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ORIGINAL ARTICLE

Caesalpinia pulcherrima Arrests Cell Cycle and Triggers Reactive Oxygen Species-Induced Mitochondrial-Mediated apoptosis and Necroptosis via Modulating Estrogen and Estrogen Receptors

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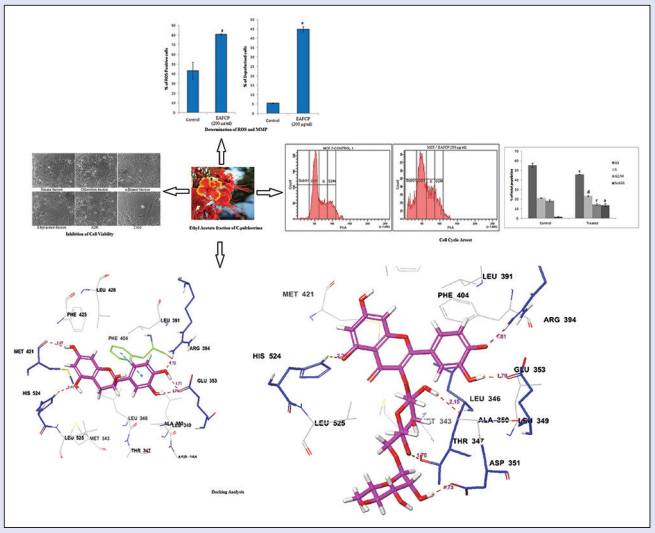
ABSTRACT

Background: *Caesalpinia pulcherrima* belonging to the family *Fabaceae* is used in India as a traditional medicine for a variety of ailments. Globally, traditional medicines are presently being used for the treatment of cancer. **Objective:** The present study was aimed at investigating the chemomodulatory potential of *C. pulcherrima* flowers in breast cancer and explaining its possible mechanism. **Materials and Methods:** The cytotoxic potential of ethyl acetate fraction of *C. pulcherrima* (EAFCP) flower was tested in MCF-12A (normal breast), MCF-7 (estrogen receptor [ER] positive), and MDA-MB-453 (human epidermal growth factor receptor 2 positive) human breast cancer cells by sulforhodamine B assay. Chemomodulatory potential was evaluated *in vivo* against N-methyl-N-nitrosourea (MNU)-induced mammary carcinoma in female Sprague Dawley® rats. The mechanism for anticancer potential was screened by *in vitro* studies involving Annexin V-FITC assay (apoptosis), cell cycle patterns, intracellular reactive oxygen species, and mitochondrial membrane potential measurement (FACS based) followed by docking study on estrogen receptor-alpha (ER-α). **Results:** The fractions showed perceptible cell growth inhibition potency (IC_{50} <50 μg/ml) in MCF-7 breast cancer cells. In MNU-treated animals, antioxidant enzymes and histological examination showed statistically significant ($P < 0.001$) changes. Treatment of MCF-7 cells with EAFCP reduced cell growth rate by a mechanism associated with both apoptotic and necrotic cell death. Molecular docking study further showed that rutin and catechin have a comparable binding affinity for the ER-α. **Conclusion:** In this study, we confirmed that EAFCP was most effective in reducing cell viability, scavenging physiological oxidant species, and causing mitochondria-mediated apoptosis and necroptosis in MCF-7 cell by selectively modulating the functions of ER-α.

Key words: Antioxidant, breast cancer, estrogen receptor modulator, high-performance liquid chromatography, histopathology

SUMMARY

- Ethyl acetate fraction of Caesalpinia pulcherrima (EAFCP) flower showed cytotoxic potential against breast cancer cells by sulforhodamine B assay. The anticancer and antioxidant potential of EAFCP flower was evaluated against N-methyl-N-nitrosourea-induced mammary cancer in female Sprague Dawley rats. The in vitro results of the present study showed that EAFCP can induce mitochondrial-mediated apoptosis and necroptosis through reactive oxygen species generation with loss of mitochondrial membrane potential in MCF-7 via modulating the functions of estrogen receptor-alpha *in silico*.



Abbreviations used: EAFCP: Ethyl acetate fraction of *Caesalpinia pulcherrima*; MNU: N-methyl-N-nitrosourea; ROS: Reactive oxygen species; ER-α: Estrogen receptor-α; HPLC: High-performance liquid chromatography; TBARS: Thiobarbituric acid-reactive substances; HP: Hydroperoxides; LPO: Lipid peroxidation; CAT: Catalase; SOD: Superoxide dismutase; NBT: p-nitro blue tetrazolium chloride; GP_X: Glutathione peroxidase; GST: Glutathione-S-transferase; CDNB: 1-Chloro-2,4-dinitrobenzene; GR: Glutathione reductase; GSH: Reduced glutathione; MMP: Mitochondrial membrane potential; H and E: Hematoxylin and eosin; TB: Toluidine blue; LBD: Ligand-binding domain; TAM: Tamoxifen; ADR: Adriamycin; ANOVA: Analysis of variance.

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INTRODUCTION

Breast cancer is one of the most common malignancies in the world and a major cause of cancer-related deaths in women. It has been observed that estrogen level is an important factor in the initiation and development of breast cancer. Estrogen receptor-alpha (ER-α) is a well-characterized mediator in breast cancer for the proliferation of cells.[1] On the other hand, estrogens and estrogenic compounds are a subject of

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considerable fear in humans and animals during critical periods of development.[2] According to the WHO, medicinal herbs have played an important role in the treatment of cancer and would be the best source to obtain a variety of anticancer drugs.[3] Phytochemicals are an alternative medicine for chemoprevention in humans. These phytochemicals have multiple mechanisms for the prevention of cancer progression.[4] The administration of chemical carcinogen triggers the oxidative stress and elevates the levels of oxygen free radicals, which directly plays a key role in the development of carcinogenesis.[5]

Caesalpinia pulcherrima is commonly known as peacock flower or "Barbados pride" and belongs to the family *Fabaceae*. The aerial parts of this species have been used in traditional medicine for the treatment of various diseases such as asthma, bronchitis, cholera, diarrhea, dysentery, and malaria. The plant is known to possess antiviral, purgative, emmenagogue, tonic, stimulant, and cathartic activities and can be used for treating pyrexia, menoxenia, wheezing, and bronchitis.[6] The presence of ellagic acid, gallic acid, quercetin, rutin lupeol, β-Sitosterol, myricetin, flavonoids, and homoflavonoids, such as (E)-7-methoxy-3-(4'-methoxybenzylidene) chroman-4-one, (E)-7-hydroxy-3-(3',4',5'-trimethoxybenzylidene) chroman-4-one, isobonducellin, bonducellin and (E)-7-hydroxy-3-(2',4'-dimethoxybenzylidene) chroman-4-one, 3-(4'-hydroxy-benzyl)-5,7-dihydroxy-6,8-dimethyl-chroman-4-one, hyperforin, and platycodigenin in its flowers are reported.[7,8]

Chemical carcinogen-induced rat mammary carcinoma model is a prime model for studying the efficacy of chemopreventive activity. N-methyl-N-nitrosourea (MNU) is a chemical carcinogen used to induce mammary cancer in a rat model. It is well known that MNU stimulates the growth of estrogen-dependent tumors.[9] Hence, in the present study, we have tried to investigate the effects of ethyl acetate (EA) fraction of *C. pulcherrima* (EAFCP) flower by *in vitro*, *in vivo,* and *in silico* methods.

MATERIALS AND METHODS

Chemicals

All the chemicals and reagents used were of analytical grade. MNU and other chemicals were purchased from Sigma-Aldrich Chemicals Private Ltd. (MO, USA).

Plant material

Fresh flowers of *C. pulcherrima* were collected from the local areas of Aurangabad, Maharashtra, India. Plant taxonomical identification was verified (accession no. 0662) by Prof. Dr. Dhabe, Head of the Botanical Department of Dr. Babasaheb Ambedkar Marathwada University, Aurangabad.

Preparation and phytochemical analysis of the plant extract

Shade-dried flowers (500 g) were powdered and soxhleted with methanol and water (MeOH) for 12 h. The MeOH was removed under vacuum in a rotary vacuum evaporator. The MeOH extract (28 g) rich in polyphenolic compounds was further suspended in 250-ml water, and fractionation was achieved by solvents of increasing polarity (n-hexane, chloroform, and EA). A part of this extract was partitioned with EA which given the fraction with yields 6.6 g. The solvent fractions were concentrated under vacuum in a rotary vacuum evaporator, and the extracts were preserved at 4°C. The flowchart of fractionation is depicted in Figure 1. EAFCP has been studied in a further aspect, and it was submitted to high-performance liquid chromatography (HPLC) analysis. The HPLC system was equipped with a dual-pump gradient system, C_{18} reversed phase column (internal diameter: 4.6 mm × 250 mm, 5 μm). The flow rate and injection volume were 0.7 ml/min and 20 μl, respectively. 0.05% orthophosphoric acid was used as a buffer. Mobile phase A was used as a mixture of buffer: acetonitrile in the ratio of 90:10 and mobile phase B was used as buffer: acetonitrile in the ratio of 10:90 with linear gradient program for elution. The column temperature was maintained at 25°C. The standard solutions of ellagic acid, gallic acid, myricetin, quercetin, rutin, and catechin were injected into the HPLC for analysis.[10] The sample and standard solutions (1 mg/mL) were prepared using appropriate amounts of ellagic acid, gallic acid, myricetin, quercetin, rutin, and catechin dissolved in methanol. The prepared solutions were filtered through a 0.45-mm Durapore® membrane filter (Millipore; Billerica, MA, USA). The samples were monitored with ultraviolet detection at 280 nm and at ambient temperature separation was achieved with a single linear gradient program. The compounds were identified by comparing the retention time of samples to standards.

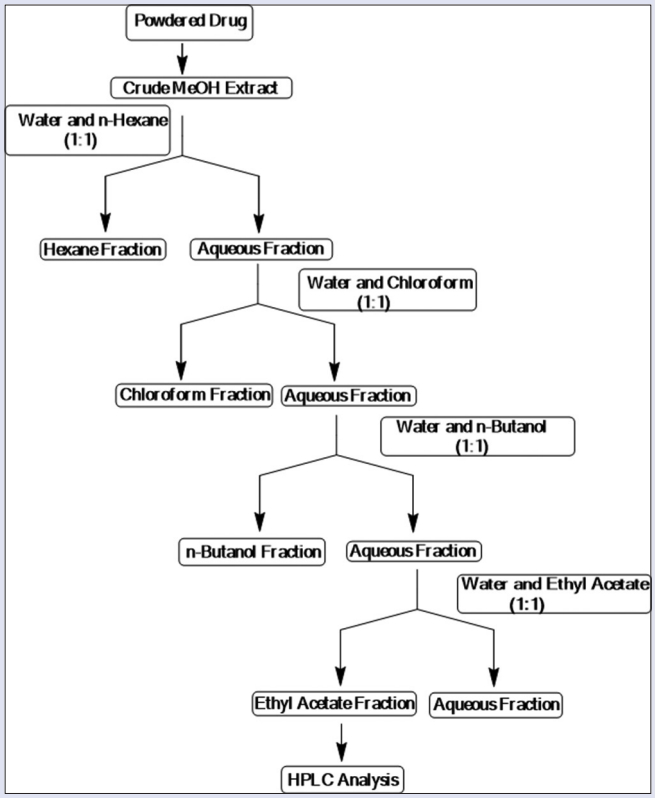

Figure 1: Scheme of extraction and fractionation of plant material with different solvents

Animals

Virgin female Sprague Dawley® rats (body weight 160–200 g) were obtained from Wockhardt Research Centre Pvt. Ltd., Aurangabad, India, and housed in standard conditions (12 h light/dark cycle, 20°C ± 2°C; 65% ± 15% relative humidity) with free access to water *ad libitum*. The entire study was carried out according to the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA/IAEC/Pharm.Chem.-31/2016-17/129), and efforts were made to reduce animal suffering and number of animals used.

Experimental design

In vitro anticancer screening

In vitro testing was done using sulforhodamine B assay protocols,[11] with each drug tested at five dose levels (10, 25, 50, 100, and 200 μg/ml).

Tamoxifen (TAM) and adriamycin (ADR) were run as positive control drugs in each experiment, and each experiment was repeated thrice. Results were presented with reference to growth inhibition (GI) of 50%, total GI, and LC_{50} values.

In vivo anticancer screening

In this study, animals were divided into the following different groups with six animals in each group: Group I: normal control (0.9% NaCl solution), Group II: cancer induced (MNU and normal saline solution treated), Group III: cancer induced + EAFCP (250 mg/kg, p.o., body weight), Group IV: cancer induced + EAFCP (500 mg/kg, p.o. body weight), and Group V: cancer induced + TAM (2 mg/kg, p.o., body weight) for 4 weeks. Animals in the normal control group and untreated MNU group were given vehicle (0.9% NaCl) according to the experimental protocol.

A fresh solution at a concentration of 10 mg/ml was prepared by wetting the MNU powder with 3% acetic acid and then dissolving it in 0.9% NaCl solution, for each injection. Rats were given intraperitoneal (i.p.) 50 mg/kg of MNU on the 50th day of age. After MNU injection, the presence of mammary tumor masses was assessed by palpation weekly and recorded. MNU gives a high incidence of ER-positive tumor masses. The latency period (time of appearance of the first tumor), tumor burden (number of tumors/rat), and relative size of each tumor were recorded. The tumor diameter was measured by a micrometer caliper, and volume was calculated by using the following formula:

$V = 4/3\ \pi\ r^3$

Where r is half of the average diameter.

At the end of the treatment, blood was collected from retro-orbital puncture for evaluating estrogen level.[12]

Biochemical assay

Preparation of tissue homogenate

Mammary tumor tissue (100 mg) homogenate was prepared in 0.1 mol/l of Tris-HCl buffer, at pH 7.4, and centrifuged at 3000 ×*g* for 15 min at 4°C. The supernatant was transferred into a new tube and used for biochemical analysis. The total protein of tumor tissue samples was estimated by Lowry's method.[13]

Lipid peroxidation analysis

Lipid peroxidation (LPO) was estimated colorimetrically by thiobarbituric acid-reactive substances by Niehaus and Samuelsson[14] and by hydroperoxides by the method of Jiang.[15]

Catalase assay

Catalase (CAT) was assayed by the method of Sinha.[16] The enzyme activity was expressed as μM of H_2O_2 consumed/min/mg protein.

Superoxide dismutase assay

Superoxide dismutase (SOD) activity was assayed according to the method of Kakkar *et al.*[17] The activity was measured of p-nitro blue tetrazolium chloride (NBT) reduction in light without protein minus NBT reduction with protein. One unit of enzyme activity was established as the amount of enzyme reaction, which gave 50% inhibition of NBT reduction in 1 min.

Glutathione peroxidase assay

Glutathione peroxidase (GP_X) assay was measured by the method described by Rotruck.[18] The activity was expressed as μg of reduced glutathione (GSH) consumed/min/mg protein.

Glutathione-S-transferase assay

Glutathione-S-transferase (GST) assay was determined by the method of Habig *et al.*[19] The activity of GST was expressed as mM of GSH-1-Chloro-2,4-dinitrobenzene conjugate formed/min/mg protein using an extinction coefficient of 9.6/mM.

Glutathione reductase assay

Glutathione reductase (GR) activity was measured by the method of Staal *et al.*[20] The GR activity was expressed as nmoles of nicotinamide adenine dinucleotide phosphate oxidized/min/mg protein.

Reduced glutathione assay

GSH was determined by the method of Ellman.[21] The values were expressed as mg/100 g tissue.

Histopathological studies

Mammary tumors were removed and washed with an ice-cold buffered saline solution (pH 7.4). They were fixed in 10% formalin for 48 h and were gradually dehydrated in alcohol, cleared of fat with toluene; rehydrated; and embedded in molten paraffin wax. The paraffin-embedded tumors were then cut into thin sections (5 μm) and stained with hematoxylin and eosin (H and E) for mammary epithelial cell architecture and toluidine blue (TB) for mast cell analysis.[22,23]

Annexin V-FITC-propidium iodide assay

MCF-7 cells were plated at 2×10^5 in a six-walled plate and treated with 200 μg/ml of EAFCP for 24 h. The cells were then trypsinized and washed by centrifugation (1200 rpm, 4 min, 4°C) with prechilled phosphate-buffered saline (PBS 1x). The cell pellet was resuspended with 100 μl of the 1x binding buffer with 5 μl of annexin V-FITC and 5 μl of propidium iodide (PI) for 15 min in dark. Then, 400 μl of the binding buffer was added, and the cells were filtered through a cell strainer. Cell cycle analysis was performed on the FACS Aria™, BD, USA scanner (BD Biosciences). Cell debris and aggregates were excluded from the analysis.[24]

Cell cycle analysis

MCF-7 cells were seeded at a density of 2×10^5 cells/ml in a six-walled plate. The cells were preincubated for 12 h and then treated with EAFCP (200 μg/ml) followed by incubation for 24 h. Cells were washed and harvested by trypsinization, collected and fixed in ice-cold 70% ethanol, and again rehydrated with PBS. The pellets were resuspended in 100 μl of PBS containing RNase A (1 mg/ml) (Sigma-Aldrich, #R6513) for 30 min and then incubated in dark at 37°C for 30 min and protected from light. A 10 μl of PI (1 mg/ml) (Sigma, #P4170) was added followed by an incubation of 15 min in dark, and analysis was carried out by BD FACS Aria™ system.[25]

Determination of reactive oxygen species and mitochondrial membrane potential

MCF-7 cells were seeded at a density of 2×10^5 cells/well in a 6-well plate and treated with 200 μg/ml EAFCP for 24 h at 37°C in 5% CO_2 and 95% air. Subsequently, the treated cells were collected, washed two times by PBS, and re-suspended in 500 μl of DCFHDA (5 μM) for reactive oxygen species (ROS) estimation and DiOC6 (3) (200 nM) in serum-free media for mitochondrial membrane potential (MMP) at 37°C in a dark room for 15 min. The analysis of samples was done using BD FACS Aria™ system.[26]

Docking methodology

Molecular docking study for rutin and catechin was performed using Glide v7.6 program interfaced with Maestro v11.3 of Schrödinger 2017 (Schrodinger, LLC, NY, USA). The crystal structure for ER-α (Protein Data Bank (PDB) ID: Estrogen-related receptor alpha (1ERR)) was taken from RCSB Protein Data Bank and prepared for docking using "protein preparation wizard." The structure of both compounds was built using Maestro build panel and optimized to

low-energy conformers using Ligprep v3.3. The docking study was performed according to previously reported[27] procedures using extra precision docking mode.

Statistical analysis

Data were analyzed using one-way analysis of variance followed by Tukey's test, and the results were expressed as mean ± standard error of the mean ($n = 6$), $^{a}P < 0.001$, $^{b}P < 0.01$, $^{c}P < 0.05$, ^{d}nonsignificant.

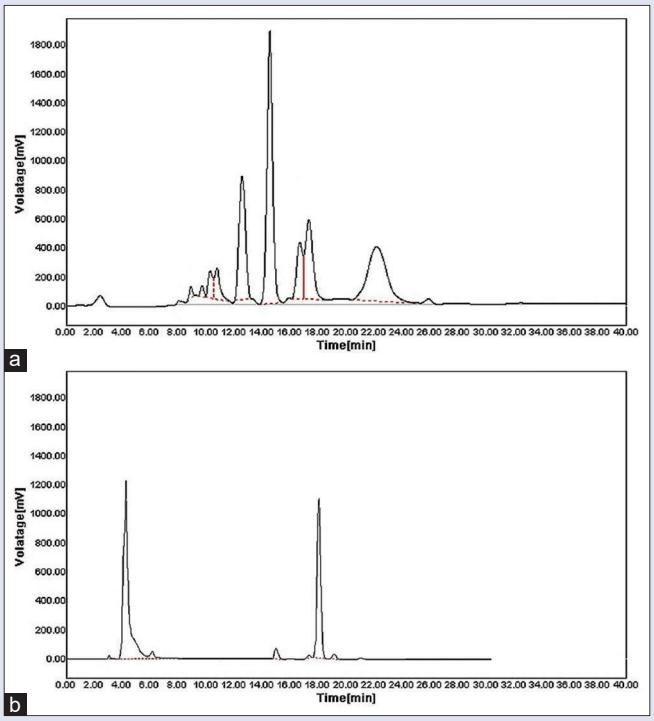

Figure 2: High-performance liquid chromatography analysis of ethyl acetate fraction of *Caesalpinia pulcherrima*: (a) reference compounds, (b) flower ethyl acetate

RESULTS

High-performance liquid chromatography analysis

The analysis of the EAFCP of the extract showed that the fraction is rich in polyphenols.[8] The HPLC profile of EAFCP showed the presence of several peaks. Chromatographic peaks were identified by comparing the Retention time (RT) of individual standards with the fraction. HPLC chromatogram [Figure 2a and b] of the extract showed sharp peaks for rutin and catechin.

In vitro anticancer activity

C. pulcherrima fractions were evaluated for their cytotoxic activity against MCF-12A, MCF-7, and MDA-MB-453 cell lines. TAM and adriamycin were taken as the standard. After treatment, EAFCP revealed inhibitory effect on the growth of MCF-7 with LC_{50} value of 23.6 µg/ml [Table 1 and Figure 3].

Effect of treatment on mammary tumorigenesis

Chemomodulatory effect was observed in the rats treated with EAFCP. Oral administration of EAFCP at doses of 250 and 500 mg/kg extended the latency and reduced the tumor load and volume significantly compared with MNU-treated rats. EAFCP at a dose of 500 mg/kg showed the highest reduction of mammary tumor incidences (66.6%) next to TAM-treated group (33.3%). The data for tumor latency, burden, and volume are summarized in Table 2.

Effect of treatment on serum estrogen levels

Estrogen levels were analyzed in rat serum after completion of treatment. The MNU and control group estrogen levels were compared which showed notable difference. The mean of the serum estrogen levels of normal and treated groups is summarized in Table 3. The treatment makes clear that EAFCP has affinity toward ER as TAM.

Effect of treatment on the oxidative markers

Enhanced LPO of cellular membranes can damage cells, tissues, and organs. The increased LPO in rats exposed to MNU might result from change in the antioxidant enzymes such as CAT, SOD, GPx, GST, GR, and GSH, compared to normal control rats. These enzymes are

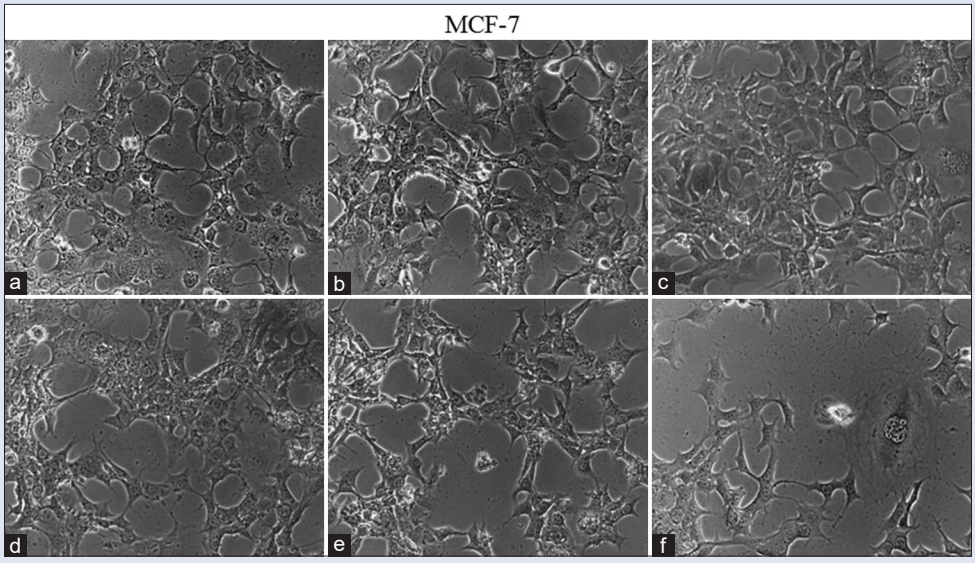

Figure 3: Morphological analysis of cytotoxicity of extract for MCF-7 cells: (a) hexane fraction; (b) chloroform fraction; (c) n-Butanol fraction; (d) ethyl acetate fraction; (e) adriamycin; (f) tamoxifen

important scavengers of superoxide ions, hydrogen peroxide, and hydroxyl free radicals. EAFCP administration significantly decreased LPO and increased the activities of antioxidants in a dose-dependent manner [Figure 4].

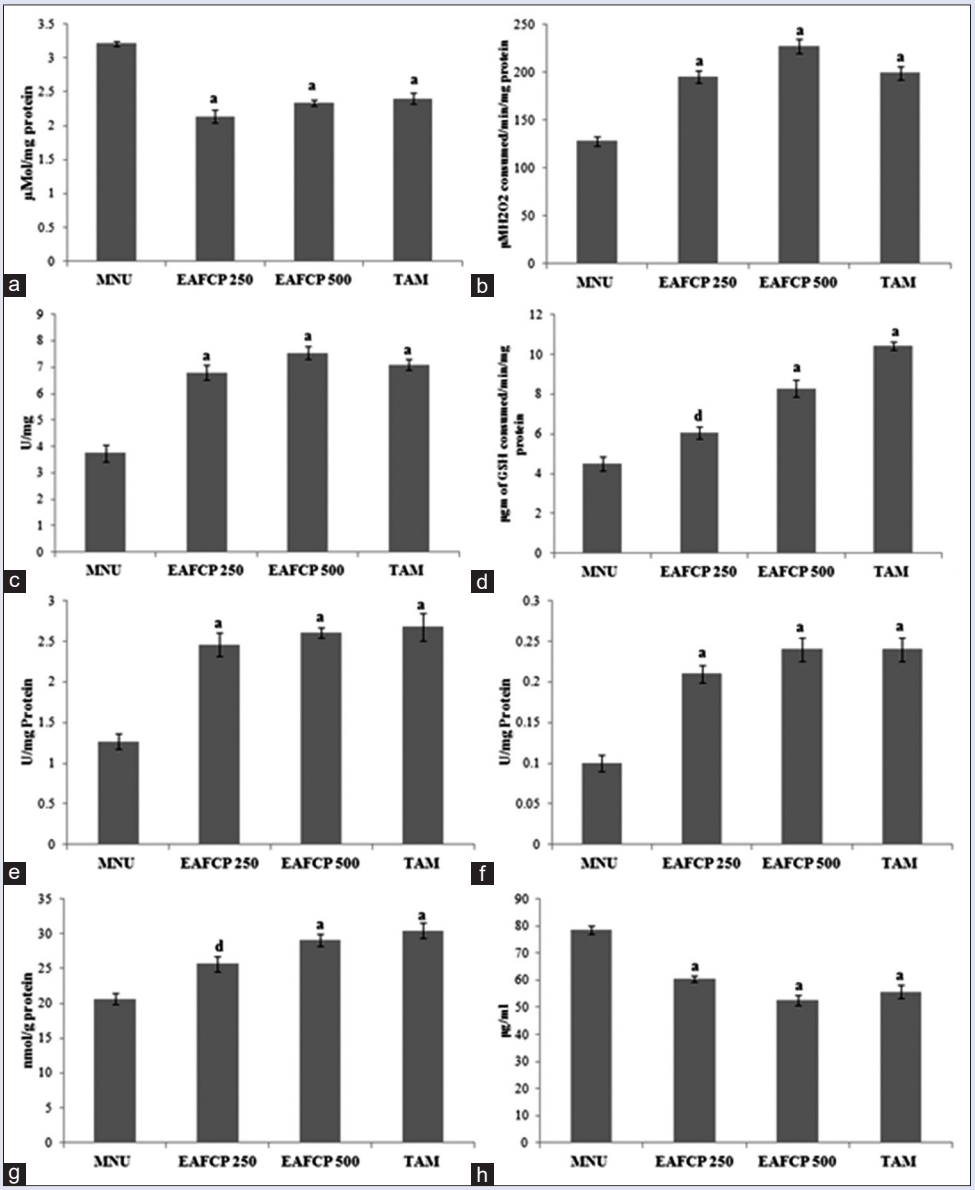

Figure 4: Biomarker levels of (a) lipid peroxidation, (b) catalase, (c) superoxide dismutase, (d) glutathione peroxidase, (e) glutathione-S-transferase, (f) glutathione reductase, (g) reduced glutathione, and (h) protein in mammary tissue of rats. The results are expressed as mean ± standard error of the mean, and the data are analyzed using one-way analysis of variance followed by Tukey's test. [a]$P < 0.001$, [b]$P < 0.01$, [c]$P < 0.05$, [d]nonsignificant

Table 1: *In vitro* anticancer activity (µg/ml) of fractions

Fractions	Cell lines								
	MCF-12A			MCF-7			MDA-MB-453		
	LC_{50} [a]	TGI[b]	GI_{50} [c]	LC_{50} [a]	TGI[b]	GI_{50} [c]	LC_{50} [a]	TGI[b]	GI_{50} [c]
Hexane fraction	>80	>80	>80	>80	>80	>80	>80	>80	>80
Chloroform fraction	>80	>80	>80	>80	>80	>80	>80	>80	>80
n-Butanol fraction	>80	>80	>80	76.8	53.2	65.9	>80	>80	>80
Ethyl acetate fraction	>80	>80	>80	23.6	12.5	<10	79.9	55.2	64.9
ADR	>80	>80	>80	>80	54.5	<10	>80	61.8	<10
TAM	>80	>80	>80	29.4	11.2	<10	54.2	21.5	<10

Most potent compounds shown by bold text as compared to standard. TAM: Tamoxifen; ADR: Adriamycin. [a]Compound concentration that produces 50% cytocidal effect, [b]Compound concentration that produces total growth inhibition, [c]Compound concentration that produces 50% growth inhibition

Table 2: Effect of treatment on mammary tumorigenesis

Group (n=6)	Treatment	Number of rats with tumor	Incidences (%)	Tumor latency (week)	Tumor burden	Tumor volume (mm^3)
I	Normal control	-	-	-	-	-
II	MNU	6/6	100	3.5±0.6	3.45±0.03	5.1±0.4
III	EAFCP (250 mg/kg)	5/6	83.3	5.5±0.7[d]	2.6±0.02[a]	3.3±0.7[d]
IV	EAFCP (500 mg/kg)	4/6	66.6	6.7±0.8[c]	2.3±0.2[a]	1.9±0.8[c]
V	TAM (2 mg/kg)	2/6	33.3	7.0±1.0[c]	1.8±0.01[a]	1.5±0.6[b]

The results are expressed as mean±SEM, (n=6). The data are analyzed using one-way ANOVA followed by Tukey's test. [a]P<0.001, [b]P<0.01, [c]P<0.05, [d]Nonsignificant. ANOVA: Analysis of variance; SEM: Standard error of mean; TAM: Tamoxifen; EAFCP: Ethyl acetate fraction of *Caesalpinia pulcherrima*; MNU: N-methyl-N-nitrosourea

Table 3: Estrogen levels (pg/ml) of animals on the 30th day

Group	Treatment	Estrogen level
I	Control	32.11±1.01
II	MNU	40.12±1.27[a]
III	EAFCP (250 mg/kg)	34.24±0.98[a]
IV	EAFCP (500 mg/kg)	32.67±2.11[a]
V	TAM	33.01±1.11[b]

The results are expressed as mean±SEM, (n=6). The data are analyzed using one-way ANOVA followed by Tukey's test. [a]P<0.001, [b]P<0.01, [c]P<0.05, [d]Nonsignificant. ANOVA: Analysis of variance; SEM: Standard error of mean; TAM: Tamoxifen; EAFCP: Ethyl acetate fraction of *Caesalpinia pulcherrima*; MNU: N-methyl-N-nitrosourea

Histopathological studies

Histopathological studies of H and E-stained mammary gland [Figure 5a-d] revealed that the normal architecture was disturbed by MNU induction. Microscopic examination of the section showed tumor tissue comprising of tubules, glandular structure, and cribriform arrangement of cells having hyperchromatic nuclei and scanty cytoplasm. Furthermore, dense lymphoid aggregates and eosinophilic secretions were seen along with fibroadipose tissue and congested blood vessels. An abnormal mitosis was not observed in microscopic analysis. EAFCP-treated rats showed the highest number of necrotic cells and reduced tumor cells. This is due to the potent inhibition of mammary tumor growth. Histopathological examination of TB staining for mast cells is shown in Figure 5e-h. MNU-treated rats (Group II) showed significant increase in mast cell population which was changed by the treatment with TAM and EAFCP.

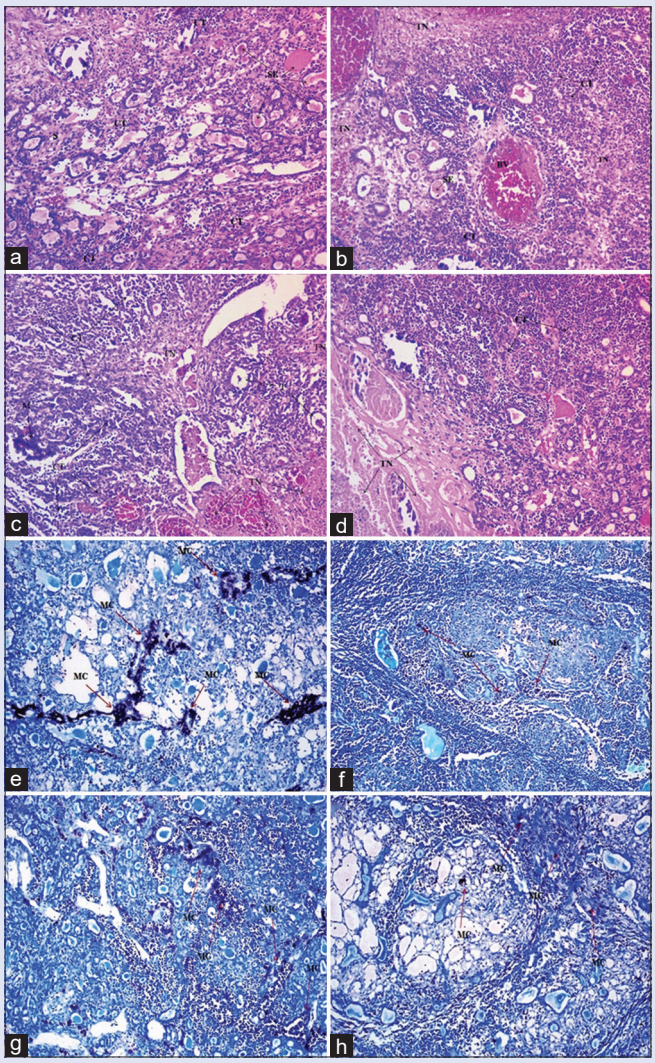

Figure 5: Representative histopathology images (sections are hematoxylin and eosin and toluidine blue stained, ×100) showing the effect of treatment on pathological analysis in rat mammary gland after 4 weeks of treatment. All the groups received intraperitoneal dose of N-methyl-N-nitrosourea at 50 mg/kg, body weight. (a-d; sections are hematoxylin and eosin stained, ×100): mammary tumor groups signifying cancerous tissue, tissue secretion, stroma, tumor tissue necrosis, congested blood vessel, and mast cells; (e-h; sections are toluidine blue stained, ×100): mammary tumor groups signifying accumulation of mast cells

Annexin V-FITC-propidium iodide assay

The FITC annexin V apoptosis assay was conducted to check whether the extract is inducing apoptosis or necrosis. Annexin staining showed that the extract induced both apoptosis and necrosis with 24-h treatment [Figure 6a-c]. The reduction in viable cells and the PI staining were significant, indicating cell death by necroptosis at 47.85% after 24 h.

Cell cycle analysis

Analysis of cell cycle was conducted to check whether the extract arrests any cell cycle phase. PI staining indicated that treatment of cells for 24 h with EAFCP (200 µg/ml) increased the percentage of cells in subG1 phase population which represents apoptotic population [Figure 7a-c]. There are few changes in various phases of cell cycle but are not significant with 24-h treatment.

Reactive oxygen species and mitochondrial membrane potential

MCF-7 was treated with EAFCP (200 µg/ml) for 24 h, and the levels of ROS and MMP were evaluated. A considerable rise in intracellular ROS and MMP level was experienced in the EAFCP-treated MCF-7 cells as compared to the control [Figure 8a and b]. The results showed that EAFCP might be inducing antiproliferative effect through ROS generation with loss of MMP 24 h after treatment, which proves the role of mitochondria in the cell death observed due to EAFCP treatment.

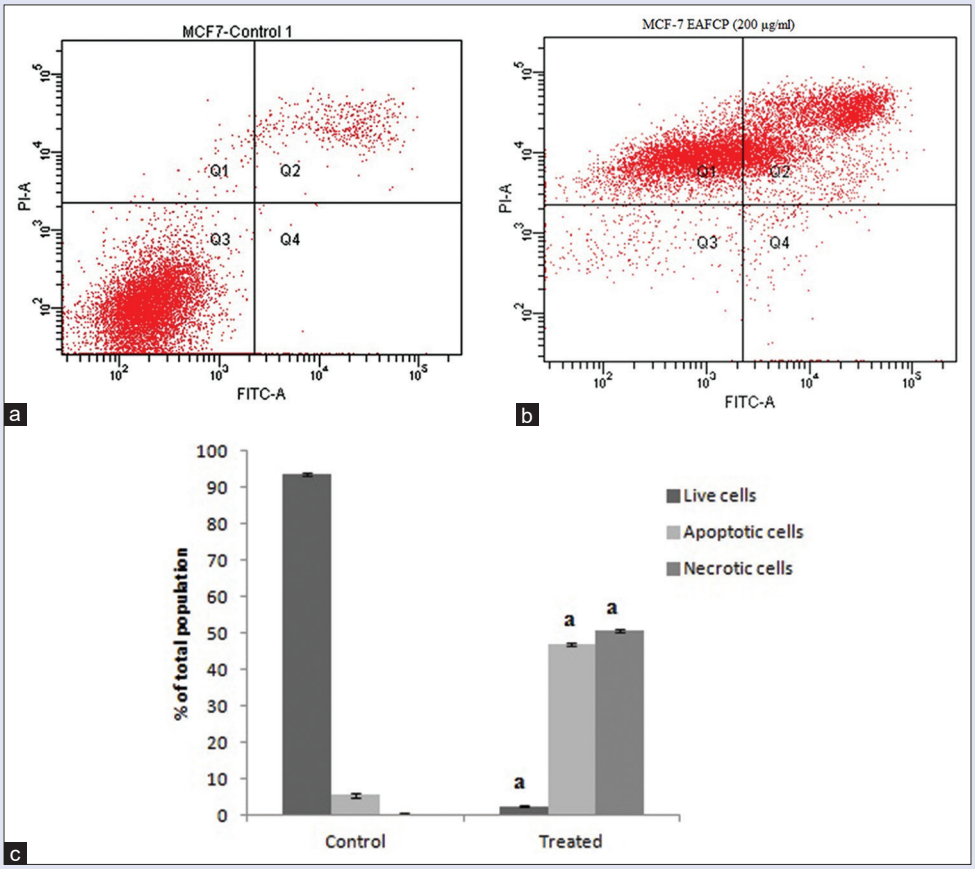

Figure 6: Assay annexin-propidium iodide flow cytometry after treatment with the ethyl acetate fraction of Caesalpinia pulcherrima. (a) Control after 12 h. (b) extract causes necroptosis in MCF-7 after 12 h of treatment. (c) Percent of total population of MCF-7 after 12 h of treatment

DISCUSSION

The present study demonstrates the chemomodulatory action of EAFCP in MNU-induced carcinogenesis in rat mammary gland. One of the ways to treat cancer is administering natural or synthetic compound for prevention, suppression, or reversion of cancer. Diet rich in vegetables and fruits is associated with reduced incidences of cancer and other diseases.[28] Herbs have been regarded as one of the most visible options for new potent anticancer agents and cancer treatment.[29] It produces diverse biological effects, such as detoxification, scavenging of ROS, and cell proliferation and its regulation.[30]

EAFCP exhibits strong cytotoxic and anti-oxidant activities,[31] which may possibly inhibit the carcinogenesis to transform a normal cell into malignant cells. The presence of rutin and catechin in the fraction is important because this fraction showed the best activity. All the treated groups with EAFCP had statistically significantly ($P < 0.001$) diminished tumor incidences and increased latency as compared to MNU-treated group. Reduced tumor volume in the treated group indicates that EAFCP seems to be reversing the tumorigenesis. Multistep carcinogenesis process has been divided into initiation, promotion, and progression.[32] The cancer-preventing agent may act dominantly at the initiation or promotion stage of carcinogenesis as an antipromoting agent. Thus, it can be presumed that EAFCP may interfere at the initiation or promotion stage to suppress the growth of the tumor. Decreased tumor burden indicates that EAFCP may also act as an antimetastatic agent. Breast cancers express the ER and progesterone receptor (PR) which respond to therapy with hormones or aromatase inhibitors and shrink breast cancer masses in patients.[33,34] Elevated serum estradiol levels (E2) are connected with higher risk of breast cancer.[35] E2 promotes cell proliferation and suppresses apoptosis by altering the genetic expression, and thus is considered a key target for treatment.[36] MNU-induced tumors are more estrogen responsive. Natural phytoestrogen may stimulate the apoptotic pathway via modulating estrogen and ERs.[37] Thus, EAFCP may act by modulating estrogen and ERs and regulate the cell death by mitochondria-mediated necroptosis in subG1 phase, which may be due to the presence of polyphenols. In the present study, the activities of antioxidant enzymes such as LPO, CAT, SOD, GPx, GST, GR, and GSH were estimated in mammary tumors of rats. The antioxidant system contributes toward the inhibition of carcinogenesis. Exogenous sources such as environmental pollutants, drugs, radiation, and pathogens are involved in the production of free radicals.[38] Increased production of ROS and a decrease in antioxidant level might be responsible for the increase in LPO. This has been associated with altered membrane structure and enzyme inactivation.[39] Data obtained revealed that a statistically significant ($P < 0.001$) increase in the antioxidant enzymes and decreased LPO level were found in treated mammary tissue homogenates. In the present study, the protein levels in mammary tumor-induced rats and EAFCP extract-treated rats had a significant difference. The impaired protein levels may influence the course of the disease.

Histopathological study of mammary tissue of rats illustrates ductal carcinoma of the cribriform type with infiltrating malignant tumor. EAFCP- and TAM-treated rats increased tumor tissue necrosis and altered inflammation and tumor tissue ratio. Inflammatory mast cells are the major effector cells and significant for the pro-angiogenic factor

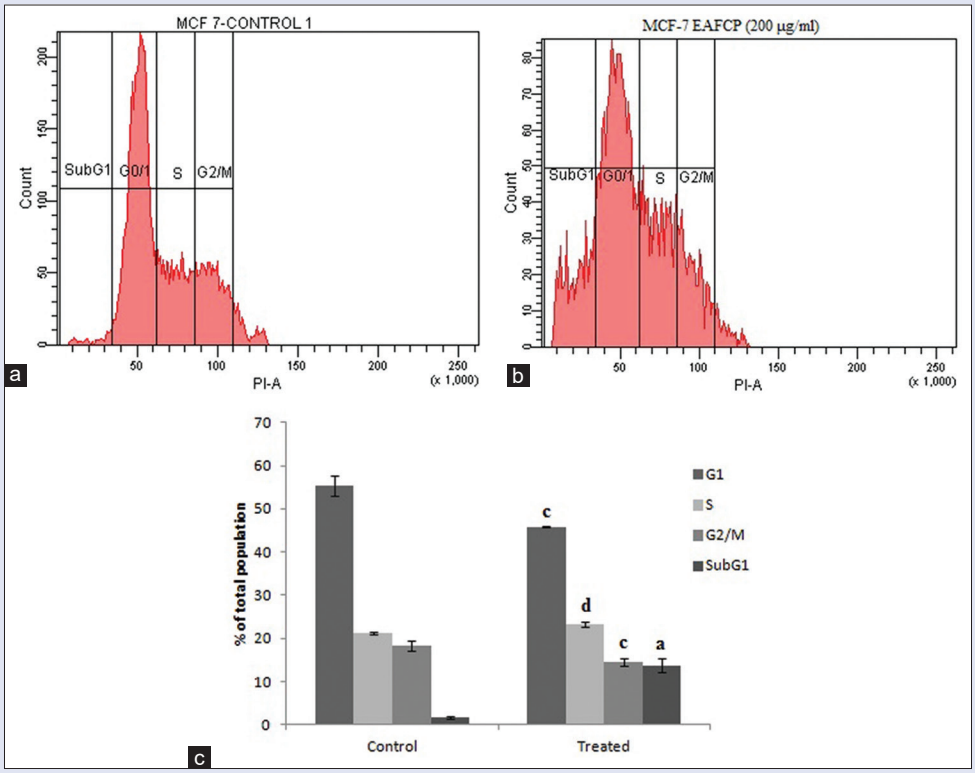

Figure 7: Flow cytometric analysis of the effect of ethyl acetate fraction of Caesalpinia pulcherrima on cell cycle of MCF-7 cells. (a) control, (b) extract arrest cell cycle at subG1 phase after treatment (c) Percent of total population of MCF-7 in different phases

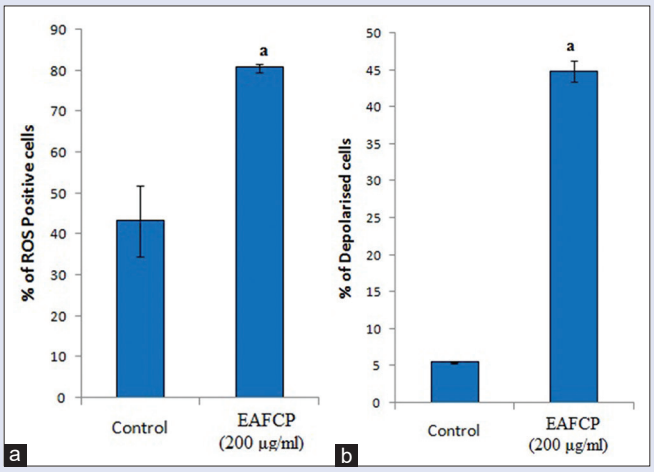

Figure 8: Determination of (a) reactive oxygen species and (b) mitochondrial membrane potential at indicated dose of ethyl acetate fraction of *Caesalpinia pulcherrima* in MCF-7 cells

to induce tumor growth.[40] An increased number of mast cells create tumor cellular environment for the progression, angiogenesis, and metastasis.[41] In the present histological analysis, mammary tissue showed an increase in mast cell population, which is in conformity with that of the preceding study observations of histological signs in mammary tissue.[42] Alteration in the mammary tissue architecture of EAFCP- and TAM-treated rats may possibly due to its anti-inflammatory activity.

In order to visualize the binding affinity, interaction, and orientation of catechin and rutin, on ligand-binding domain (LBD) of ER-α, the docking studies were performed using Glide and docking scores of catechin and rutin which were found to be −12.626 and −7.262, respectively. Upon ligand binding, the flexible and hydrophobic nature of LBD of ER-α remodels their shapes and stabilizes the ligand–receptor complex by making specific hydrogen bonds and complementary hydrophobic interactions. Thus, both the compounds, catechin and rutin, bind to LBD of ER-α with a comparable binding affinity [Figure 9a and b]. The hydroxyl groups of both catechin and rutin formed hydrogen bonds with amino acid residues Leu346, Thr347, Asp351, Glu353, Arg394, Met421, and His524 in the LBD of ER-α, which stabilizes the ligand–enzyme complex. Apart from hydrogen bonds, catechin also formed pi–pi bonding with Phe404 to enhance hydrophobic interaction with LBD of ER-α.

CONCLUSION

The results of this study showed that the number of phytoconstituents of EAFCP may have synergistically participated in the preventive MNU-induced mammary carcinogenesis in rats. A positive correlation was found between catechin, rutin, and binding affinity for the ER-α of EAFCP, which indicates that it could be the major contributors in the chemoprevention. Our results strongly highlight the possible use of EAFCP as an economic value added in ingredients in nutraceutical products to prevent/treat mammary carcinoma.

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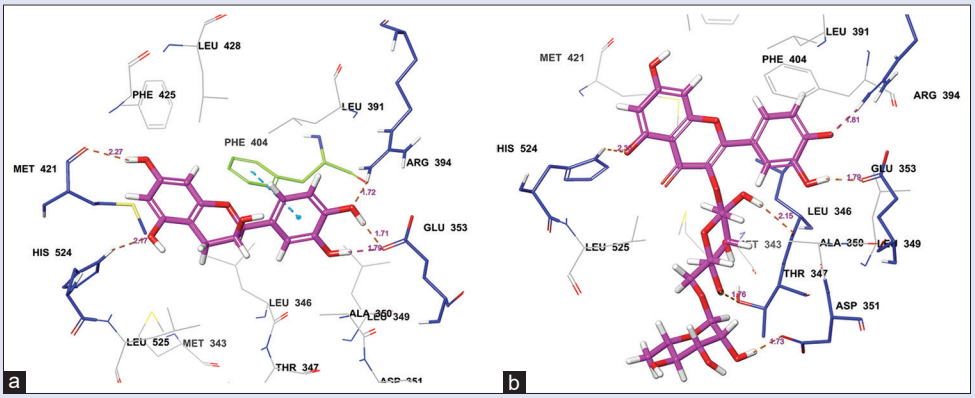

Figure 9: Binding pose of compounds in ligand-binding domain of estrogen receptor-alpha (a) catechin and (b) rutin. Pink dotted lines indicate H-bond interaction and cyan color dotted lines indicate π–π interaction

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Conflicts of interest

There are no conflicts of interest.

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