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Induction of DNA damage by the leaves and rhizomes of *Curcuma amada* Roxb in breast cancer cell lines

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ABSTRACT

Objective: To evaluate DNA damage inducing effect of the methanolic extract of both the leaves and the rhizomes of *Curcuma amada* (*C. amada*) against breast cancer cell lines MCF-7 and MDA MB 231 and analyze the active components present in the methanolic extract of the leaves and the rhizomes.
Methods: The DNA damage induced in yeast was assessed using diphenylamine method. The DNA damage induced by the extracts in cell lines was assessed using single cell gel electrophoresis (Comet assay). Various phytochemicals present in the leaves and the rhizomes were analysed using various chromatographic and spectral studies. A normal non-cancer cell line HBL-100 and an eukaryotic model organism yeast was also used for comparison.
Results: The results indicated that the methanolic extract of both the leaves and the rhizomes of *C. amada* induced cell death in the breast cancer cell lines MCF-7 and MDA MB 231. The extracts showed less DNA damage in yeast and HBL-100 cells. The phytochemical investigation revealed the presence of more amounts of terpenoids and steroids in both the leaves and rhizomes.
Conclusions: The results indicated that the methanolic extract of leaves of rhizomes of *C. amada* possess genotoxic and cytotoxic activity against the breast cancer cell lines.

1. Introduction

Cancer represents a multidimensional spectrum of diseases characterized by malignancies. It is a major health disorder occurring in almost every part of the world. Oxidative stress is involved in the pathophysiology of various types of cancers. Human system is constantly exposed to both exogenous and endogenous free radicals. Oxidative stress causes gene mutation, which leads to alterations in the signal transduction pathways, which ultimately leads to the development of cancer. Antioxidants protect the cells from these free radical mediated damage and aid in the treatment of cancer[1].

Breast cancer refers to tumour that occurs in the tissues of the breast. Breast cancer is the most common type of cancer among women worldwide. More than one million breast cancer cases are newly diagnosed globally each year[2]. Breast cancer is the most complex and heterogenous disorder. Based on the expression of receptors in the breast cancer tissues, the breast cancer is categorized into 3 types *viz* ER/PR positive, Her2 positive and triple negative[3].

Curcuma amada Roxb (*C. amada*) is a well known rhizomatous herb which is commonly known as mango-ginger that belongs to the family Zingiberaceae (Ginger family) that is widely cultivated in various parts of South India. *C. amada* rhizomes have been used for culinary

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purposes and pickle preparations in South India. Our previous study have shown that the methanolic extract possess good antioxidant activity and also did not induce cytotoxicity towards yeast cells. Hence, the present study was formulated to evaluate the DNA damage inducing ability of the methanolic extract of leaves and rhizomes against ER positive and Triple negative breast cancer cell lines MCF-7 and MDA MB 231, respectively. A non-cancerous breast cell line HBL-100 and eukaryotic model organism yeast was also used for comparison.

2. Materials and methods

2.1. Plant material

C. amada Roxb rhizomes were procured from Arya Vaidya Pharmacy, Centre for Indian medicinal plant heritage, Kanjikode, Kerala and were grown as pot culture in our university herbal garden and were identified by Botanical Survey of India, Southern circle. Both leaves and rhizomes were collected fresh for the study. Previous studies conducted by us showed that the methanolic extract of the leaves and rhizomes were rich in antioxidants.

2.2. Extract preparation

The leaves and rhizomes collected fresh were rinsed with tap water blotted dry using a filter paper and used for extract preparation. The components present in the leaves and rhizomes were extracted using methanol. The methanolic extract prepared after evaporation of methanol was dissolved in dimethylsulfoxide (0.2 mg/mL- IC_{50} dose).

2.3.Culturing of cell lines and yeast

All the cell lines MCF-7, MDA MB 231 and HBL-100 were purchased from National Centre for Cell Science (NCCS), Pune, India. The cell count was done and the cell viability was tested by trypan blue using haemocytometer. The cells were grown in dulbecco's modified eagle medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, 1% non-essential amino acids in tissue culture flasks and incubated in a CO_2 incubator in a 5% CO_2 and 95% humidity atmosphere. Once the cells attained confluent growth, the cells were trypsinized using trypsin-EDTA (PAA) and the required number of cells 10^6/mL was seeded into 6-well for carrying out various assays. In each well of the 6-well plates, a clean, dry, sterile cover slip was placed before the cells were seeded, followed by incubation in a CO_2 incubator in a 5% CO_2 and 95% humidity atmosphere (Innova CO-170, United States)for comet assay. Yeast was cultured in yeast peptone dextrose broth and incubated at 30 °C and overnight grown culture was used for the assay.

2.4. Treatment groups

MCF-7, MDA MB 231 and HBL-100 were treated with the leaf and rhizome extract (0.2 mg/mL) for 24, 18 and 12 h, respectively which was the optimal treatment time of the extracts in each of the cell lines. The effect induced was also compared to the standard drugs used *viz* tamoxifen for ER positive MCF-7 and HBL-100 cells and etoposide for triple negative MDA MB 231 cells. The yeast cells were treated for 1 h and H_2O_2 was used as oxidative stress inducing agent. The following treatment groups are set up of the study. Negative control: cells alone; Positive control: cells+tamoxifen/etoposide/H_2O_2; Test groups: cells+methanolic extract of *C. amada* leaves (CAL); cells+CAL+tamoxifen/etoposide/H_2O_2; cells+methanolic extract of *C. amada* rhizomes (CAR); Cells+CAR+tamoxifen/ etoposide/H_2O_2.

2.5. Detection of DNA damage

2.5.1. Diphenylamine method

The cells were taken equally in tubes labeled B and treatments were performed according to treatment groups. After that the tubes were centrifuged at 1 300 r/min at 4 °C for 10 min. The supernatants were transferred to new tube labeled S. To the pellets in tube B, 1.0 mL of TTE solution was added and vortexed to release the fragmented chromatin from nuclei. To separate the fragmented DNA from intact chromatin, centrifuged tubes B at 13 000 r/min for 10 min at 4 °C. From the tubes B, supernatant were carefully transferred to new tube labeled T. To the small pellets in tubes B, 1.0 mL of TTE solution was added. Then 1.0 mL of 25% TCA was added to tubes T, B and S and vortexed vigorously. Precipitation was allowed to proceed overnight at 4 °C. After the incubation, the precipitated DNA was recovered by pelleting for 10 min at 13 000 r/min at 4 °C. The supernatants were then discarded by aspiration. Then the DNA was hydrolyzed by adding 160 μL of 5% TCA to each pellet and heating for 15 min at 90 °C. A blank was also prepared having 160 μL of 5% TCA alone. Freshly prepared

DPA solution (320 μL) was added to all tubes. The tubes were incubated for 4 h at 37 °C or overnight at room temperature for colour development. A liquots of 200 μL coloured solution were transferred from each tube to wells of a 96–well microtitre plate[4]. The percent fraction of fragmented DNA was calculated using the formula.

Percent fragmentation=(T+S)/(T+S+B) ×100

2.5.2. Comet assay

The treated cells were harvested by trypsinization, washed with phosphate buffer solution and resuspended in ice–cold phosphate buffer solution. All steps were performed at room temperature under dimmed or yellow light to prevent DNA damage, unless otherwise specified. About 25 μL of the resuspended cells was mixed with 75 μL 0.5% low melting agarose at 37 °C and spread uniformly with the side of a microtip on preheated (37 °C) microscopic slides coated with normal melting point agarose. The slides were placed at 4 °C in the dark until gelling occurred and then immersed in prechilled lysis solution at 4 °C. After 1 h of incubation, the buffer was drained from the slides and immersed in freshly prepared alkaline–unwinding solution at room temperature for 30–60 min. After lysis and unwinding, the slides were placed in a horizontal electrophoresis tank filled with freshly prepared alkaline electrophoresis buffer, and electrophoresis was carried out at 30 V for 15 min. After electrophoresis, the slides were covered with neutralization buffer for 5 min and washed twice by immersing in distilled water for 5 min each, then in 70% ethanol for 10 min. Thereafter, the slides were dried at room temperature for 10 to 15 min and stained with ethidium bromide solution. The slides were washed to remove excess ethidium bromide and air–dried. They were then scored for the presence of comet 'tails' under oil immersion in a fluorescent microscope (Nikon, Japan). Totally 100 cells per slide were scored and the frequency of DNA damage, as the number of comet bearing cells, was noted. Tail moment for each comet was also calculated using Casp comet scoring software[5].

2.6. Phytochemical analysis

2.6.1. Preliminary phytochemical screening

The methanolic extract of the leaves and the rhizomes of *C. amada*, were screened for the presence of phytochemicals according to the method of Khandelwal[6].

2.6.2. Spectral analysis

2.6.2.1. UV absorption spectral analysis

A preliminary absorption spectral analysis was done by a survey scan of the methanolic extract of the leaves and the rhizomes of *C. amada* in a nanospectrophotometer (Optizen, Korea). The instrument was set to scan mode and the absorption spectrum of the methanolic extract of the leaves and the rhizomes was obtained in the range of 190–400 nm.

2.6.2.2. Thin layer chromatography (TLC) of the methanolic extract of the leaves and rhizomes of C. amada

The methanolic extract of the leaves and the rhizomes were subjected to TLC on silica gel G60 F_{254} plates (Merck) using different solvent systems specific for the phytochemical constituent and were developed by spraying with specific developing agents (Table 1). The R_f values of the spots were calculated as the ratio of the distance travelled by the solute to that travelled by the solvent front.

2.7. Statistical analysis

The parameters of the experiment are expressed as mean ±SD. Statistical evaluation of the data was done using One–way ANOVA with the level of significance at P<0.001 using Sigma stat package version 3.1.

3. Results

3.1. Effect of C. amada leaf and rhizome extract on non–cancerous and cancerous cells

The methanolic extract of leaves and rhizomes of *C. amada*

Table 1

TLC of the methanolic extract of the leaves and rhizomes of *C. amada*.

Phytochemicals	Solvent system	Developing agent
Alkaloids	Ethyl acetate–Methanol–Water (10 : 1.35 : 1)	Dragendroff's reagent followed by ethanol sulphuric acid
Phenolics	Toluene–Acetone–Formic acid (4.5 : 4.5 : 1)	20% Sodium carbonate reagent followed by Folin–Ciocalteau reagent
Flavonoids	Ethyl acetate–Butanone–Formic acid–Water (5 : 3 : 1 : 1)	1% Ethanol aluminium chloride reagent
Saponins	Chloroform–Glacial acetic acid–Methanol–Water(6.4 : 3.2 : 1.2 : 0.8)	Anisaldehyde sulphuric acid reagent
Steroids	Toluene–Methanol (9 : 1)	Anisaldehyde sulphuric acid reagent
Tannins	Toluene–Ethyl acetate–Formic acid–Methanol (3 : 3 : 0.8 : 0.2)	5% Ferric chloride reagent
Terpenoids	*n*–Hexane–Ethyl acetate (7.2 : 2.9)	Anisaldehyde sulphuric acid reagent

induced less DNA damage in non–cancerous cells yeast (analysed by DPA method) and HBL–100 whereas in breast cancer cell line MCF–7 and MDA MB 231 it induced more DNA damage as evidenced by increase in the tail length of the comets (Figure 1, Figure 2 and Table 2). The results also indicated that the extracts protected the normal cells from drug/oxidant induced damage. In case of cancerous the co–treatment of extracts along with standard chemotherapeutic drugs increased the percent of DNA damaged.

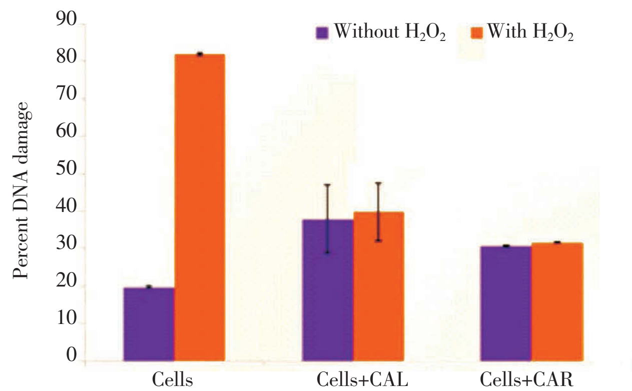

Figure 1. Effect of *C. amada* leaf and rhizome extracts on the percent DNA damage in *Saccharomyces cerevisiae* (yeast) cells determined by diphenylamine method.

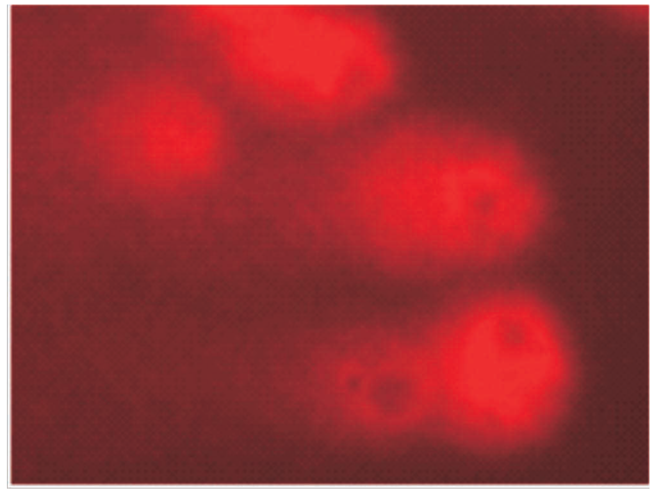

Figure 2. Photograph showing comet bearing cells in MCF–7.

3.2. Phytochemical analysis

The result of phytochemical analysis revealed the presence of following components (Table 3). The absorbance survey scan of the methanolic extract of the leaves and the rhizomes of *C. amada* in the wavelength ranging from 190–400 nm respectively was performed(Figure 3). The results revealed the presence of six major peaks between the wavelength 290–360 nm in the leaves and the presence of

Table 2

Effect of *C. amada* leaf and rhizome extracts on the number of comet bearing cells, tail length and olive tail moment of comet bearing cells.

Treatment groups		No. of comets/100 cells			Tail length of comets (μm)			Olive tail moment of comets (arbitrary units)		
		Cells	CAL	CAR	Cells	CAL	CAR	Cells	CAL	CAR
HBL–100	Control	9±1	25±4[a]	25±2[a]	3.00±0.00	7.40±1.14[a]	6.80±0.84[a]	0.011±0.002	1.120±0.140[a]	1.020±0.090[a]
	Tamoxifen treated	49±2[a]	42±1[a,b,c,d]	38±1[a,b,c]	8.40±3.04[a]	23.00±2.55[a,b,c]	21.60±2.07[a,b,c]	2.020±0.120[a]	1.910±0.590[a]	1.430±0.160[a,b,c]
MCF–7	Control	13±1	47±1[a]	49±1[a]	3.00±0.00	12.80±1.92[a]	11.80±2.17[a]	0.008±0.006	1.510±0.100[a]	1.690±0.510[a]
	Tamoxifen treated	51±2[a]	56±1[a,b,c]	57±1[a,b,c]	9.60±3.21[a]	22.80±2.77[a,b,c]	24.00±3.16[a,b,c]	1.350±0.450[a]	2.320±0.200[a,b,c]	2.400±0.300[a,b]
MDA MB 231	Control	7±1	59±2[a]	57±2[a]	3.00±0.00	12.00±1.58[a]	10.60±1.82[a]	0.011±0.006	1.560±0.110[a]	1.690±0.160[a]
	Tamoxifen treated	44±2[a]	64±1[a,b,c]	59±3[a,b,c]	10.60±2.41[a]	21.40±2.51[a,b,c]	21.00±3.16[a,b,c]	1.320±0.200[a]	2.190±0.140[a,b,c]	2.190±0.150[a,b,c]

Values are expressed as mean±SD of triplicates; [a]: Statistically significant ($P<0.05$) compared to untreated control; [b]: Statistically significant ($P<0.05$) compared to drug alone treated group; [c]: Statistically significant ($P<0.05$) compared to the respective extract treated group; [d]: Statistically significant ($P<0.05$) compared to the rhizome extract treated group.

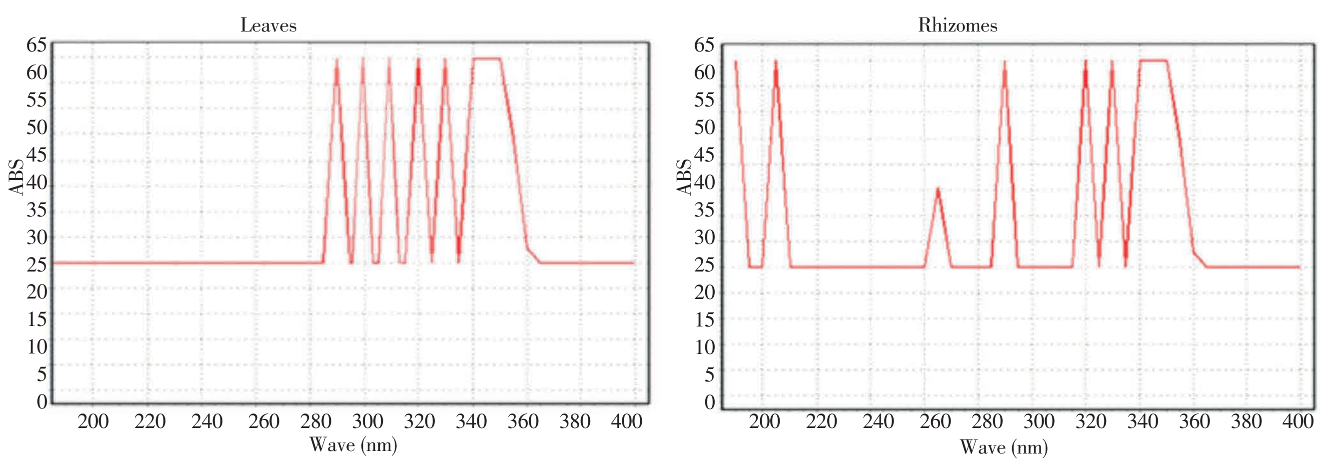

Figure 3. UV absorption spectrum of the methanolic extracts of leaves and rhizomes of *C. amada* leaves

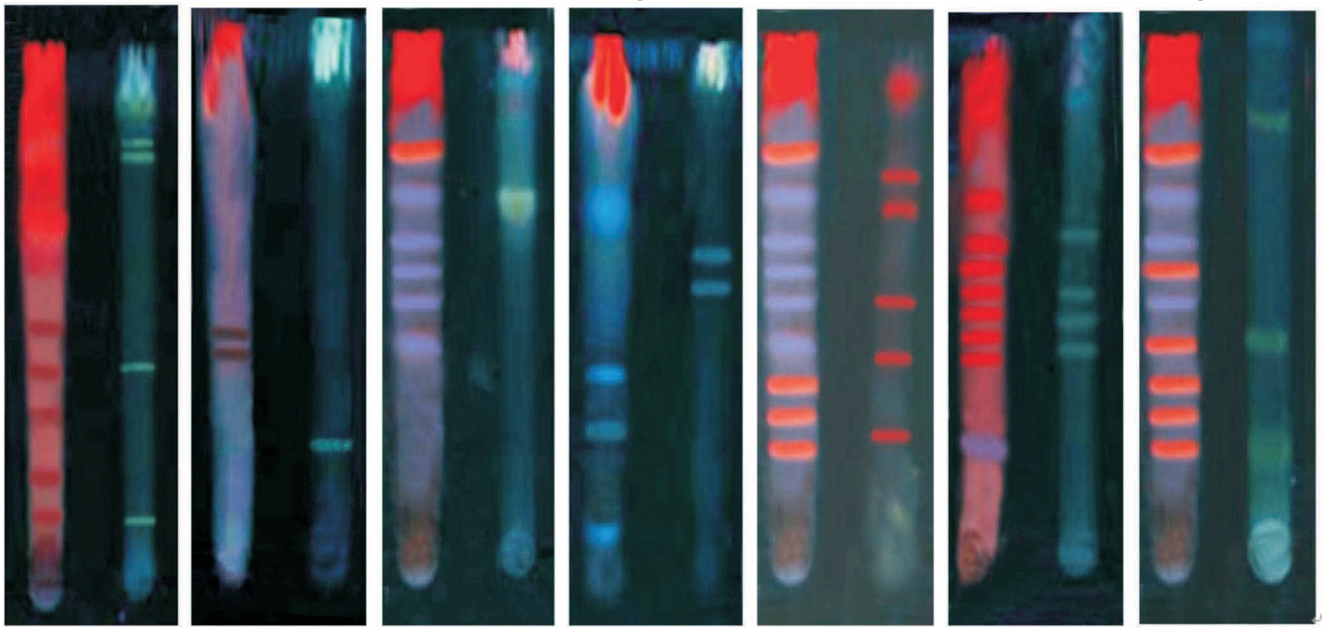

Figure 4. TLC of the methanolic extracts of leaves and rhizomes of *C. amada*.

Table 4
R_f values of TLC of the methanolic extracts of leaves and rhizomes of *C. amada*.

Phytochemicals	Leaves		Rhizomes	
	No. of bands	R_f values	No. of bands	R_f values
Alkaloids	8	0.05, 0.12, 0.2, 0.25, 0.43, 0.54, 0.63, 0.71	5	0.08, 0.14, 0.26, 0.57, 0.75
Flavonoids	2	0.34, 0.46	1	0.85
Phenolics	6	0.23, 0.61, 0.64, 0.67, 0.77, 0.81	1	0.62
Saponins	4	0.10, 0.22, 0.33, 0.74	2	0.17, 0.37
Steroids	9	0.33, 0.43, 0.5, 0.55, 0.61, 0.65, 0.71, 0.76, 0.83	5	0.3, 0.47, 0.62, 0.78, 0.85
Tannins	8	0.19, 0.29, 0.43, 0.55, 0.61, 0.67, 0.72, 0.79	4	0.43, 0.49, 0.54, 0.76
Terpenoids	9	0.14, 0.27, 0.37, 0.49, 0.54, 0.59, 0.64, 0.73, 0.85	3	0.16, 0.74, 0.89

five major peaks, four between 280–360 nm, one major peak between 200–210 nm and one minor peak between 260–270 nm in the rhizomes. The TLC analysis revealed the presence several phytoconstituents in both leaves and the rhizomes with different R_f values (Figure 4 and Table 4).

Table 3
Qualitative phytochemical analysis of leaf and rhizome extracts of *C. amada.*

Phytochemicals	Test	Leaf	Rhizome
Alkaloids	Mayer's test	+	+
	Dragendroff's test	+	+
	Wagner's test	+	+
Phenolics	Ferric chloride test	+	+
	Lead acetate test	+	+
Flavonoids	Aqueous NaOH test	+	+
	Concentrated H_2SO_4 test	+	+
	Schinodo's test	+	+
Saponins	Froth test	+	+
	Emulsion test	+	+
Tannins	Braemer's test	+	+
Steroids	Libermann–Buchard test	+	+
	Salkowski test	+	+
Terpenoids	H_2SO_4 test	+	+

4. Discussion

The results of our experiments also correlated with the below findings by other researchers. The administration of 70% ethanolic extract of Moringa oleifera leaves prevented the DNA damage in lymphocytes against H_2O_2[7]. The hexane, dichloromethane, ethyl acetate, methanol and water extracts obtained from the powdered roots, leaves and stems of Coriandrum sativum by sequential extraction prevented the DNA damage in the 3T3–L1 fibroblast cells induced by H_2O_2 treatment[8]. Diisopropyl fluorophosphates, a serine protease inhibitor, prevented the DNA fragmentation in the neutrophils in culture[9]. The ethanolic fraction of *Fructus rhodomyrti* whole plant protected the lymphocytes from DNA damage induced the strong oxidant H_2O_2[10]. The treatment with the methanolic extract of *Euphorbia hirta* increased the comet tail length and tail moment in human breast cancer cell line MCF–7[11]. The active fraction from the hexane extract of *Zornia diphylla* (L) Pars induced DNA damage in Dalton's lymphoma ascites tumour cells[12]. The comets were observed in MCF–7 cells exposed to aqueous extract of *Fagonia cretica* in a dose and time dependent manner[13]. The pre–administration of isoliquiritigen before cyclophosphamide treatment in mice protected the blood cells from DNA damage induced by cyclophosphamide. The tail moment was also found to be decreased in isoliquiritigen treated groups[14]. The treatment with 4–fluoro benzofuran phenyl methyl imidazole induced genotoxicity in mammary carcinoma cells MCF–7 as evident by increase in tail length in comet assay[15]. The pre–treatment of melatonin to methyl methanesulfonate exposed MCF–7 cells caused an increase in tail moment[16].

C. amada leaves and rhizomes was found to possess cytotoxic properties which is mediated through damaging intracellular DNA. The extract also showed differential response towards cancerous and non–cancerous cells.

Conflict of interest statement

The authors report no conflict of interest.

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