Screening of extracts from selected medicinal plants based on their cytotoxic activity against breast cancer cell lines

3.1. Introduction

Natural compounds of plant origin play an important role in the progress of new and safer drugs in India as well as worldwide [1-4]. Medicinal plants are rich in bioactive molecules which may be beneficial for the treatment of various human diseases [3,5]. In the last few decades, numerous traditional knowledge-based drugs have been isolated and commercialized [6-9]. Secondary metabolites of plants like flavonoids, alkaloids, glycosides and tannins have evolved as potent candidates against various cancers [10,11]. Plant-derived compounds like vincristine, vinblastine, taxol, paclitaxel, and podophyllotoxin are already marketed as successful anticancer agents [12]. Exploring the huge biodiversity of Northeast India can be important to the discovery of novel lead molecules with therapeutic potential against cancer [13,14]. In this study, we focus to find potent natural lead compounds for the development of a drug against breast cancer. Based on an extensive literature survey and traditional knowledge, we selected three plants for our study. Six extracts from various parts of three plants were prepared and activity of the individual extracts was tested.

3.2. Material and methods

3.2.1. Cell lines and reagents

The cell lines MCF-7, MDA-MB-231, and HEK293 were purchased from NCCS Pune, India and MEF was a gift from Dr. Sougata Saha, Department of MBBT, Tezpur University, India. DMEM (Dulbecco's Modified Eagle Medium) and FBS (Fetal Bovine Serum) were purchased from Life Technologies, USA. MTT (3-(4, 5-dimethyl thiazolyl-2)-2, 5-diphenyltetrazolium bromide), DMSO, Doxorubicin and all the polyphenols used in this study were purchased from Sigma Aldrich. Other chemicals used in various in-vitro assays in this chapter were purchased either from Himedia, India, Merck, India or Sisco Research Laboratories Pvt. Ltd. (SRL), India.

3.2.2. Preparation of polyphenol solutions

Towards the establishment of the MTT assay commercially available polyphenols like Ascorbic acid, Apigenin, Chrysin, Ellagic acid, Morin, Pyrogallic acid, Rutin were used as controls in this study. Stock solutions were prepared in the manufacture's directed solvent. Working solutions were prepared in incomplete DMEM for treatment in the cell culture system. The polyphenols used in this study are presented in **Table 3.1.**

Table 3.1.: Polyphenols used for the establishment of MTT assay

Sl no.	Polyphenol name	Origin	Source	Abbreviation	References
1.	**Ascorbic acid**	Plant	Commercial	**Aa**	[15-17]
2.	**Apigenin**	Plant	Commercial	**Ap**	[18-21]
3.	**Chrysin**	Plant	Commercial	**Ch**	[22-25]
4.	**Ellagic acid**	Plant	Commercial	**Ea**	[26-29]
5.	**Morin**	Plant	Commercial	**Mo**	[20,30,31]
6.	**Pyrogallic acid**	Plant	Commercial	**Pa**	[32]
7.	**Rutin**	Plant	Commercial	**Ru**	[33,34]
8.	**Quercetin**	Plant	Commercial	**Qu**	[35,36]

3.2.3. Preparation of plant extracts

Fresh parts from three medicinally important plants like leaves, fruits, stem, roots, tuber were collected from the Golaghat district of the state of Assam, India and each part extracted individually with 50% denatured ethanol at room temperature for 48hr for cold as well as hot extraction (by soxhlet) method. The extracts were filtered and then concentrated under reduced pressure to remove excess ethanol and finally was lyophilized to obtain a dried and powdered hydro-ethanolic extract of the parts used separately. All the extracts were stored at 4°C and checked for the biological activity. The plants, its parts used, the extraction process and abbreviations for each extract are given in **Table 3.2.**

Table 3.2.: Extracts used for screening

Sl no	Plant name	Part used	Type of solvent used	Extraction type	Abbreviation	Traditional use	References
1.	***Ricinus communis* L.**	Fruit	50% EtOH	Cold	**RCFE**	Pain, paralysis, constipation, gastritis, warts, anti-fungal, anti-tumor, anti-inflammatory	**[37,38]**
2.	***Ricinus communis* L.**	Leaf	50% EtOH	Hot	**RCLE**		
3.	***Amorphophallus paeoniifolius* (Dennst.) Nicolson**	Tuber	50% EtOH	Cold	**APTE**	Anthelmintic, analgesic, antitumor, anti-inflammatory	**[39-41]**

4.	***Smilex perfoliata* Lour.**	Leaf	50% EtOH	Cold	**SPLE**	Anti-diabetic, rheumatism, anti-ulcer, nourishing the functions of spleen, stomach, muscle and bone	**[42-44]**
5.	***Smilex perfoliata* Lour.**	Stem	50% EtOH	Cold	**SPSE**		
6.	***Smilex perfoliata* Lour.**	Root	50% EtOH	Cold	**SPRE**		

3.2.4. Cell culture

MCF-7, MDA-MB-231, HEK293, and MEF cell lines were maintained in complete Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% fetal bovine serum and 1% antibiotic. Cell lines were incubated in a CO_2 incubator at 5% CO_2 and 37° C temperatures.

3.2.5. *MTT assay for cytotoxicity determination by measuring cellular metabolic activity*

5000 cells/well were plated in a 96 well plate and incubated for 48 hr. Cells were treated with different concentrations of each test sample followed by further incubation of 24 and 48 hr. Following incubation, cells were treated with MTT and incubated for 3.5 hr. MTT quantitates ATP present in metabolically active cells, which indicate viable cells. NAD(P)H-dependent cellular oxidoreductase enzymes reduce MTT to insoluble formazan, a purple colour dye, which is then measured spectrophotometrically. MTT dissolving solution was added after the media was removed carefully and absorbance of the purple dye in the cell lysate was measured at 590 nm wavelength using UV-Vis spectrophotometer (Multiscan Go, ThermoScientific) which corresponds to the percentage of viable cells [45,46].

3.2.6. In vitro free radical scavenging activity

3.2.6.1. DPPH radical scavenging assay

The scavenging activity of stable free radicals of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) by the extract was measured in this assay. For this 1 ml of different concentrations of extracts (25-400 µg/ml) and 1 ml of 0.8 mM DPPH solution in methanol were mixed. This mix was shaken vigorously and allowed to react for 30 min. Absorbance was read at 517 nm against a blank. Ascorbic acid was used as a standard. The percentage of radical scavenging activity was calculated as

$$\%\ of\ scavenging\ activity = \left[1 - \left(\frac{A_{sample}}{A_{control}}\right)\right] \times 1$$

3.2.6.2. Hydroxyl radical scavenging assay

In this assay, 500 µl of 2-deoxyribose (2.8 mM) in phosphate buffer (50 mM, pH 7.4) was mixed with 200 µl of premixed $FeCl_3$ (100 mM) and EDTA (100 mM) in the ratio 1:1. To this reaction mixture, 100 µl H_2O_2 (200 mM) was added and then 100 µl of various concentrations of extract (25-400 µg/ml) were added. 100 µl of 300 mM ascorbate was added to the above mixture and incubated at 37^0 C for 1 hr. From the reaction mixture, a 0.5 ml solution was added with 1ml TCA (2.8%w/v) and 1 ml of 1% aqueous Thiobarbituric acid (TBA). This mixture was placed in a boiling water bath for 15 min. This mix was then cooled and absorbance was read at 532 nm against a blank. Quercetin was used as a standard. The percentage of radical scavenging activity was calculated as

$$\%\ of\ scavenging\ activity = \left[1 - \left(\frac{A_{sample}}{A_{control}}\right)\right] \times 100$$

3.2.6.3. ABTS radical scavenging assay

The 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid), also known as ABTS radical scavenging assay is done by allowing to react ABTS solution (7 mM) with potassium persulfate (2.6 mM) in 1:1 ratio for 16 hr in dark to yield ABTS cation radicals. Then 0.54 ml of this radical solution is added with 4 ml of extract (25-400 µg/ml) and 0.5 ml of 100 mM phosphate buffer was added and incubated for 2 hr. Absorbance was taken at 734 nm against a blank. Ascorbic acid was used as a standard. The percentage of radical scavenging activity was calculated as

$$\%\ of\ scavenging\ activity = \left[1 - \left(\frac{A_{sample}}{A_{control}}\right)\right] \times 100$$

3.2.6.4. Nitric oxide radical scavenging assay

To measure nitric oxide scavenging activity of the extract Griess reagent was used. In this assay 3 ml, sodium nitroprusside (10 mM) in phosphate buffer saline (pH 7.4) is mixed with 2 ml of different concentrations of extract (25-400 µg/ml) and incubated at 25^0 C for 60 min. After this 5 ml of Griess reagent is added and absorbance was taken at

546 nm against a blank. Ascorbic acid was used as a standard. The percentage of radical scavenging activity was calculated as

$$\%\ of\ scavenging\ activity = \left[1 - \left(\frac{A_{sample}}{A_{control}}\right)\right] \times 100$$

3.2.7. Total antioxidant activity (FRAP)

The FRAP method is used to determine the total antioxidant activity of a sample where the reduction of ferric ion to ferrous form is measured in the presence of the antioxidant sample. For this FRAP reagent was made by adding 2.5 ml of 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ) in HCl (40 mM), 2.5ml of ferric chloride (20 mM) and 25 ml of acetate buffer (0.3 M, pH 3.6). 20 µl of the extract was mixed with 180 µl of FRAP reagent and absorbance was read at 595 nm against a blank. The total antioxidant activity of the extract was expressed as milligrams of ascorbic acid equivalents (AAE) per g of dry weight of the extract using ascorbic acid as a standard.

3.2.8. Determination of phytochemicals in RCFE and APTE

Qualitative phytochemical analysis of the extracts was performed for the determination of the presence of secondary metabolites by following the standard methods [47-49].

3.2.8.1. Test for Carbohydrates:

About 10 mg of the extract was separately solubilised in 5 ml of water and then filtered, which was used to do the following test.

Molish's test: To 2-3 ml of the test solution, a few drops of 1-naphthol solution were added. The mixture was shaken well and about 1 ml of concentrated H_2SO_4 was added from the sides of the test tube. The formation of a violet ring at the junction of the two liquids indicates the presence of carbohydrates.

3.2.8.2. Test for Reducing Sugars:

About 10 mg of the extract was separately solubilised in 5 ml of water and then filtered, which was used to do the following test.

Fehling's test: 1 ml of Fehling's A solution was mixed with 1ml of Fehling's B solution and the mixture was boiled for 1 min. An equal volume of test solution was added to the

mixture and then heated in a boiling water bath for 5 -10 min. Formation of a yellow first and then a red ppt. indicates the presence of reducing sugar.

Benedict's test: An equal volume of Benedict's reagent was mixed with the test solution and then the mixture was heated in a boiling water bath for 5 min. The appearance of green or yellow color indicates the presence of reducing sugar.

3.2.8.3. Test for Non Reducing sugars:

About 10 mg of the extract was separately solubilised in 5 ml of water and then filtered, which was used to do the following test.

Iodine test: To 3 ml of the test solution, a few drops of iodine solution were added. The appearance of blue color which appears on boiling and disappears on cooling indicates the presence of non-reducing sugar.

3.2.8.4. Test for Proteins:

About 10 mg of the extract was separately solubilised in 5 ml of water and then filtered, which was used to do the following test.

Biuret test: To 3 ml of the test solution, 1 ml of 4% NaOH and a few drops of 1% $CuSO_4$ were added. The appearance of violet or pink color indicates the presence of protein.

Ninhydrin test: 2 ml of test solution was mixed with 0.2% ninhydrin and the mixture was boiled in boiling water bath for 10 min. The appearance of purple or bluish color indicates the presence of amino acid.

3.2.8.5. Test for Cardiac Glycosides:

About 10 mg of the extract was separately solubilised in 5 ml of water and then filtered, which was used to do the following test.

Keller- Killani test: To 2 ml of the extract a few drops of glacial acetic acid, one drop of 5% $FeCl_3$ and concentrated H_2SO_4 were added. The appearance of reddish-brown color at the junction of two liquid layers and bluish-green color in the upper layer indicates the presence of cardiac glycosides.

3.2.8.6. Test for Saponin Glycosides:

Foam test: The extract was shaken vigorously with water for 5 min. The appearance of persistent foam indicates the presence of saponin glycosides.

3.2.8.7. Test for Alkaloids:

Wagner's test: To 3 ml of the test solution, a few drops of Wagner's reagent were added. Formation of reddish-brown ppt. indicates the presence of alkaloids.

3.2.8.8. Test for Flavonoids:

Zinc hydrochloride reduction test: To a small quantity of the extract, 0.5 gm of Zn dust and few drops of concentrated HCl was added. The formation of red color indicates the presence of flavonoids.

Sodium hydroxide test: To a small quantity of extract, an increasing amount of sodium hydroxide (NaOH) was added. The appearance of yellow color which decolorizes after the addition of a dilute acid indicates the presence of flavonoids.

3.2.8.9. Test for Tannins and Phenolic compounds:

Ferric chloride test: To 3 ml of extract, 2-3 drops of $FeCl_3$ were added. The appearance of a deep blue-black color indicates the presence of tannins and phenolic compounds.

Dilute iodine solution test: To the small amount of extract, few drops of dilute iodine solution were added. The appearance of a transient red color indicates the test positive.

Dilute HNO_3 acid test: To the small quantity of the extract, few drops of dilute HNO_3 were added.

Potassium Dichromate solution: A small quantity of the extract was dissolved in water; few drops of potassium dichromate solution were added. Formation of red ppt. indicates the test positive.

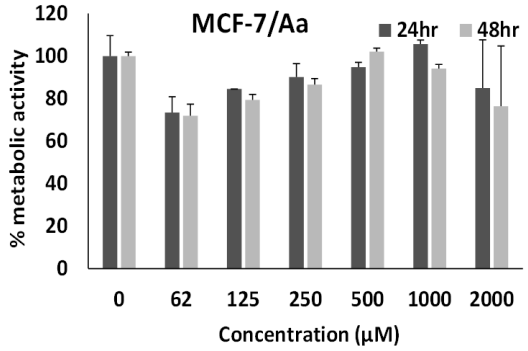
3.3. Results

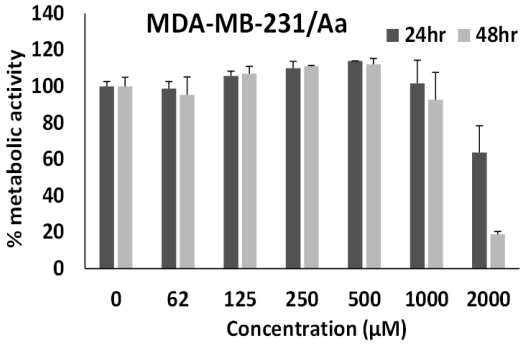
3.3.1. Establishment of cytotoxicity (MTT) assay method using commercial polyphenols

Eight polyphenols were selected on the basis of reported literature for their anti-cancer

as well as other biological activity in different diseases. Two breast cancer cell lines (MCF-7 and MDA-MB-231) were treated with indicated concentrations of the eight commercially obtained polyphenols viz. Ascorbic acid, Apigenin, Chrysin, Ellagic acid, Morin, Pyrogallic acid, Rutin and Quercetin for 24 and 48 hr **(Fig 3.1.A-H)**. The percentages of metabolic activity (which corresponds to viable cells) with respect to untreated control were determined by quantification with MTT. The results suggested differential cytotoxicity in the cells.

A





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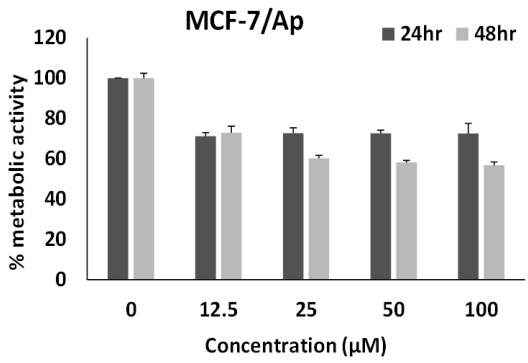

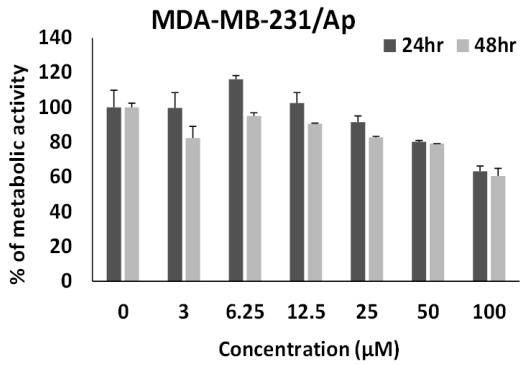

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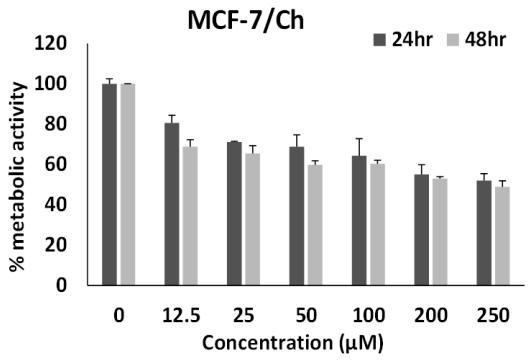

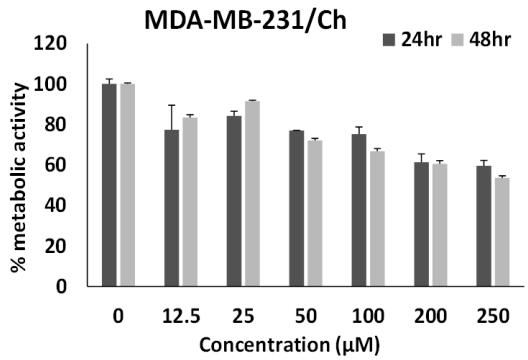

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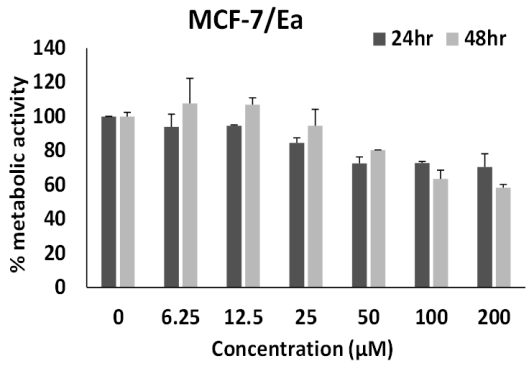
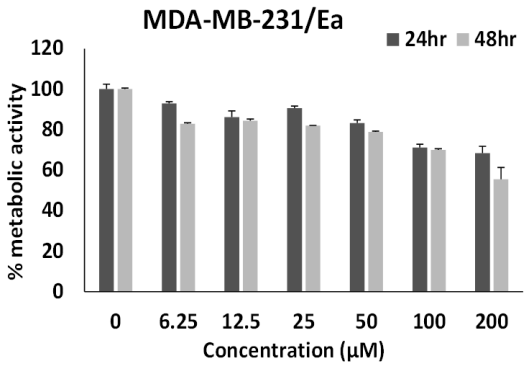

MCF-7/Ea
24hr
48hr
% metabolic activity
0
20
40
60
80
100
120
140
0
6.25
12.5
25
50
100
200
Concentration (μM)


MDA-MB-231/Ea
24hr
48hr
% metabolic activity
0
20
40
60
80
100
120
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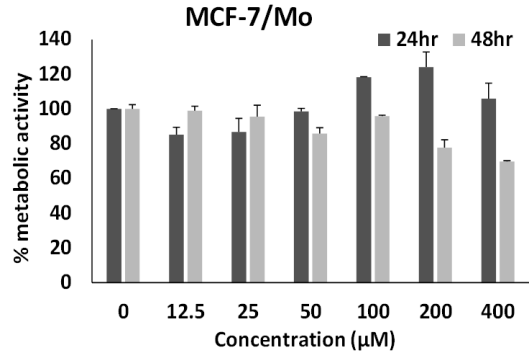
6.25
12.5
25
50
100
200
Concentration (μM)

E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48hr
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100
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140
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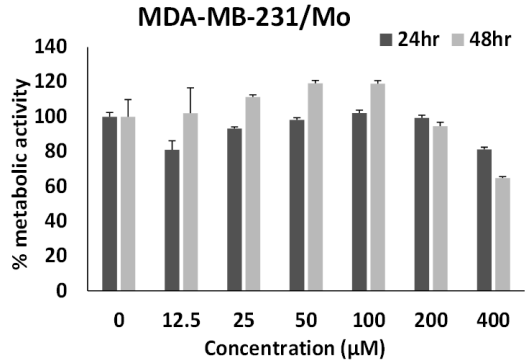
25
50
100
200
400
Concentration (μM)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24hr
48hr
% metabolic activity
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40
60
80
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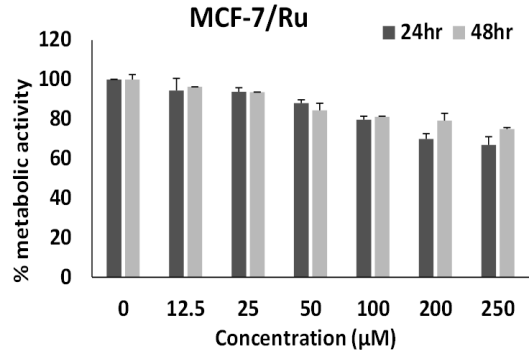
120
140
0
12.5
25
50
100
200
400
Concentration (μM)

F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48hr
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100
120
0
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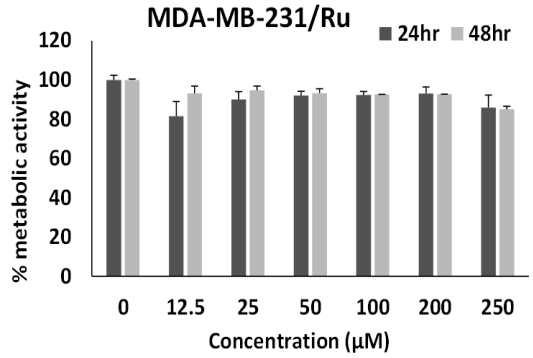
25
50
100
200
250
Concentration (μM)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24hr
48hr
% metabolic activity
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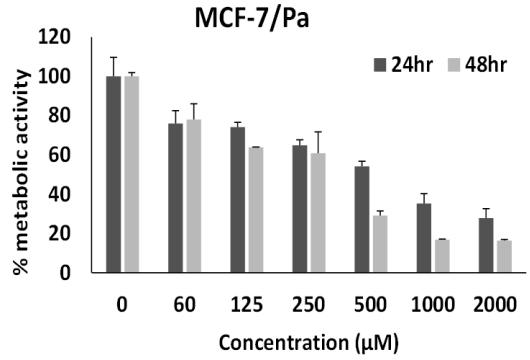
20
40
60
80
100
120
0
12.5
25
50
100
200
250
Concentration (μM)

G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48hr
% metabolic activity
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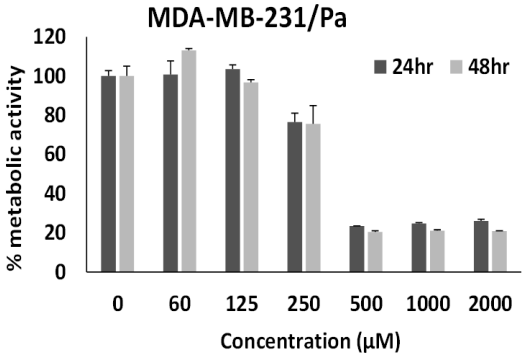
20
40
60
80
100
120
0
60
125
250
500
1000
2000
Concentration (μM)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24hr
48hr
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40
60
80
100
120
0
60
125
250
500
1000
2000
Concentration (μM)

H

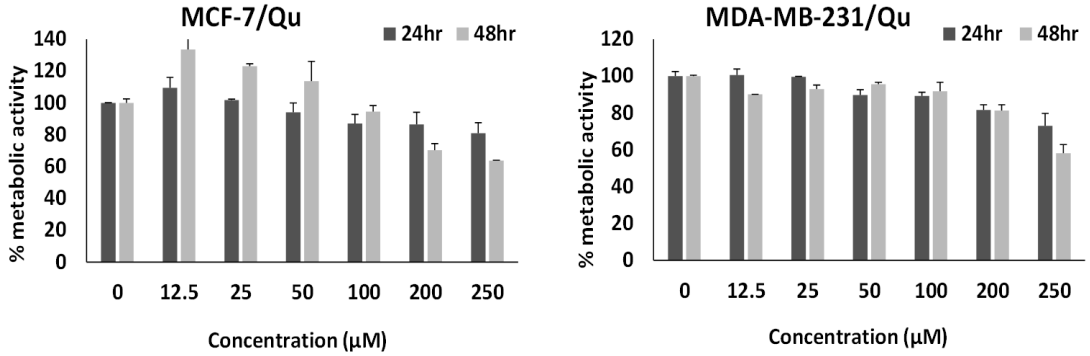


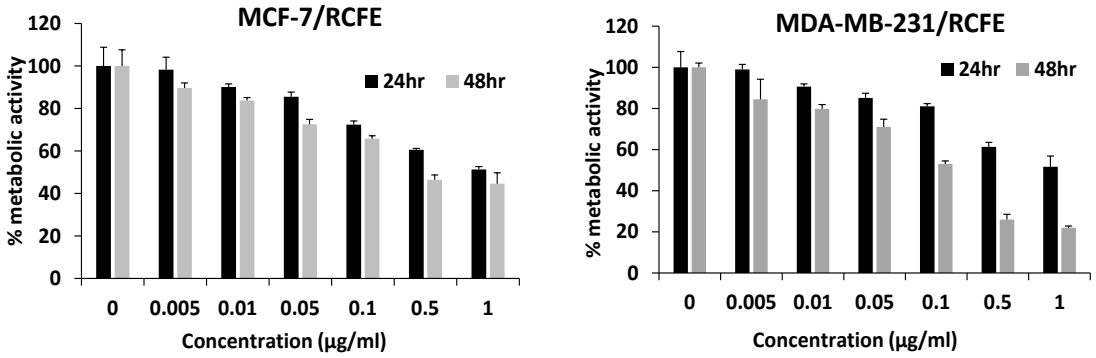
Figure 3.1.: Screening of eight polyphenols in MCF-7 and MDA-MB-231 cells by MTT assay. Cells were treated with various concentrations of Ascorbic acid **(A),** Apigenin **(B),** Chrysin **(C),** Ellagic acid **(D),** Morin **(E),** Pyrogallic acid **(F),** Rutin **(G),** Quercetin **(H)** for 24 and 48 hr. variable effects were observed in each polyphenol. Data represent the mean ± SEM of three independent experiments.

Ascorbic acid was found to be effective at a high concentration of 2000 µM in both 24 and 48 hr in MCF-7 and MDA-MB-231 whereas apigenin and chrysin were effective at a much lower concentration of 100 µM in both the cells. Other polyphenols like ellagic acid, morin, rutin, pyrogallic acid and quercetin were effective in the concentration range 250-500 µM at 24 and 48 hr in both MCF-7 and MDA-MB-231.

3.3.2. Cytotoxic activity of plant extracts

Six extracts viz. *Ricinus communis* fruit extract (RCFE), *Ricinus communis* leaf extract (RCLE), *Amorphophallus paeoniifolius* tuber extract (APTE), *Smilex perfoliata* leaf extract (SPLE), *Smilex perfoliata* stem extract (SPSE) and *Smilex perfoliata* root extract (SPRE) were used to treat MCF-7 and MDA-MB-231 cells for 24 and 48 hr and percentage of metabolic activity were quantified which indicated percentage of metabolically active viable cells **(Fig 3.2.A-F)**.

A



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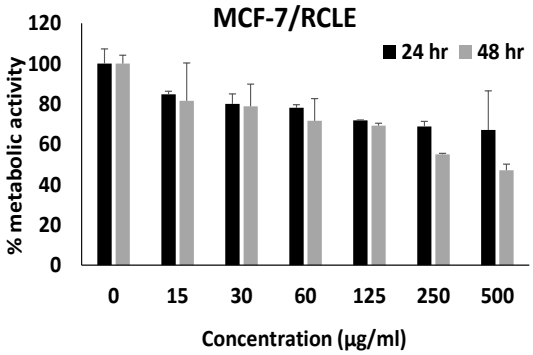
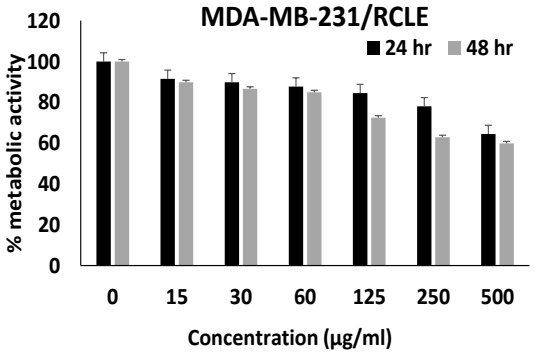

MCF-7/RCLE
24 hr
48 hr
% metabolic activity
120
100
80
60
40
20
0
0
15
30
60
125
250
500
Concentration (µg/ml)


MDA-MB-231/RCLE
24 hr
48 hr
% metabolic activity
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100
80
60
40
20
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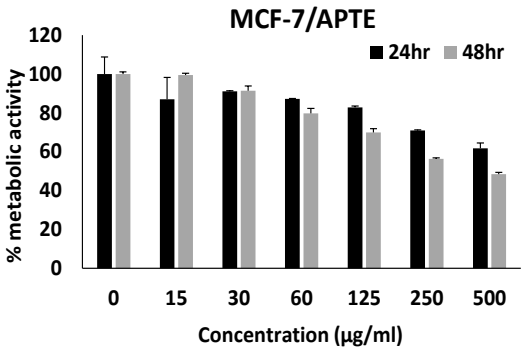
0
15
30
60
125
250
500
Concentration (µg/ml)

C


MCF-7/APTE
24hr
48hr
% metabolic activity
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80
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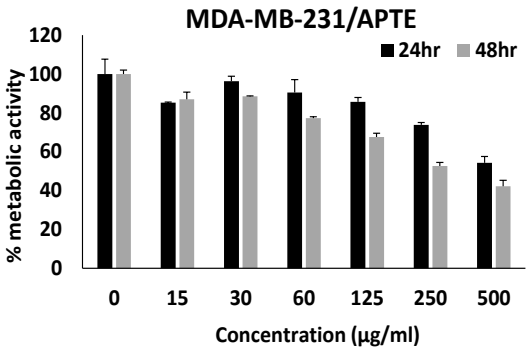
125
250
500
Concentration (µg/ml)


MDA-MB-231/APTE
24hr
48hr
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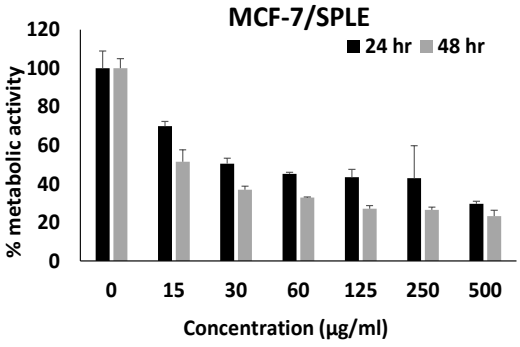
Concentration (µg/ml)

D


MCF-7/SPLE
24 hr
48 hr
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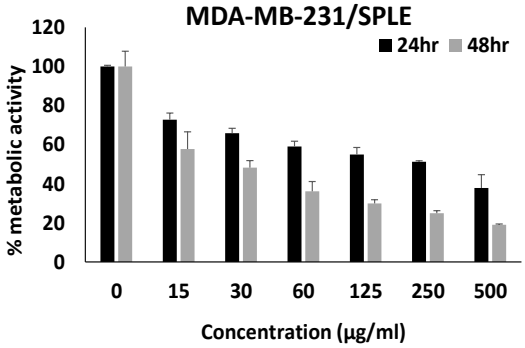
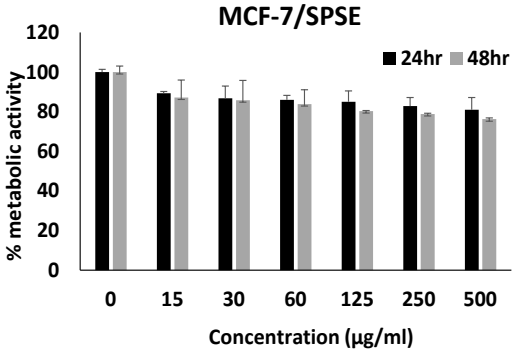
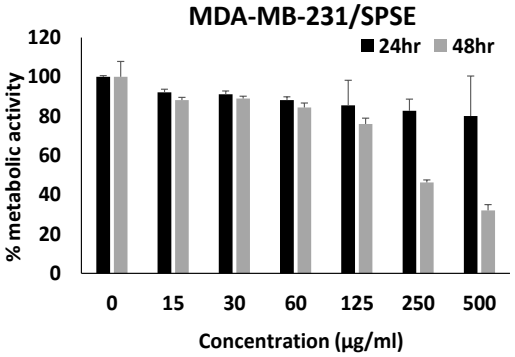
125
250
500
Concentration (µg/ml)


MDA-MB-231/SPLE
24hr
48hr
% metabolic activity
120
100
80
60
40
20
0
0
15
30
60
125
250
500
Concentration (µg/ml)

E


MCF-7/SPSE
24hr
48hr
% metabolic activity
120
100
80
60
40
20
0
0
15
30
60
125
250
500
Concentration (µg/ml)


MDA-MB-231/SPSE
24hr
48hr
% metabolic activity
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Concentration (µg/ml)

F

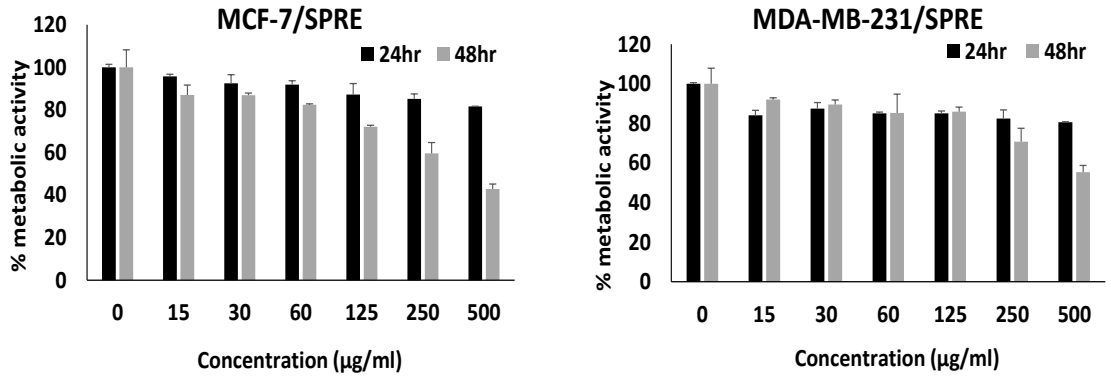


Figure 3.2.: Screening of eight polyphenols in MCF-7 MDA-MB-231cells by MTT assay. Cells were treated with various concentrations of RCFE **(A),** RCLE **(B),** APTE **(C),** SPLE **(D),** SPSE **(E),** SPRE **(F)** for 24 and 48hr. Data represent the mean ± SEM of three independent experiments.

RCFE was found to be most effective among all the extracts under study where it induced more than 50% cytotoxicity in both MCF-7 and MDA-MB-231 cells at a very low concentration of 1 µg/ml. RCLE showed less than 50% cytotoxicity in both the cells at a concentration as high as 500 µg/ml. This concentration is significantly high when as compared to RCFEAPTE showed almost 50% induction of cytotoxicity in these cells at 500 µg/ml. Among the three extracts from the plant *Smilex perfoliata,* SPLE showed highest activity i.e. more than 50% cytotoxicity at 500 µg/ml. However, activities of SPSE and SPRE were negligible at this concentration.

On the basis of the results of preliminary screening, availability of the samples and lesser toxicity on normal cell lines two plant extracts RCFE and APTE were considered for further study.

3.3.3. RCFE and APTE are non-toxic to normal cells

To understand the cytotoxic specificity of RCFE and APTE, additional cell lines of normal origin were treated with the extracts. Treatment with similar doses of RCFE and APTE showed the minimal effect on the cytotoxicity of the human embryonic kidney (HEK-293) and mouse embryonic fibroblast (MEF) cells **(Fig 3.3.A** & **B)** suggesting the extract's specific cytotoxic efficacy against cancer cells only without harming the nearby normal cells and tissues.

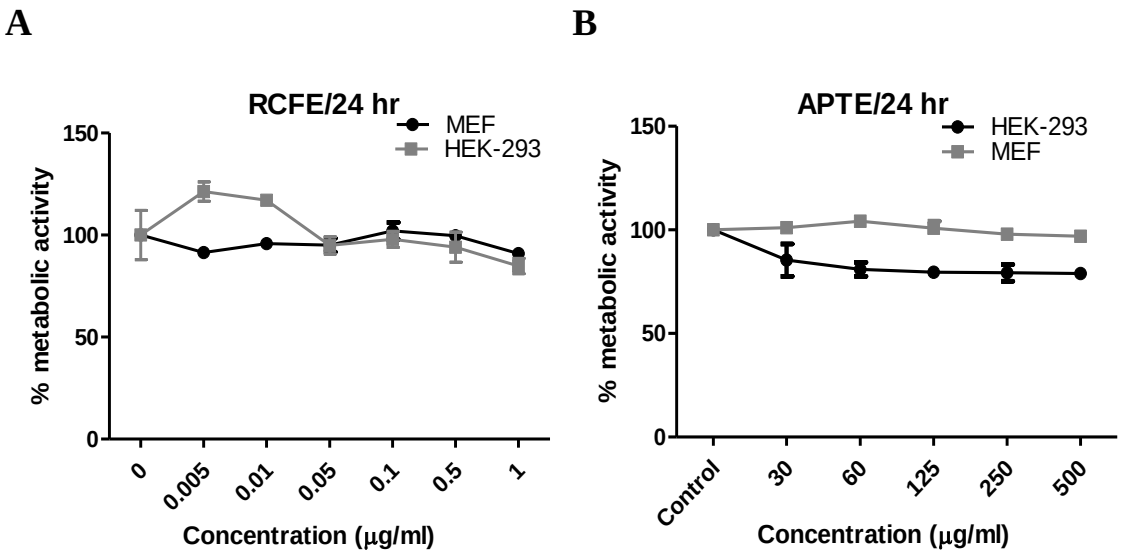
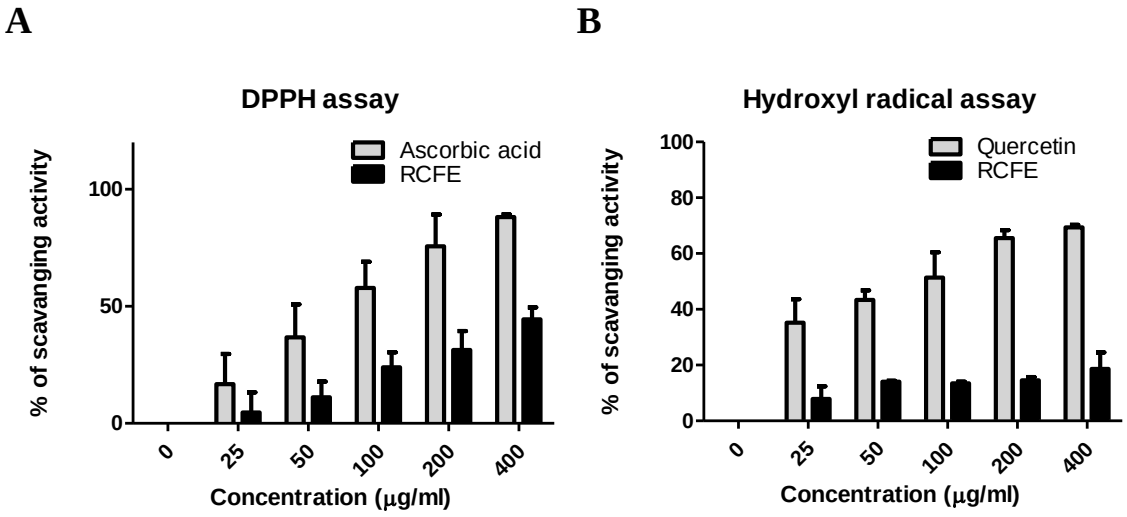


Figure 3.3.: RCFE and APTE do not induce cytotoxicity in HEK-293 and MEF. Various concentrations of RCFE **(A)** and APTE **(B)** were treated to HEK-293 and MEF cells for 24 hr but the same concentrations of RCFE and APTE were not toxic HEK-293 and MEF cells in MTT assay. Data represent the mean ± SEM of three independent experiments.

3.3.4. In vitro free radical scavenging activity

To understand the role of the extracts in scavenging free radicals, four different *in vitro* assays were performed [50,51]. Free radicals and ROS are important factors in cancer initiation and progression and can act as a target for cancer remediation. RCFE and APTE showed a dose-dependent activity in scavenging DPPH, hydroxyl, ABTS and nitric oxide radicals in the respective scavenging assays. The data showed comparatively higher free radical scavenging activity by RCFE than APTE but the



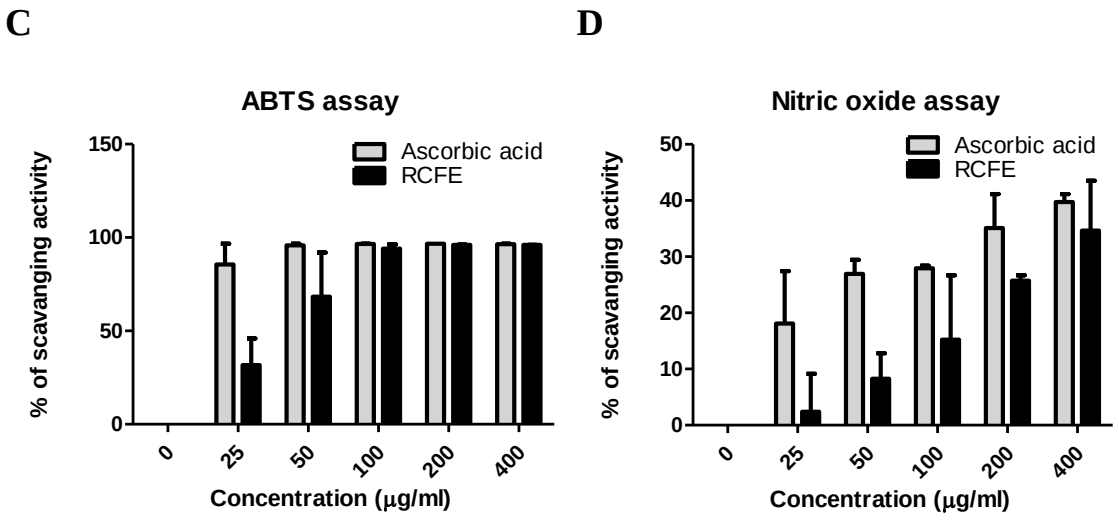
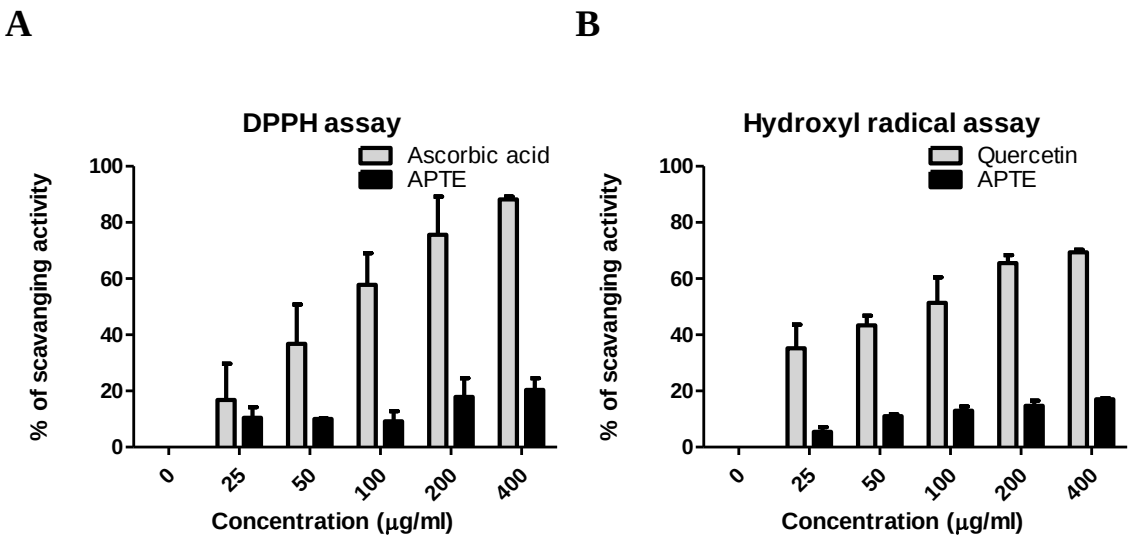


Figure 3.4.: In vitro free radical scavenging activity of RCFE. (A) DPPH radical scavenging activity of the RCFE compared to ascorbic acid **(B)** hydroxyl radical scavenging activity of the RCFE compared to quercetin **(C)** ABTS radical scavenging activity of the RCFE compared to ascorbic acid **(D)** Nitric oxide radical scavenging activity of the RCFE compared to ascorbic acid.

effects were comparable to the standard ascorbic acid in DPPH, ABTS, and nitric oxide assay and quercetin in hydroxyl radical assay (as ascorbic acid is a component of the reaction mixture we have not used ascorbic acid as control) **(Fig 3.4.A-D** & **3.5.A-D).** Total antioxidant activity of the extracts was determined by FRAP assay taking ascorbic acid as standard and it was found to be 139.53±3.18 Ascorbic Acid Equivalent (AAE)/g of extract in RCFE and 2.6±2.2 mg AAE/g of extract in APTE.



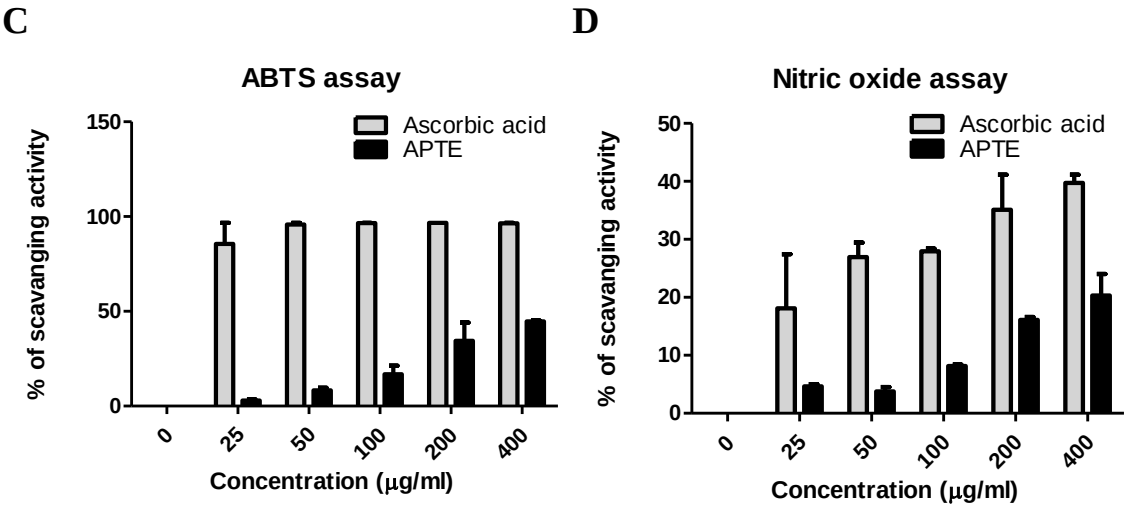


Figure 3.5: In vitro free radical scavenging activity of APTE. (A) DPPH radical scavenging activity of the APTE compared to ascorbic acid **(B)** hydroxyl radical scavenging activity of the APTE compared to quercetin **(C)** ABTS radical scavenging activity of the APTE compared to ascorbic acid **(D)** Nitric oxide radical scavenging activity of the APTE compared to ascorbic acid.

3.3.5. Phytochemicals and bioactive compounds in RCFE and APTE

Phytochemicals are the natural metabolites of the plants with numerous preventive and medicinal properties. These metabolites include different classes of compounds like flavonoid, phenol, alkaloids, and glycosides. Efforts were made for qualitative and quantitative phytochemical analysis of the extracts. The qualitative assessment suggested the presence of reducing sugar, amino acids, tannins, phenols, flavonoids and tannins **(Table 3.3.)** in the two plant extracts under study.

Table 3.3.: Preliminary phytochemical analysis of RCFE and APTE

Constituents	**Chemical test**	**Result**	
		RCFE	**APTE**
Reducing sugars	Fehling's test	+ve	+ve
Non-reducing sugars	Iodine's test	-ve	-ve
Proteins	Biuret test	-ve	-ve
	Ninhydrin test	+ve	-ve
Cardiac Glycosides	Keller-Killani test	-ve	+ve
Saponin Glycosides	Foam test	-ve	-ve
Alkaloids	Wagner's test	-ve	-ve

Flavonoids	Zinc hydrochloride reduction test	+ve	-ve
	Sodium hydroxide test	+ve	+ve
Tannins and Phenolic compounds	Ferric chloride test	+ve	+ve
	Dilute iodine solution test	+ve	+ve
	Dilute HNO_3 acid test	+ve	-ve
	Potassium Dichromate solution	+ve	-ve

3.4. Discussion

The use of traditional medicinal plants for the treatment of various human ailments dates back to the seventeenth century. The two plants selected for this study, *Ricinus communis* and *Amorphophallus paeoniifolius* have been reported to possess anti-inflammatory, anti-diabetic, hepatoprotective and anti-fungal activities [41,52-57]. Reports on its anticancer role are limited. Some preliminary observations on its cytotoxic activity against cancer cells have been reported [58-60]. However, no mechanistic details for the activity are available and hence, it becomes important to explore the mechanism of their anticancer activities. Two highly potential extracts from these two plants *viz.* RCFE and APTE were selected for further study. Treatment of RCFE and APTE on two breast cancer cell lines, MCF-7 and MDA-MB-231 showed time and concentration-dependent inhibition of viability in both the cell lines. RCFE was effective at a concentration as low as 1 µg/ml inducing cell death by 48.7% and 55.4% in MCF-7 cells and 48.4% and 78.5% in MDA-MB-231 cells after 24 and 48 hr of incubation, respectively **(Fig 3.2.A)**. RCFE showed near similar cytotoxicity at 24 hr in both the cells but almost 20% more efficacy at 48 hr in MDA-MB-231 suggested the RCFE-mediated cell death might be a hormone (Estrogen, Progesterone, HER) receptor-independent event or may be due to additional reasons like interactions of pro-apoptotic BCL-2 family protein and anti-apoptotic Bax family proteins, p53 dependence for its differential action [61-63]. In contrast to its effect on cancer cell lines, treatment with similar doses of RCFE showed a minimal effect on the HEK293 and mouse embryonic fibroblast (MEF) cells suggesting the extract's specific cytotoxic efficacy against cancer cells **(Fig 3.3.A)**. The second extract, APTE, also induced significant cytotoxicity in MCF-7 and MDA-MB-231 cells in a dose and time-dependent fashion. Treatment with the highest experimental dose of 500 µg/ml APTE showed 38.3% and

51.6% cell death after 24 and 48 hr incubation, respectively in MCF-7 cells **(Fig 3.2.C)**. The same concentration induced 45.8% and 57.8% cell death in MDA-MB-231 cells at those time points, respectively **(Fig 3.2.C).** Near similar cytotoxicity in both cells suggested the APTE-mediated cell death might not be an estrogen receptor-dependent event [62]. Treatment with similar doses of APTE also showed a minimal effect on the HEK293 and mouse embryonic fibroblast (MEF) cells suggesting the extract's specific cytotoxic efficacy against cancer cells **(Fig 3.3.B)**.

There is a relationship between the antioxidant property and the anti-cancer activity of plant polyphenols [64,65]. Plant extracts with higher antioxidant activity are reported to be more effective against cancer cells, though the actual mechanism is still unknown. This may be due to the presence of the phenolic compounds in the plants which give the protection to normal cells against the free radicals and exert the anticancer effect selectively to the cancer cells [65]. To understand the antioxidant role of RCFE and APTE, radical scavenging activity of the extracts was measured using four *in vitro* assays [50,51]. Although RCFE showed comparatively high antioxidant activity than APTE, both RCFE and APTE showed a dose-dependent activity in scavenging DPPH, hydroxyl, ABTS and nitric oxide radicals **(Fig 3.4. and 3.5.)**. Antioxidant properties of plant extracts are known to vary widely depending on its flavonoid and phenolic content [65]. These flavonoid and phenolic compounds are generally the secondary metabolites of plants. Plants produce a large number of primary and secondary metabolites which are generated as end products of a metabolic reaction. Primary metabolites are unswervingly used in reproduction, growth and development. These include carbohydrates, lipids, proteins and minerals necessary for basic cellular function and reproduction and basically utilized as food by human. Secondary metabolites are not directly involved in these physiological processes but exert some therapeutic effects in humans. These include alkaloids, glycosides, flavonoids, tannins, phenolic compounds, etc. These secondary metabolites are the by-products of primary metabolites i.e. they are biosynthetically derived from primary metabolites and are more limited in distribution, usually being restricted to a taxonomic group [66,67]. In general, lower phenolic content and antioxidant activity in tubers are expected as reported for other tubers like potato [68]. This may be the reason for comparatively higher antioxidant activity of RCFE than APTE.

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