been found to morphologically alter the cytoskeleton and thereby affect the plasma membrane and intracellular membranes<sup>[24]</sup>.

Natural products have been used as anticancer agents, such as vincristine and vinblastine from *Catharanthus roseus*, taxol and docetaxel from *Taxus brevifolia* and

camptothecins from *Camptotheca acuminata*[25].

In recent years, plant derived–bioactive substances that are capable of selectively arresting cell growth in tumor cells have received considerable attention in cancer chemopreventive approaches[26]. Many studies have been reported that the active phytochemicals with anti–cancer, anti–invasive and anti–metastatic activities in cancer cells[27,28].

The anti–tumor properties of rhein have been reported in many types of cancer cells, such as SCC–4 human tongue cancer cells[29–31]. In our experiment, we found higher toxicity in rhein (anthraquinone) against COLO 30DM cells. It induced apoptosis at 6.25  $\mu$ g/mL. Rhein–induced apoptosis of human tongue cancer cells, promyelocytic leukemia cells and nasopharyngeal carcinoma cells[32].

Anthraquinone compounds, anthracyclines and anthracenediones, have long been used as effective anticancer drugs against a broad spectrum of tumours[33–34]. Depending on their chemical structure, anthraquinone drugs can kill tumor cells by diverse mechanisms, involving different initial intracellular targets that normally contribute to drug–induced toxicity and the induction of apoptosis[35].

Under normal conditions, the cells in which the DNA or other components are irreversibly damaged by various causes undergo apoptotic cell death, which is a self–destructive metabolism according to the genetically encoded cell death signal[36,37]. However, cancer cells, which are already irreversibly developed, obtain the capability to escape apoptosis by various ways. The aim of anticancer agents is to trigger the apoptosis signaling system in these cancer cells whilst disturbing their proliferation[38]. For many years, the cytotoxic actions of the chemotherapeutic drugs were ascribed solely to their ability to induce genotoxic death[39]. However, there were accumulating evidences that these agents exert their cytotoxic effects mainly by inducing apoptosis in tumor cells. Impairment of apoptosis is known to be related to cell immortality and carcinogenesis and the induction of apoptosis in neoplastic cells, therefore, is vital in cancer treatment. Recently several plant derived natural compounds have been screened for their anticancer activity in order to identify putative compounds with novel structures or mechanism of action. Therefore many plants have been examined to identify new and effective antioxidant and anticancer compounds, as well as to elucidate the mechanisms of cancer prevention and apoptosis[40]. The compound (Rhein) showed good activity significantly killing the COLO 30DM cells at 200  $\mu$ g/ml. Previously several studies reported that Rhein suppresses the phorbol ester–induced tumor promotion in mouse epidermal cell line JB6[41].

Among the many promising strategies currently under investigation for cancer chemoprevention, selective apoptosis induction is regarded as one of the best ways to remove tumor cells[42]. Results from apoptosis analyses suggest that Rhein could be used as a cancer chemopreventive and therapeutic agent.

Other investigators have shown that Rhein can suppress the growth of Caco–2 human adenocarcinoma cells[43], breast cancer cells[44] and human hepatocellular carcinoma BEL–7402 cells[45].

Here we report that Rhein induces apoptosis in human adenocarcinoma cancer cells (COLO 320 DM) at 6.25  $\mu$ g/mL for the first time. Rhein showed cytotoxic activity against human colon cancer cells (COLO 320 DM) and it also induced apoptosis at 6.25 mg/mL. It can be a useful to treat colon cancer studies.

### Conflict of interest statement

We declare that we have no conflict of interest.

### Acknowledgements

Authors are thanks to Entomology Research Institute, Loyola College, and Chennai for the collection of the plant and financial support (Grant No.ERI/02/2007).

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