Cancer-specific chemoprevention and anti-metastatic potentials of *Rheum emodi* rhizome ethyl acetate extracts and identification of active principles through HPLC and GC-MS analysis

Devanga Ragupathi Naveen Kumar[1], Vazhapilly Cijo George[1], Palamadai Krishnan Suresh[1] and Rangasamy Ashok Kumar[2]*

[1]School of Bio Sciences and Technology, VIT University, Vellore, India
[2]Department of Zoology, Government Arts College, Dharmapuri, India

Abstract: *Rheum emodi* Wall. ex Meissn. (Polygonaceae) is a Himalayan perennial herb which has been cultivated over 5000 years for its medicinal properties by rural and tribal people of Kashmir, and has great significance for its traditional use in Ayurvedic, Unani and folk systems of medicine for cancer treatments. However, there is lack of reports pertaining to specific-chemopreventive properties of *R. emodi* rhizome. The present study investigates *R. emodi* rhizome hot and cold ethyl acetate extracts (EHR and ECR) for specific-chemopreventive properties. The extracts were found to be effective antioxidant sources, and showed significant ($P<0.05$) cancer-specific cytotoxicity towards MDA-MB-231 cells (when compared to WRL-68 [non-tumoral cells]) with IC_{50} values of 56.59±1.29 µg/ml (EHR) and 152.38±1.45 µg/ml (ECR) respectively, and induced apoptosis significantly ($P<0.05$) high in MDA-MB-231 cells (estrogen receptor-(ER)-negative) when compared to MCF-7 cells (ER-positive). Extracts also demonstrated evident anti-metastatic activity. Further, the extracts were chemically characterized through HPLC analysis which revealed major polyphenolics and the GC-MS analysis of the effective extract EHR unveiled (Methyl 6,7-dideoxy-6-C-methyl-2,3-di-O-methyl-à-D-gluco-oct-6-eno-1,5-pyranosid)urono-8,4-lactone,Chrysophanol, derivatives of cyclopropanes and a quinazoline derivative. Overall, EHR exhibited significantly better results on par with ECR, and thus could be considered for their use in designing cancer-specific chemopreventive agents against ER-negative breast cancer.

Keywords: Apoptosis; anti-metastasis; HPLC; GC-MS; *Rheum emodi.*

INTRODUCTION

Recent progress in tumour targeting technology has augmented the likelihood that cancer prevention relies increasingly on interventions collectively termed 'chemoprevention' (Tamimi *et al*., 2002). Cancer chemoprevention employs the use of agents to inhibit, delay or reverse the process of carcinogenesis (Banerjee *et al*., 2006) by selectively targeting cancer cells either by its antioxidant potential (Khan *et al*., 2008) and/or its cytotoxic (Paliwal *et al*., 2005)/ apoptosis-inducing property (Chen *et al*., 1998). Moreover, the potential drug is also expected to combat tumour cells in advanced stages of malignancy (metastasis), which is a major cause of mortality in cancer (Fidler, 2003). Recent scientific reports have proved that a wide variety of naturally occurring substances render protection against carcinogenic exposure (Sultana and Saleem, 2004; Guha *et al*., 2010). Plants are considered as the best sources for drug components, since it exhibits lesser side effects (Balekar *et al*., 2012). Numerous plant products in the form of decoction, tincture, tablets and capsules have been clinically used for the treatment of different kinds of cancer (Ram and Kumari, 2001).

Rheum emodi Wall. ex Meissn. is a perennial stout herb belonging to the family Polygonaceae, has been cultivated for over 5000 years for its medicinal properties, and is distributed in the temperate and subtropical regions of Himalaya ranging from Kashmir to Sikkim, at a height of 2800 and 3800 m above mean sea level (Nautiyal *et al*., 2003). *R. emodi* was recorded as an important food source of rural and tribal people in Kashmir, believed to act as an antipyretic, antihelminthic, laxative and to treat atonic indigestion, constipation, jaundice and liver disorder (Kounsar *et al*., 2011). The rhizomes of *R. emodi,* is officially listed in the Indian Pharmacopoeia, and have great significance for its traditional use in Ayurvedic, Unani and folk systems of medicine, and as an ingredient in many polyherbal formulations used for the regulation of blood fat, hepatitis and cancer (Singh *et al*., 2005; Singh *et al*., 2010). There are also scientific reports subsequent to the traditional use of *R. emodi,* where crude polar extracts of *R. emodi* has been reported for its promising antioxidant effects and cytotoxicity (Rajkumar *et al*., 2010). Some of the well known therapeutic compounds such as chrysophanol, physcion and emodin were also found to be present in *R. emodi* (Babu *et al*., 2003). One of our previous studies investigated the chloroform extracts of *R. emodi* for its bioactivities (Naveen Kumar *et al*., 2012). However, further exploratory studies are obligatory, in order to isolate additional bioactive constituents responsible for its therapeutic potentials.

**Corresponding author:* e-mail: ashoku_2000@yahoo.com

Correspondingly, reports on the evaluation of polar aprotic (ethyl acetate) counterparts of *R. emodi* for its cancer-specific chemopreventive properties are currently not available and some of the important compounds in investigated crude extracts may be overseen. Thus, the present study focuses on the evaluation of hot and cold ethyl acetate extracts of *R. emodi* for their selective-chemopreventive properties by estimation of its antioxidant potential, capacity for inhibition/annihilation of cancer cells and induction of apoptosis by cellular DNA fragmentation. The extracts were also tested for its anti-metastatic potentials by conventional cell migration assay and characterized by quantifying polyphenols which were subsequently validated by HPLC and GC-MS analysis.

MATERIALS AND METHODS

Chemicals

2,2-Diphenyl-1-picrylhydrazyl (DPPH), trichloroacetic acid (TCA), ascorbic acid, gallic acid, phenazine methosulfate (PMS) (also known as N-methylphenazonium methosulfate), L-15 (Leibovitz) cell culture medium (with L-glutamine) and MEM (minimal essential medium) cell culture medium (with Earle's salt, NEAA and L-glutamine) were purchased from Himedia Laboratories Pvt. Ltd. (India). XTT {2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide} was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Folin–Ciocalteau reagent was procured from Sisco Research Lab (India). Cellular DNA fragmentation ELISA (Cat. NO. 11 585 045 001) to determine apoptosis was procured from Roche Diagnostics, Germany. The remaining chemicals and solvents used were of standard analytical grade and HPLC grade, respectively.

Plant Material

Rheum emodi rhizomes were collected from their natural habitat in the Garhwal Himalayas at Chamoli (30º24' N, 79º21'E), Uttaranchal, India. Collected rhizome specimens were shade-dried, powdered and used for solvent extraction. Voucher specimens were maintained at our laboratory for future reference (Accession NO.: VIT/SBCBE/CCL/07/6/04; Date: June 11, 2007).

Solvent extraction

Hot extraction

Ethyl acetate was used as a solvent to extract the rhizome powder of *R. emodi* using a Soxhlet apparatus in a ratio of 1:6 [powder (in g): solvent (in mL]. The extract obtained was evaporated to dryness at 40ºC under reduced pressure (ethyl acetate: 240 mbar in a rotary evaporator (BUchi, Switzerland). Fifty grams of rhizome powder yielded 5.03 g (percentage extract yield: 10.16% of dry weight) of crude ethyl acetate extract (EHR). The sample was stored in a vacuum desiccator at room temperature until further use.

Cold extraction

Ethyl acetate was mixed with the rhizome powder of *R. emodi* and extracted with a ratio of 1:6 [powder (in g):solvent (in ml)] at room temperature with the flask shaken at regular intervals. Fifty grams of rhizome powder yielded 1.18 g (percentage extract yield: 2.36% of dry weight) of crude ethyl acetate extract (ECR). The sample was stored in a vacuum desiccator at room temperature until further use.

Estimation of antioxidant potentials

Reducing power assay

Reducing power (Fe^{+3}–Fe^{+2}) of the extracts were accessed by the method of Yildirim *et al*., (2001). Amount of 20, 40, 60, 80 and 100 µg of extracts from the stock solution were mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6; 1.79% NaH_2PO_4 and 1.89% Na_2HPO_4) and 2.5mL of 1% potassium ferricyanide in test tubes, which were then incubated at 50ºC for 30 min. Subsequently, 2.5ml of a 10% trichloroacetic acid solution was added to all the tubes and centrifuged at 3000 rpm for 10min. An aliquot of 2.5ml of the upper layer solution was then mixed with an equal volume of distilled water and 0.5mL of 0.1% ferric chloride. Absorbance was recorded at 700 nm using Cary 50 UV-Vis spectrophotometer (Varian, Inc., CA, USA).Increase in absorbance of the reaction mixture indicates a steady increase in reducing power of the extracts.

DPPH radical scavenging assay

The $DPPH^{\bullet}$ radical scavenging assay was performed according to the method of Blois (1958) with some modifications. Varying concentrations (20, 40, 60, 80 and 100µg) of extracts were taken in separate test tubes and made it up to 0.5ml using ethanol. This free radical generator ($DPPH^{\bullet}$ **-** 3ml of a 0.1mM solution), in ethanol, was added to all the tubes. The tubes with ethanol and $DPPH^{\bullet}$ solution alone were maintained as controls. The tubes were incubated in the dark for 30min and the absorbance was measured at 517nm with ethanol as blank. The percentage radical scavenging (RS%) was calculated using the formula: $RS\ \% = [(Ac-At)/ Ac] \times 100$ Where Ac and At are the absorbance of the control and treated samples respectively.

Cell lines and maintenance

MDA-MB-231 (human breast carcinoma), MCF-7 (human breast carcinoma) and WRL-68 (normal human liver embryonic) cell lines were obtained from National Centre for Cell Science (Pune, India). MDA-MB-231 cells were maintained in L-15 (Leibovitz's) culture medium. MCF-7 and WRL-68 were maintained in Minimum essential medium (MEM) (Eagle) with Non-essential amino acids with 10% serum in a humidified atmosphere at 37ºC (with 5% CO_2 for MCF-7 and WRL-68 only). The cell lines were maintained in their growing phase at 70% confluency with regular passaging. All the experiments were performed with the cells at 70-80% confluency.

Cytotoxicity assessment-XTT assay

Cytotoxic efficacy of the extracts was tested using XTT-formazan dye formation assay with 10% serum (Weislow *et al*., 1989). MDA-MB-231 and WRL-68 cells were seeded in their respective culture medium (200 µl, 1 x 10^4 cells/well and 6 x 10^3 cells/well respectively) in a 96-well plate and incubated at 37ºC for 24 h with/without 5% CO_2 supply. After incubation, the control wells were replenished with fresh medium and the test wells were treated with 25, 50, 100 and 200 µg/ml of extracts. The cells were further incubated for 24 h maintaining the same conditions. After the treatment incubation period, medium in each well was replenished with 200 µl of fresh medium plus 50 µl of XTT (0.6 mg/ml containing 25 µM PMS). The plate was then re-incubated for 4 h in the same conditions, after which the absorbance was measured at 450 nm (with a 630 nm reference filter) in a Dynex Opsys MR™ Microplate Reader (Dynex Technologies, VA, USA). Percentage of cytotoxicity was calculated by the following formula: *% Cytotoxicity*=[(Ac-At)/Ac]×100 Ac is the mean absorbance of the control wells and At is the mean absorbance of test wells with a particular extract dosage.

Determination of apoptotic induction

The cellular DNA fragmentation ELISA is a photometric enzyme-linked immunosorbent assay (ELISA) in culture supernatants. It employs measurement of apoptotic cell death by detection of Bromodeoxyuridine (BrdU)-labelled DNA fragments in the cytoplasm of affected cells. The experiment was performed as per the supplier's instructions. Cells (MDA-MB-231 and MCF-7) were labelled with 10 µM BrdU at 1 x 10^5 cells/ml density. 1 x 10^4 BrdU-labeled cells in 100 µl medium were treated with varying concentrations (12.5, 25, 50, 100 and 200 µg/ml) of the extracts for a period of 4 h. The cells were then lysed and the supernatant containing apoptotic fragments were obtained after centrifugation at 1500 rpm for 10 min and analyzed in the ELISA procedure. Obtained sample (100 µl) was transferred to anti-DNA coated 96-well flat-bottom microplates. The plates were incubated for 90 min at 15-25°C. The DNA bound to coated microplates was then denatured by microwave irradiation (500 W for 5 min). The assay plates were washed with washing buffer followed by addition of 100 µl anti-BrdU-POD (Peroxidase) conjugate. The plates were further incubated for 90 min and were washed three times with washing buffer. An amount of 100 µl substrate [3,3′,5,5′-Tetramethylbenzidine (TMB)] solution was then added and the plates were shaken until colour development is sufficient. The absorbance was read at 450 nm after addition of 25 µl stop solution.

Cell migration inhibition assay

Cell migration inhibition efficiency of the extract was tested according to the method described by Dimmeler *et al*., (2000) with some modifications. MDA-MB-231 cells (6 × 10^5 per well) were cultured on 6-well plates and incubated at 37ºC to form a confluent monolayer. After 24 h of incubation, *In vitro* `scratch' wounds were created by scrapping the cells using sterile cell scrapper on monolayer. Subsequently, the wells were gently washed with growth medium to remove dislodged cells. The control wells were further added with fresh medium while the treatment wells were added with medium containing EHR and ECR (56.59 µg/ml and 152.38 µg/ml respectively). The plates were re-incubated at 37ºC and observed for migration of cells at every 4 h intervals (4, 8, 12 and 16 h). The migration of cells was then monitored by a decrease in distance between wounded edges in a computer-attached inverted phase contrast microscope (Hund wetzlar, Germany).

Assessment of phenolic contents

Phytochemical screening

Phytochemical screening was performed to analyse the class of compounds present in the crude extracts. The method employed to screen for different class of chemicals are as described by Trease and Evans (1989) and Ayoola *et al*. (2008).

Estimation of total phenolic content

Total phenolic content was determined by the method described by Rajkumar *et al*. (2010). Amounts of 20, 40, 60, 80 and 100 µg of extracts were made it up to 0.5ml with distilled water in separate tubes. An amount of 2.5 ml Folin–Ciocalteau reagent (1:10 dilution) and 2 ml of sodium carbonate (7.5% w/v) were added to the tubes and incubated at 45ºC for 15 min. Absorbance was then read at 765 nm. A standard polyphenolic compound, gallic acid, was used to express the results in terms of gallic acid equivalence (GAE) in micrograms.

HPLC analysis for phenolic compounds

HPLC analysis was performed using a Waters 2487 HPLC system consisting of a dual λ detector and a Waters 1525 binary pump, equipped with a Waters Symmetry® C18 column (5 µm, 4.6 mm x 150 mm) with Waters Sentry™ universal guard column (5 µm, 4.6 mm x 20 mm) (Waters Corporation, Milford, MA, USA). EHR and ECR extracts of *R. emodi* were analyzed for the presence of phenolic contents as per our previously reported protocol (Naveen Kumar *et al*., 2012), using the library for phenolic compound standards (Sakakibara *et al*., 2003) as a reference.

GC-MS analysis

Instrument specifications

The analysis was performed on Thermo GC-Trace Ultra ver 5.0 coupled with Thermo MS DSQ II, fitted with a DB 5- MS Capillary Standard column (30 m, i.d.: 0.25 mm and film thickness: 0.25 µm).

Analytical conditions

Helium was used as a carrier gas (flow-1.0 ml/min). Injector temperature was 260ºC and oven temperature

raised from 80ºC to 260ºC (5ºC/min). 1 µl of sample was injected through injection port and the individual compounds were identified based on their retention times and standard matching spectral peaks in Wiley mass spectral library.

STATISTICAL ANALYSIS

All experimental analyses carried out in this study were in triplicates and the data was expressed as mean ± SD. One-way ANOVA was performed for statistical analyses and significant differences between groups were determined at P<0.05. Results were also scrutinized for correlation and tested for significance by Student's t-test (P<0.05) to assess relationships between experimental parameters. MATLAB ver. 7.0 (Natick, MA, USA), GraphPad Prism 5.0 (San Diego, CA, USA) and Microsoft Excel 2007 (Roselle, IL, USA) were used for the graphical and statistical evaluations.

RESULTS

Antioxidant potential

The most widely employed DPPH• radical scavenging antioxidant assay was performed to analyse the free radical scavenging ability of the *R. emodi* extracts. EHR and ECR showed a concentration dependent increase in DPPH• scavenging activity. EHR was found to be efficient when compared to ECR which was graphically represented as % Radical scavenging (mean±SD at P < 0.05) with equivalence to ascorbic acid in micrograms (fig. 1). The ability of extracts to reduce Fe^{+3}-Fe^{+2} is measured spectrophotometrically by formation of Perl's Prussian blue due to reduction reaction. Dose dependent increase in reducing power of the extracts was illustrated by a steady increase in absorbance of the reaction mixture (fig. 2). Wherein, EHR was found to be superior to the standard, BHT (Butylated hydroxytoluene), in its ability.

Cytotoxic potential

The present study employed two cell types, MDA-MB-231 (cancer cells) and WRL-68 (normal cells) to determine the selective toxicity of the extracts towards cancer. The cytotoxicity of EHR and ECR was exemplified by a steady increase in the percentage cytotoxicity with respect to extract dosage (fig. 3). Both the extracts showed significantly (P<0.05) higher toxicity towards cancer cells when compared to normal cells.

Apoptosis induction

Apoptosis induction is one of the hallmarks of cancer treatment. The ability of extracts to induce apoptosis in cancer cells were analysed by quantifying the amount of apoptotic DNA fragments present in the cytoplasm of treated cells. The extracts EHR and ECR showed a dose-dependent increase of apoptotic fragments in both MDA-MB-231 and MCF-7 cells (fig. 4), in which, the activity was better in the former cell type than the latter.

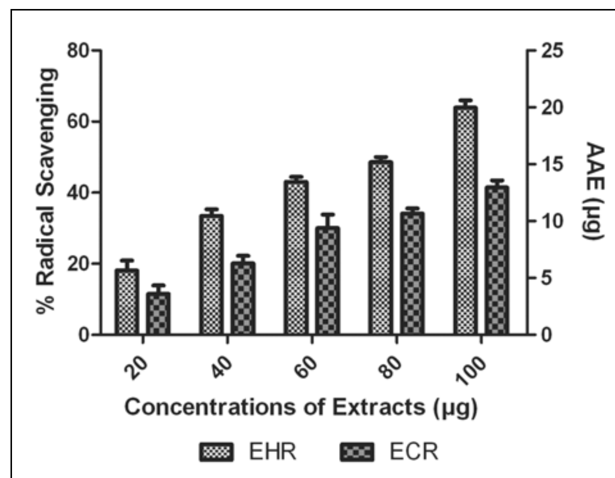

Fig. 1: DPPH• scavenging ability of EHR and ECR expressed in AAE (Ascorbic acid equivalence). Data expressed as percentage radical scavenging (mean ± SD, n=3, P<0.05).

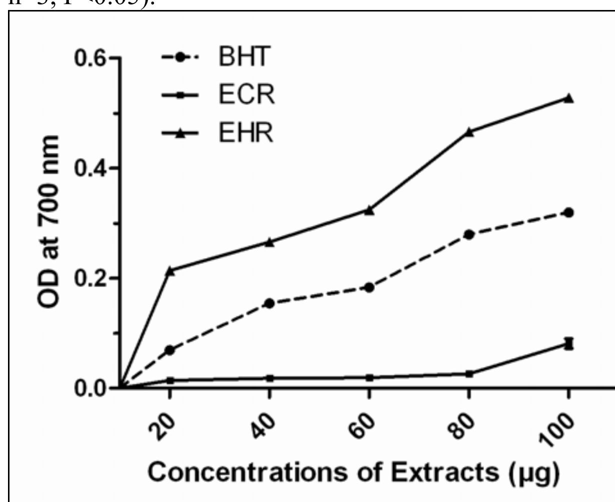

Fig. 2: Reducing power of EHR and ECR with BHT as standard. Data points are mean values ± SD (n=3, P<0.05).

Anti-metastatic potential

EHR and ECR have inhibited cell migration in MDA-MB-231 cells, which is evident from the observations made at every 4 h intervals (4, 8, 12 and 16 h) after addition of the extracts (fig. 5A). At the same time, cells in the control wells migrated to cover up the space created by scratch wound. The pattern of percentage cell migrations depicted in fig. 5B clearly portrays the significant (P<0.05) decrease of cell migration in treated cells when compared to control.

Characterization of the extracts

Phytochemical screening

The extracts were also characterized for the nature of phytochemicals present and were found to contain various groups. EHR was found to possess additional number of phytochemicals when compared to ECR (table 1).

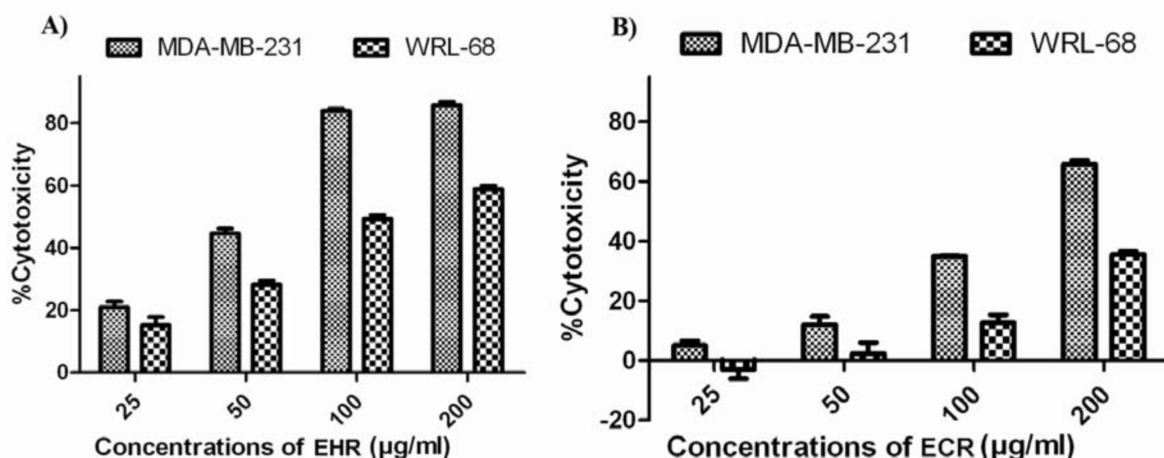

Fig. 3: Percentage cytotoxicity of EHR (A) and ECR (B) in MDA-MB-231 and WRL-68 cells. Data expressed as mean ± SD of n = 3 samples (P < 0.05).

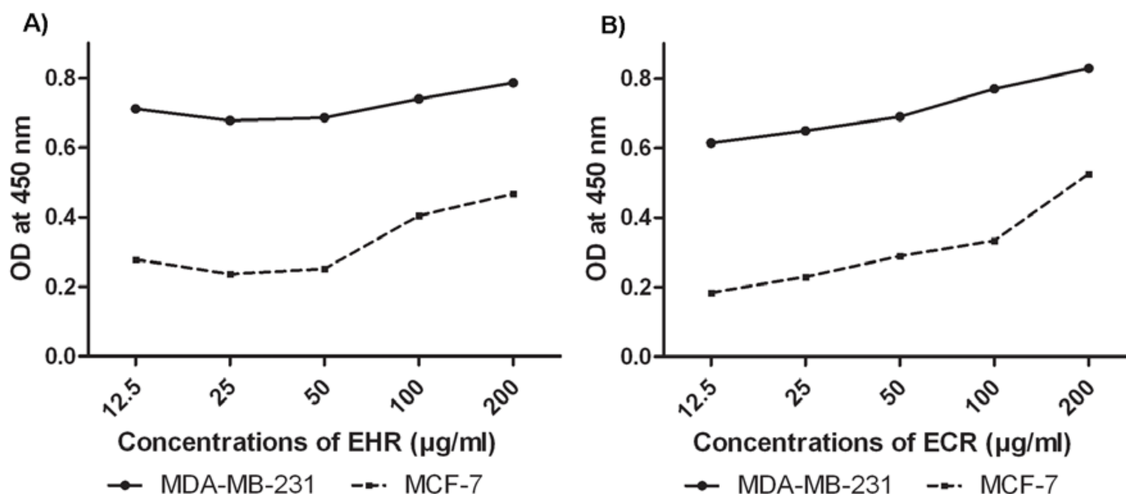

Fig. 4: Dose-dependent increase of apoptotic fragments in EHR (A) and ECR (B) treated cells (MDA-MB-231 and MCF-7) as determined by cellular DNA fragmentation ELISA.

Total phenolics determination

The total phenolic contents of the extracts were quantified spectrophotometrically and expressed in GAE (Gallic acid equivalence) (in micrograms) as depicted in table 2. Among the extracts, EHR was found to contain higher amounts of polyphenolics in comparison with those in ECR. The results obtained in both the antioxidant assays showed significant difference (P<0.05) between each treatment groups. Statistical correlations have been performed to confirm that antioxidant activity might be due to the polyphenol presence in the extracts. Total phenolic content of both the extracts showed strong positive correlation (at P<0.05) with $DPPH^{\bullet}$ radical scavenging and reducing power assays (table 3), revealing that the counteraction of free radicals by the extracts might be due to the presence of polyphenolics.

HPLC analysis

Due to the diversity and complexity of natural phenolic compounds, it is difficult to characterize every compound present in the crude extract to elucidate its structure (Surveswaran *et al*., 2007). Various classes of polyphenolics were identified in hot and cold ethyl acetate extracts of *R.emodi* using the established library of more than 100 phenolic standards as a reference for the analytical characteristics (λ_{max}, retention time, determining λ, slope and limit calibration) (Sakakibara *et al*., 2003). Table 4 depicts the polyphenolics identified in the different extracts of *R. emodi*. Collectively, nineteen known compounds were identified in EHR and ECR along with few unknown compounds as apparent from the HPLC chromatograms.

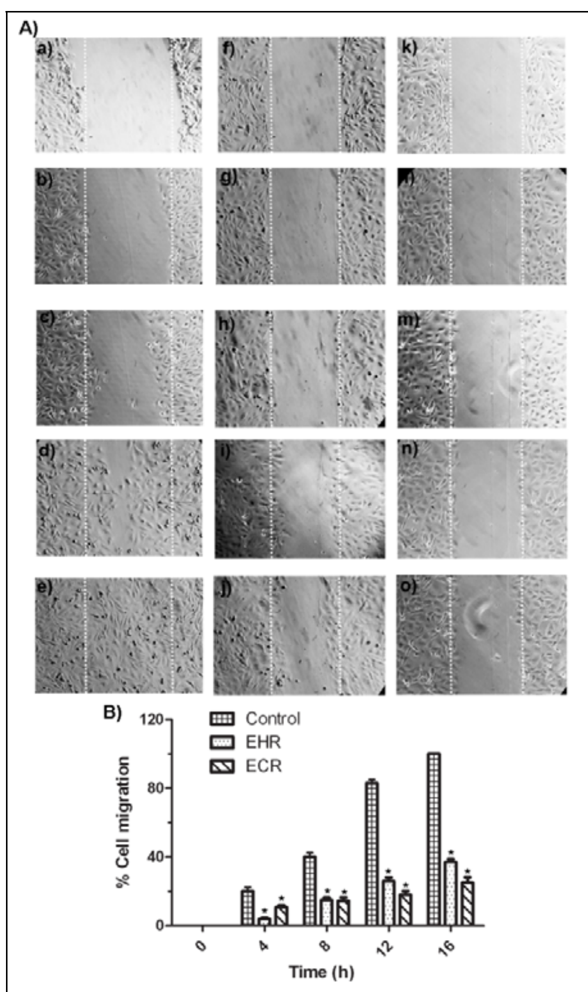

Fig. 5: (A) Demonstrating the migration of MDA-MB-231 cells from edges of the scratch wound after 0, 4, 8, 12 and 16 h in control well (a, b, c, d, e), EHR treated well (f, g, h, i, j), and ECR treated well (k, l, m, n, o) respectively (n=3). (B) % Cell migration of MDA-MB-231 cells, showing significantly ([P<0.05] marked by Asterisk [*]) less migration in treated cells compared to control cells.

GC-MS analysis

The GC-MS analysis of the effective extract EHR has revealed the presence of 30 compounds with 99.9% of the total composition (table 5). The major components identified were (Methyl 6,7-dideoxy-6-C-methyl-2,3-di-O-methyl-à-D-gluco-oct-6-eno-1,5-pyranosid)urono-8,4-lactone - 45.38%, Chrysophanol - 7.68%, 1-(2-Ethoxyethenyl)cyclopropane – 4.56%, 1-Hexadecene - 5.36%, Cyclohexadecane - 4.65%, (2R,5S)-5-Allyl-2,5-dihydro-3,6-dimethoxy-5-(2-hydroxyethyl)-2-isopropylpyrazine - 2.73%, Octadecanoic acid - 2.71%, 1-Tetradecene - 2.68%, Cyclopropane, 1-methyl-2-octyl-, cis- 2.54%, 4-Ethoxycarbonyl-2,2,5-trimethyl-3-(n-decyl)-2H-pyrrole1-oxide - 2.52%. Few other components identified in minor quantities were, Octadecane - 1.96%, 1-Dotriacontanol - 1.53%, Tricosane - 1.42%, N-[Thiophene-2-carbonyl) piperazine - 1.30%, 1-(2-thienyl)-3-pentanone - 1.15%, 8-Pentadecanone - 0.99%, Neopentyl 2-hydroxy-2-methylpropanoate - 0.95%, 5-Methyl-3-phenylbenzo [h]quinazoline-2,4 (1H,3H)-dione - 0.95%, 1-phenyl-Ethanone (acetophenone) - 0.92%, Pent-4-enal - 0.89%, dicarbonyl(trimethylphosphine)(ü(5)-2,5-dimethylpyrro-lyl) manganese - 0.75% along with other minor components.

Table 1: Semi-quantitative identification of various phytoconstituents identified from *R. emodi* extracts.

Components	Ethyl acetate extracts	
	HOT	COLD
Saponins	-	-
Flavonoids	++	++
Terpenoids	++	++
Reducing Sugar	-	-
Cardiac Glycosides	++	++
Tannins	+	+
Anthraquinones	++	-
Steroids	++	++
Phlobatannins	-	++
Oils	++	-

+ = mildly present, ++ = strongly present, - = absent

Table 2: Total phenolic content of *R. emodi* extracts given in mean ± SD (n = 3, P<0.05). GAE of the extracts is expressed in micrograms.

Amount (μg)	*GAE ± SD (in μg)	
	EHR	*ECR*
20	12.03 ± 0.034	9.27±0.03
40	16.22 ± 0.37	12.66±0.31
60	21.09 ± 0.003	15.43±0.07
80	24.99 ± 0.29	17.68±0.06
100	28.75 ± 0.11	22.24±1.34

*GAE ±SD at 95% confidence interval.

Table 3: Correlations between experimental results (of total phenolic, reducing power, DPPH) tested for significance. R^2 denotes coefficient of determination.

Extracts	Correlations	R^2
EHR	Total phenolics and DPPH• scavenging	0.986**
	Total phenolics and reducing power	0.978**
	DPPH and Reducing power	0.949*
ECR	Total phenolics and DPPH• scavenging	0.986**
	Total phenolics and reducing power	0.964**
	DPPH and Reducing power	0.911*

**Correlation is significant at the 0.01 level
*Correlation is significant at the 0.05 level

DISCUSSION

Since years, natural products have been under research to formulate novel chemopreventive agents. Such new

Table 4: Polyphenols identified in extracts of *R. emodi* employing HPLC analysis

Polyphenols	λ[a] (nm)	Et_R[b] (min)		Rt_R[c] (min)
		EHR	ECR	
Benzoic acids				
1. *p*-hydroxybenzoic acid	250	14.556	-	13.8
2. protocatechuic acid	250	10.200	-	9.7
3. Gallic acid	250	6.081	6.283	5.8
Flavones				
4. Flavone	280	-	87.820	88.8
5. Chrysin	320	-	89.394	88.8
6. Luteolin	320	78.474	-	78.9
7. Luteolin-4'-*O*-glucoside	320	54.7	-	54.9
8. 5,7-dihydroxy-3',4',5'-trimethoxyflavone	320	87.783	-	88.3
Flavonols				
9. Galangin	370	88.969	90.286	89.9
10. Kaempferol-3-*O*-glucoside (astragalin)	370	55.009	56.193	55.6
11. Kaempferol-7-*O*-neohesperidoside	370	-	52.739	53.2
Flavanones				
12. Hesperetin	280	-	78.550	79.4
Isoflavones				
13. Genistein	250	80.379	-	79.8
14. Glycitein	250	72.910	-	73.1
15. Biochanin A	250	-	88.696	88.8
Catechins				
16. (-)-Epigallocatechin	280	-	12.331	13.1
Theaflavins				
17. Theaflavin-3-gallate	280	80.381	-	80.4
Chalcones				
18. Phloretin	320	-	80.098	80.3
Anthraquinones				
19. Rhein	250	86.045	-	85.3

[a] Wavelength for determination. [b] Experimental retention time. [c] Reference retention time (Sakakibara *et al*., 2003).

agents could serve as exemplars for the synthesis of analogues or hybrid molecules that may be more efficacious. Any anticancer drug is expected to possess few characteristics such as antioxidant activity (Jing *et al*., 2010), cytotoxicity (Ashidi *et al*., 2010) and apoptosis induction ability (Sreelatha *et al*., 2011) to be effective against cancer and its progression. Recently, focus of researchers entail on the drugs which target cancer cells specifically without harming the normal cells (Paliwal *et al*., 2005). This study has critically analyzed the hot and cold ethyl acetate rhizome extracts of *R. emodi* for the aforesaid properties.

The antioxidant activity of a drug is validated by the ability of it to scavenge free radicals thereby reducing the stress generated (Gill and Tuteja., 2010). Plant sources are now considered as best sources of drug components with antioxidant properties, since it exhibits lesser side effects (Krishnaiah *et al*., 2007; Rates, 2001). As a medicinal plant, *R. emodi* has already been reported for its promising antioxidant effects in crude extracts using methanol and aqueous solvents (Rajkumar *et al*., 2010). The present study employs two antioxidant assays, DPPH radical scavenging assay and reducing power assay for analyzing antioxidant properties of ethyl acetate extracts of *R. emodi*. In DPPH assay, both EHR and ECR showed a remarkable activity with a dose dependent increase in $DPPH^{\bullet}$ scavenging potential. Of which EHR exhibited significantly ($P<0.05$) high radical scavenging potential when compared to ECR.

The reducing power of a compound depends on the ability to donate an electron to break the free radical chain, which could be related to the antioxidant property as it prevents oxidation (Duh, 1998). The results in reducing power assay were found similar to that of DPPH assay, in which, EHR showed high efficiency in reducing Fe^{+3} to Fe^{+2} than ECR. These results indicate that the hot extract of *R. emodi* has high efficiency as an antioxidant than its cold counterpart. The results obtained were concurrent with that of Afify *et al*. (2011)

Table 5: Identification of phytoconstituents in ethyl acetate hot extract of *Rheum emodi* through GC-MS analysis

S. No.	Rt[a] (min)	Compound	Amount of compound (area %)	Molecular Weight
1.	3.04	(Methyl 6,7-dideoxy-6-C-methyl-2,3-di-O-methyl-à-D-gluco-oct-6-e no-1,5-pyranosid)urono-8,4-lactone	45.38	258
2.	5.11	Acetophenone	0.92	120
3.	9.27	1-(2-Thienyl)-3-pentanone	1.15	168
4.	10.43	4,5-Dibromo-2-penten-4-ilide	0.66	254
5.	11.57	Azetidine-D1	0.74	57
6.	13.21	N-[Thiophene-2-carbonyl)piperazine	1.30	196
7.	13.74	1-Tetradecene	2.68	196
8.	14.98	cis 3-Hexenyl tiglate	0.77	182
9.	15.25	2,5-Cyclohexadiene-1,4-dione, 2,6-bis(1,1-imethylethyl)-	0.71	220
10.	17.27	Tricosane	1.42	324
11.	17.84	Cyclohexadecane	4.65	224
12.	18.43	(2R,5S)-5-Allyl-2,5-dihydro-3,6-dimethoxy-5-(2-hydroxyethyl)-2-isopropylpyrazine	2.73	268
13.	19.40	8-Pentadecanone	0.99	226
14.	20.01	Pent-4-enal	0.89	84
15.	21.57	(S)-2-Diethoxyphosphorylpyrrolidine	0.63	207
16.	22.26	1-Hexadecene	5.36	224
17.	23.04	1-Octadecanol	0.87	270
18.	24.09	Octadecane	1.96	254
19.	26.03	4-Ethoxycarbonyl-2,2,5-trimethyl-3-(n-decyl)-2H-pyrrole1-oxide	2.52	337
20.	26.70	1-(2-Ethoxyethenyl)cyclopropane	4.56	112
21.	27.48	Decane 3-cyclohexyl-, 3-cyclohexyl-	0.67	224
22.	28.34	Neopentyl 2-hydroxy-2-methylpropanoate	0.95	174
23.	29.04	dicarbonyl(trimethylphosphine)(ü(5)-2,5-dimethylpyrrolyl) manganese	0.75	281
24.	30.20	Octadecanoic acid	2.71	284
25.	30.98	Cyclopropane, 1-methyl-2-octyl-, cis-	2.54	168
26.	31.61	5-Methyl-3-phenylbenzo[h]quinazoline-2,4(1H,3H)-dione	0.95	302
27.	33.71	Chrysophanol	7.68	254
28.	35.04	1-Dotriacontanol	1.53	466
29.	38.99	Bis-(3,5,5-trimethylhexyl) ether	0.61	270
30.	41.52	Neopentyl hydroxyacetate	0.71	146
Sum of Percentage area identified 99.99				

[a] Retention time

The ability of extracts to annihilate cancer cells was studied by employing XTT assay. Specificity in the treatment of cancer is of utmost importance in the present situation, so as to not cause any damage to normal cells and to ensure the specificity for site of action of the drug (Wu *et al*., 2011). Hence cancer specific drugs are in need for the current research problem in cancer treatment. In all doses, both the extracts analysed in this study shows specific cytotoxicity towards cancer cells (MDA-MB-231) which is significantly ($P<0.05$) higher than in normal cells (WRL-68). Of which, EHR exhibited better activity when compared to ECR, as apparent from their IC_{50} values in MDA-MB-231 cells [56.59±1.29 μg/ml (EHR) and 152.38±1.45 μg/ml (ECR)] and WRL-68 cells [102.60±1.61 μg/ml (EHR) and 242.34±2.72 μg/ml (ECR)]. Similar observations concerning to cancer-cell-specific cytotoxicity were also made by Itharat *et al.* (2004), Kobeasy *et al*. (2011) and Naveen Kumar *et al.* (2012).

Apoptosis or planned cell death is necessary in all cells for continued growth or elimination of damaged or malfunctioning cells. Most often, improper regulation of critical proteins and/or their genes involved in apoptotic cascade leads to malfunction of the apoptotic pathway leading to cancer. Thus inducing apoptosis in cancer cells will lead to cell death thereby decreasing or eliminating the cancer cell population (Li *et al*., 1999). In this study,

MCF-7 appeared to be more resistant to apoptosis than MDA-MB-231 which was concomitant to the results reported by Calcabrini *et al*. (2006) and Zhong *et al*. (2009). This might be because of the difference in origin and pathology of the cells, as MDA-MB-231 cells are ER-negative adenocarcinoma cells from pleural effusion while MCF-7 are ER-positive invasive ductal carcinoma cells from pleural effusion (Burdall *et al*., 2003). This experimental data implies that these extracts may have potential to fervently target ER-negative breast cancer.

Metastasis of tumour is a multistep process which requires modulation of cells ability to adhere, proteolytic degradation of ECM, cell migration, and growth of tumor at the metastatic site. Therefore, inhibition of one or more of these checkpoints is an approach towards anti-metastasis (Yodkeeree *et al*., 2010). Cellular migration during metastasis is actually driven through series of proteins such as Cdc42, mPar6/PKCζ complex (Manneville and Hall, 2001). Cell migration *in vitro* system is initiated upon a scratch, which is followed by the protrusion of cells in direction perpendicular to the scratch wound. There have been reports on several phytochemicals, which was proven to inhibit metastasis of breast cancer cells (Balaji *et al*., 2009). In present study, EHR and ECR have significantly ($P<0.05$) inhibited cell migration in MDA-MB-231 cells. Since the extracts demonstrate the possession of the ability of an anti-metastatic agent, these could be considered in developing agents to treat advanced stages of breast cancer.

Phenolic compounds are the molecules contained with aromatic ring, bearing hydroxyl group and other functional derivatives, which are well recognized for their antioxidant, antimutagenic and anti-tumor activities (Guha *et al*., 2010). The efficiency of these extracts in our study is unconditionally because of its obvious presence of phytochemicals. Extracts were thus characterized for the nature of phytochemicals present and was found to contain various groups. The total phenolics present in the extracts were quantified spectrophotometrically and expressed in GAE. EHR was found to contain high amount of polyphenolics on par with ECR. A significant ($P<0.05$) positive correlation was extrapolated between the amount of total phenolic content and results of antioxidant activities. Similar statistical correlations, as reported in this study, have also been previously reported (Guha *et al*., 2010).

Moreover, the total phenolic results were validated by analysing the extracts through HPLC. This analysis revealed the polyphenolics present in the two extracts. Different compounds were present in hot or cold counterparts of *R. emodi* extracts except for gallic acid, galangin and kaempferol-3-*O*-glucoside (astragalin), which were found to be present in both EHR and ECR. Also few unknown compounds were present in the extracts as evident from HPLC chromatogram whose isolation and identification is in prospect. These phytoconstituents which are well known to hold high therapeutic potentials might have positively contributed to the documented bioactivities in this study.

Furthermore, the GC-MS analysis of the effective extract EHR had revealed 30 compounds. To the best of our knowledge, except chrysophanol, all other compounds are reported for the first time as a component of *R. emodi* in this study.

(Methyl 6,7-dideoxy-6-C-methyl-2,3-di-O-methyl-à-D-gluco-oct-6-eno-1,5-pyranosid)urono-8,4-lactone, a derivative of glucoside was found to be the profusely present component which is not previously reported for any bioactivities, while derivatives of glucurono lactones were known to be present in the gums of plant and the lactone derivatives were reported to encompass anticancer properties (Zhang *et al*., 2005; Robinson *et al*., 2008). Chrysophanol the second major compound identified is a well-known anthraquinone derivative reported to be present in *R. emodi* (Malik *et al*., 2010). Chrysophanol was usually known to cause cell death by increased ROS production (Lu *et al*., 2010), which might have contributed to the high cytotoxicity of EHR. The extract has also contained few fatty acids, where high concentration of certain fatty acids was known to cause cell death via apoptosis or necrosis (Andrade *et al*., 2005). One such interesting fatty acid, Octadecanoic acid, was found to be a component of an active anticancer fraction, but the mechanism behind was not well studied (Yoo *et al*., 2007).

Derivatives of cyclopropanes, 1-(2-Ethoxyethenyl) cyclopropane and Cyclopropane, 1-methyl-2-octyl-,cis-, were also identified in the extract. The cyclopropane derivatives were previously known for their biological properties, which also includes anticancer potentials (Salaun and Baird, 1995; Salaun, 2000). Interestingly, a quinazoline derivative 5-Methyl-3-phenylbenzo[h]quinazoline-2,4(1H,3H)-dione was also found to be present with a relatively small quantity of 0.95%. Yet, the quinazoline derivatives have now been identified as new class of chemotherapeutic agents with potent ability to inhibit epidermal growth factor receptor (EGFR), which is expressed in several types of human tumours including breast cancer (Noolvi *et al*., 2011).

Nevertheless, few studies represent that the isolated phytoconstituents were relatively less potent than the crude extract itself, which enunciates that the proven activity of whole extract may be due to a synergistic action of several phytochemical constituents (Chaiyana and Okonogi, 2012; Orhan *et al*., 2008; Savelev *et al*., 2003).

CONCLUSION

In conclusion, the study indicated that EHR and ECR were able to cause cancer-cell-specific cell annihilation along with considerable antioxidant property. Its annihilation potential could be attributed to its capacity to induce apoptosis. In addition, the extracts were also able to exert inhibitory effects on the migrating cells in culture. As evidenced from the above mentioned bioactivities and the phytochemical composition, the ethyl acetate extracts (EHR and ECR) of *R. emodi* possess various therapeutic properties and might be considered as an important candidate for isolating an effective anticancer agent against malignant ER-negative breast cancer. The isolation and identification of the unknown compounds are in prospect which may further unleash compounds with more interesting therapeutical properties.

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