ANTICANCER AND ANTI-METASTATIC ACTIVITIES OF *RHEUM EMODI* RHIZOME CHLOROFORM EXTRACTS

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ABSTRACT

In spite of advancements in cancer treatments, there is still a need for effective drugs against metastatic stages of breast cancer. *Rheum emodi*, a well known medicinal plant is not however extensively studied for its specific anticancer and anti-metastatic potentials. The present study evaluates the hot (CHR) and cold (CCR) chloroform extracts of *R. emodi* for its antioxidant properties through DPPH and reducing power assay, cytotoxicity in MDA-MB-231 and WRL-68 cells, and apoptosis inductivity in MDA-MB-231 and MCF-7 cells. Further, the anti-metastatic potential of CHR was also analysed by cell migration inhibition in MDA-MB-231 cells which is reported for the first time in this study. Both CHR and CCR exhibited efficient antioxidant properties, and significant cytotoxicity and apoptosis induction abilities. CHR had an exceptional ability to inhibit the migration of MDA-MB-231 cells. The extracts were then characterized semi-quantitatively and quantitatively to reveal its phytochemicals subsequent to HPLC analysis. Both CHR and CCR have demonstrated affirmative anticancer abilities with CHR also possessing capability to inhibit metastasis. Thus chloroform extracts of *R. emodi* could be considered in designing effective anticancer drugs especially against metastatic stages of breast cancer.

Keywords: oxidative stress, cytotoxicity, apoptosis, anti-metastasis, phenolics, HPLC.

INTRODUCTION

Rheum emodi Wall. ex Meissn. (Polygonaceae) is a Himalayan herb well known for its medicinal properties since decades. *R. emodi* has been extensively used in Ayurvedic and Unani systems of medicine[1] and is known as a purgative and stomachic as mentioned in the Chinese herbal Pen-King[2]. It is also officially enlisted in the Indian Pharmacopoeia[3] and has been in use as an ingredient for several polyherbal formulations to treat varied ailments including cancer[4]. The plant was reported to contain characteristic phytochemicals such as revandchinone, b-asarone, chrysophanol, physcion and emodin possessing biological activities[5].

Oxidative stress continues to be a great challenge for human health-related researchers[6]. Oxidative damage at cellular level compromises cellular integrity and/or damages the nuclear components. Prevention of this process or protection against such nuclear damage is obligatory in a cell system, for which an anti-oxidant is indispensable[7] to maintain a balanced ROS cycle in a cell[8-10]. The increased ROS levels are due to oncogenic stimulation, increased metabolic activity and mitochondrial malfunction, ultimately leading to genomic instability thereby promoting carcinogenesis and drug resistance in such cells[11]. Carcinogenesis is a process of deregulation of live and dead cell ratio, where the cells undergo mutation or due to oncogenic precursion, fails to eliminate the damaged cells due to lack of apoptosis. The damaged cells thus under selection, overgrow to form tumor and further leads to invasion and metastasis which is a mortal state of cancer[12,13]. In spite of advances in clinical treatment and in cure of cancer malignancies, the reduction in rate of such cancer mortality is not satisfactory as per National Cancer Institute[14]. Plant sources are being considered as best sources of drug components with anticancer properties, since it exhibits higher efficacies with lesser side effects[15-17].

An ideal chemopreventive agent is expected to inhibit, delay or reverse the process of carcinogenesis[18] through its antioxidant potential[19] and/or its cytotoxicity[20] and/or apoptosis-inducing property[21]. In this context, *R.emodi* has been reported for promising antioxidant and cytotoxic effects in its polar crude extracts[22]. The extracts of *R.emodi* were also reported to possess hepatoprotective activity in CCl_4-treated male rats, and in CCl_4-treated cultured primary rat hepatocytes[23]. It showcased also an immuno-enhancing effect through the release of various cytokines[24]. However, reports were lacking towards its cancer-specific chemopreventive properties on non-polar counterparts and some of the important phytoconstituents may have been undetected in other studies. All the aforesaid data about its therapeutic potential provided a basis for further exploratory studies, in order to critically evaluate and identify the various bioactive constituents responsible for its activity[4].

In this study, chloroform extracts of *R. emodi* were scrutinized for its various biological activities such as antioxidant property, cytotoxicity, apoptosis inductivity and anti-metastatic property. Further, the constituents of the extracts (believed to be responsible for bioactivities) were quantified by total phenolics assay and confirmed by HPLC analysis.

MATERIALS AND METHODS

Chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH), trichloroacetic acid (TCA), ascorbic acid, gallic acid, phenazine methosulfate (PMS) (also known as N-methylphenazonium methosulfate), dimethyl sulfoxide (DMSO), 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 (30º 24' N, 79º 21' E), Uttaranchal, India. Collected 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; Dated: June 11, 2007).

Cell lines for the study

MDA-MB-231 (human breast carcinoma), MCF-7 (human breast carcinoma) and WRL-68 (human liver embryonic) cell lines were obtained from National Centre for Cell Science (Pune, India). The cells MDA-MB-231 was maintained in L-15 (Leibovitz's) culture medium, and MCF-7 and WRL-68 were maintained in Minimum essential medium (MEM) (Eagle) with Non-essential amino acids

respectively with 10% serum in a humidified atmosphere at 37 ℃ (with 5% CO_2 for MCF-7 and WRL-68 only).

Extraction

Hot extraction

Rhizome powder was extracted with chloroform using a Soxhlet apparatus in a ratio of 1:6 [powder (in grams): solvent (in milliliters)]. The resulted extract was evaporated to dryness at 40 ℃ under reduced pressure (chloroform: 118 mbar in a rotary evaporator, BUchi, Switzerland).

Cold extraction

Rhizome powder was extracted with chloroform in a ratio of 1:6 [powder (in grams):solvent (in ml)] at room temperature with the flask shaken at regular intervals.

Estimation of antioxidant potentials

Reducing power assay

The ability of the extracts to reduce Fe^{+3}– Fe^{+2} was accessed using a reported method[25]. 20, 40, 60, 80 and 100 μg of the extracts 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 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50 ℃ for 30 min. 2.5 mL of 10% trichloroacetic acid was later added and the tubes were centrifuged at 3000 rpm for 10 min. 2.5 mL of the upper layer solution was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% ferric chloride. Absorbance was then measured at 700 nm using Cary 50 UV-Vis spectrophotometer (Varian, Inc., CA, USA). Increasing absorbance values of the reaction mixture indicated increasing reducing power of the extracts.

DPPH radical scavenging assay

The DPPH· radical scavenging assay[26] was performed with varying concentrations (200, 400, 600, 800 and 1000 μg) of extracts taken in separate test tubes and made up to 0.5 ml using ethanol. 3 ml of 0.1 mM DPPH· in ethanol was added to all the tubes. The tube with ethanol and DPPH· solution alone was maintained as controls. The tubes were incubated in the dark for 30 min. and the absorbance was read at 517 nm 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 culture

The cell lines were maintained in their growing phase at 70% confluency with regular passaging. All the experiments which include cell culture were performed when the cells were at 70-80% confluency.

Cell annihilation assessment

Cytotoxic efficacy of the extracts was tested using XTT-formazan dye formation assay[27]. MDA-MB-231 and WRL-68 cells cultured in their respective culture medium (with 10% serum) were seeded (200 μl, 6×10^3 cells/well and 1×10^4 cells/well respectively) in a 96-well plate and incubated at 37 ℃ 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 cytotoxicity was calculated by the following formula:

$$\% \text{ Cytotoxicity} = (Ac- At/ Ac) \times 100$$

Ac is the mean absorbance of the control wells and At is the mean absorbance of test wells with a particular extract dosage.

Apoptotic induction assay

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 BrdU-labeled 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 labeled with 10 μM BrdU at 1×10^5 cells/ml density.

BrdU-labeled cells (1×10^4) in 100 μl culture 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. 100 μl of this obtained sample 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 conjugate. The plates were further incubated for 90 min and were washed three times with washing buffer. 100 μl substrate (TMB) solution was then added and the plates were shaken until color 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 previous reported literature with some modifications[28]. MDA-MB-231 cells (6×10^5 in a well) were cultured on 6-well plates and allowed 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 cells. Subsequent to wounding, cells were gently washed with growth medium to remove dislodged cells. The cells were again added with the fresh medium in control wells and medium containing CHR (16.7 μg/ml) in treatment wells. The plates were incubated at 37 ℃ to further observe migration of cells at every 4 h intervals (4, 8, 12 and 16 h). The migration of cells was then observed by the 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 previously described[29,30].

Estimation of Total Phenolic Content

Total phenolic content estimation[31] was performed with amounts of 20, 40, 60, 80 and 100 μg of extracts taken in separate tubes and were made up to 0.5 ml with distilled water. 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 45 ℃ 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, and equipped with a Waters Symmetry® C18 column (5 mm, 4.6 x 150 mm) with Waters Sentry™ universal guard column (5 mm, 4.6 x 20 mm) (Waters Corporation, Milford, MA, USA). Phenolic compounds in the chloroform extracts of *R. emodi* were analyzed using the library for phenolic compound standards[32] as a reference. Gradient elution was performed at 35 ℃ with Solution A (50 mM sodium phosphate (pH 3.3) and 10% methanol) and Solution B (70%

methanol) in the following gradient elution program: 0–15 min— 100% of Solution A; 15–45 min—70% of Solution A; 45–65min— 65% of Solution A; 65–70min—60% of Solution A; 70–95 min—50% of Solution A; 95–100min—0% of Solution A. Flow rate was 1 ml/min and injection volume was 20 µl. Detection was monitored at diverse wavelengths (around λ_{max}) for various phenolic compounds, i.e. 250 nm for benzoic acids, isoflavones and most anthraquinones; 280 nm for some flavones, flavanones, catechins, theaflavins and some anthraquinones; 320 nm for cinnamic acids, most flavones and chalcones; 370 nm for flavonols; 510 nm for anthocyanins.

Statistical Analysis

All analyses were carried out in triplicates. Data were presented as mean ± SD. Statistical analyses were performed by one-way ANOVA. Significant differences between groups were determined at P<0.05. To evaluate relationships between experimental parameters, results were analyzed for correlation and tested for significance by Student's t-test (P<0.05). 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 statistical and graphical evaluations.

RESULTS AND DISCUSSION

Yield of extracts

Hot extraction

Fifty grams of rhizome powder yielded 0.41g (percentage extract yield: 0.82% of dry weight) of crude chloroform extract (CHR). The samples were stored in a vacuum desiccator at room temperature until further use.

Cold extraction

Fifty grams of rhizome powder yielded 0.80 g (percentage extract yield: 1.60% of dry weight) of crude chloroform extract (CCR). The samples were stored in a vacuum desiccator at room temperature until further use.

Reducing power assay

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[33]. The extracts CHR and CCR were assessed for their reducing power to reduce Fe^{+3} to Fe^{+2} which is indicated by the change in colour from yellow to blue due to formation of Perl's Prussian blue. The dose-dependent increase in absorbance corresponds to increase in the reducing power of the extracts and is depicted in figure 1 with BHT (Butylated hydroxytoluene) as a standard.
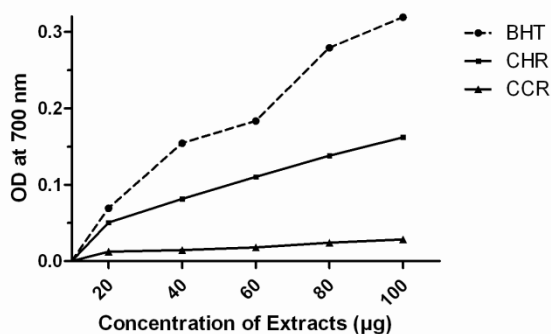

Fig 1: Reducing power of *R.emodi* chloroform extracts with BHT as standard on measurement of Fe^{+3}–Fe^{+2} transformation. Data points are mean values ± SD (n=3, P<0.05).

CHR was significantly better than CCR in reducing power (Figure 1). As the amount of reductones present in the extract is responsible for the reducing power efficacy, accordingly CHR might contain higher amount of reductone followed by CCR.

DPPH assay

Both the extracts showed a concentration-dependent increase in $DPPH^{\bullet}$ scavenging activity. The IC_{50} of the extracts (µg/ml) for $DPPH^{\bullet}$ scavenging are CHR - 927.5±0.83, and CCR - 324.79±1.36. The results were graphically represented as % radical scavenging with equivalence to ascorbic acid (AAE) in micrograms (Figure 2) where the extracts exhibited superior activity in its cold counterpart.
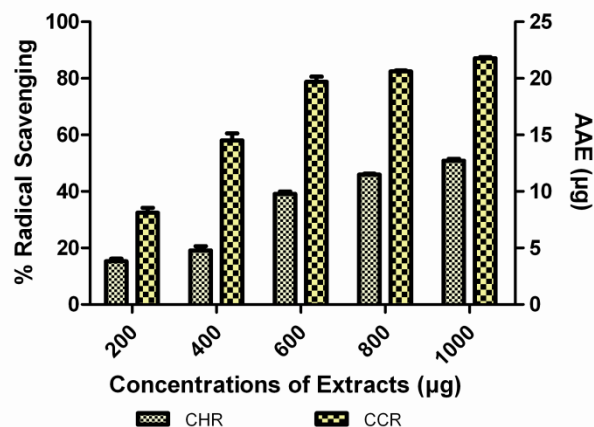

Fig 2: Comparison of $DPPH^{\bullet}$ scavenging ability of hot and cold chloroform extracts of *R.emodi* expressed in AAE. Data expressed as percentage radical scavenging (mean ± SD, n=3, P<0.05).

Cell annihilation assay

The cytotoxicity of CHR and CCR was characterised by a steady increase in the percentage cytotoxicity with respect to extract dosage (Figure 3). CHR and CCR showed toxicity towards non-tumor cells also which may be due to high toxicity of the components present in it. The drugs with the ability to exterminate cancer cells are efficient in chemotherapy of cancer[34]. Hence these extracts were further studied for its apoptotic and anti-metastatic abilities.
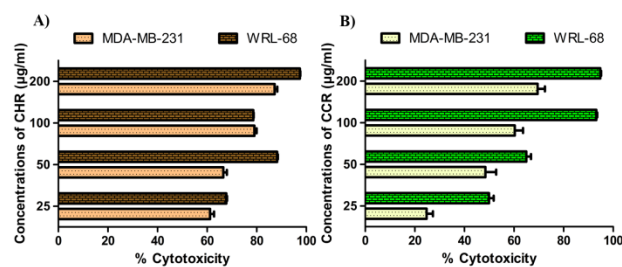

Fig 3: %Cytotoxicity of CHR (A) and CCR (B) on MDA-MB-231 and WRL-68 cell lines expressed as mean ± SD (n=3; P<0.05).

Apoptotic induction

In cancer, there is a lack of apoptosis due to dysfunction of the cell cycle regulation along with either an overproliferation of cells and/or decreased removal of cells[35]. This suppression of apoptosis in carcinogenesis plays a central role in the development and progression of cancer[36]. Tumor cells use a variety of molecular mechanisms to suppress apoptosis[13]. Hence induction of apoptosis in tumor cells is a specific therapeutic approach towards cancer chemotherapy. Consequently, 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 CHR and CCR entrenched a dose dependent increase in apoptotic fragments in both MDA-MB-231 and MCF-7 cells respectively (Figure 4) in which, CHR had significantly higher induction levels of apoptosis than CCR.
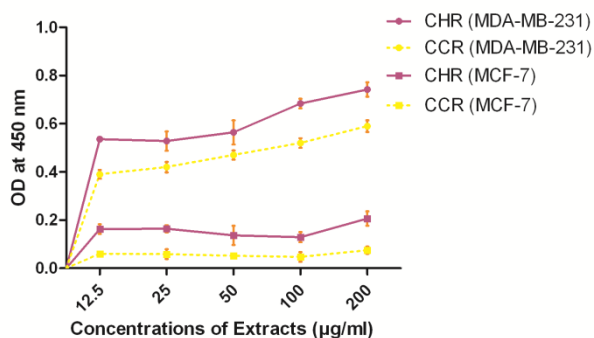
Fig 4: Induction of apoptosis in MDA-MB-231 and MCF-7cells by
***R. emodi* extracts expressed as OD ± SD (n=3; P<0.05).**

Both extracts were found to be efficient against MDA-MB-231 cells on contrast to MCF-7 in apoptosis inductivity. MCF7 cells appeared to be more resistant to apoptosis than MDA-MB-231 cells, which is concomitant to the previously reported results[37,38]. This might be because of the difference in origin and pathology of the cell lines, as MDA-MB-231 is an estrogen receptor (ER)-negative adenocarcinoma cell line from pleural effusion and MCF-7 is an ER-positive invasive ductal carcinoma cell line from pleural effusion[39].

Cell migration inhibition

Cell migration is considered to be an important step in the process of carcinogenesis. As invasion and metastasis of carcinogenesis involves several phenomena like degradation of extra cellular matrix (ECM), cell adhesion and cell migration. Inhibition of either of these steps is one of the efficient approaches in anti-metastatic therapy[40]. In a study system, cell migration is initiated upon morphological damage to the cells as effect of a scratch, and is progressed by the protrusion of cells in direction perpendicular to the scratch wound. The migration is characterized by generation of cell elongation with attain of polarity through Cdc42, mPar6/ PKCζ complex, microtubule motor dynein which results in reorientation of the Golgi and microtubule skeleton to face and act towards the direction of migration[41].

In the current study, cells in the control wells were observed to have migrated from the edges with protrusion into the open space. It is evident from the gradual decrease of distance between the edges with increase in time, (4, 8, 12 and 16 h) to attain a complete confluency at 16th h. Cells in the treatment wells did not show any progress of migration over a time period of 8 h, after which the migration is not apparent as compared with the control (Figure 5). Hence, CHR effectively inhibits the cell migration, a process which is predominantly due to the elevation of ECM degrading enzymes (mostly uPA and MMP)[42]. Similar observations were also made by Yodkeeree[40], who demonstrated suppression of migration and invasion in MDA-MB-231 cells with use of a phytochemical. Thus CHR could possibly serve as a source or precursor in designing efficient drugs against advanced stages of breast cancer.
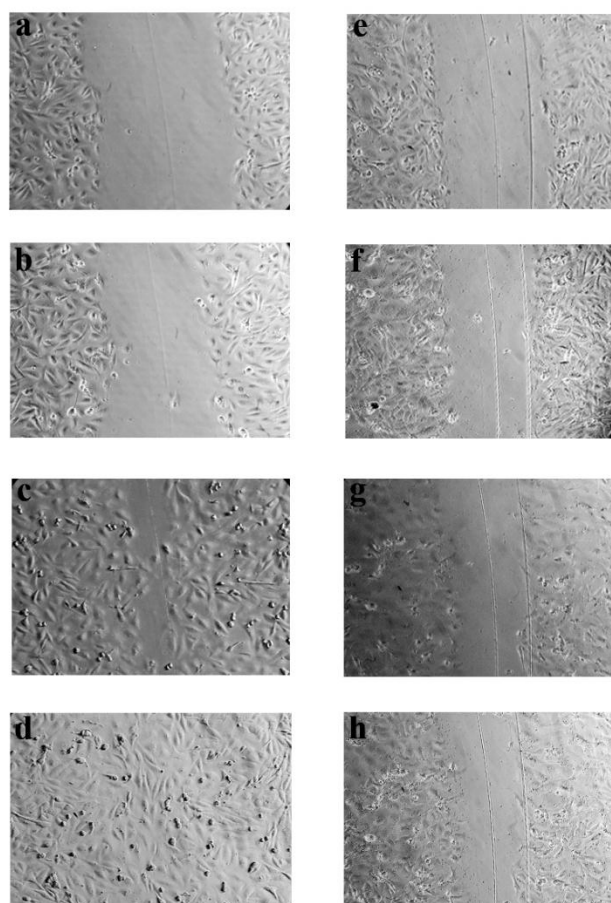

Fig 5: Illustrating the migration of MDA-MB-231 cells from edges of the wounds towards open space after 4, 8, 12 and 16 h upon creation of scratch wound, in control well (a, b, c, d) and CHR treated well (e, f, g, h) respectively.

Phenolic content analysis

The basis for the efficiency of these extracts can further be confirmed by subsequent screening, identification and quantification of the responsible phytochemicals[31].

Phytochemical screening

Screening of the extracts revealed various groups of chemical compounds present. The CHR was found to possess additional number of chemical groups when compared to CCR (Table 1).

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

Components	Chloroform extract	
	Hot	*Cold*
Saponins	-	-
Flavonoids	++	++
Terpenoids	++	++
Reducing Sugar	++	+
Cardiac Glycosides	++	++
Tannins	+	-
Anthraquinones	++	-
Steroids	++	++
Phlobatannins	++	+
Oils	++	-

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

Estimation of Total Phenolic Content

When the extracts were tested for its total phenolic contents, CHR contained quantitatively high phenolics compared to CCR. The total phenolic contents of the extracts were expressed in GAE (in micrograms) as depicted in table 2.

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 Of Extracts (µg)	Gallic Acid Equivalence (GAE) ± SD in µg	
	Chloroform Extract	
	Hot	*Cold*
20	8.29 ± 0.73	6.96±0.09
40	10.3 ± 0.13	8.25±0.14
60	13.02 ± 0.26	9.49±0.06
80	14.27 ± 0.09	10.76±0.03
100	16.23 ± 0.03	11.98±0.01

Results obtained in both the antioxidant assays showed significant differences (p<0.05) between each of the treatment groups. The strength of the evidence due to statistics-based correlation methodologies imply that antioxidant activity is due to the polyphenols in the extracts. Total phenolic content of both the extracts showed strong positive correlation 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.

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

Extracts	Correlations	R^2
CHR	Total phenolics and DPPH$^\bullet$ scavenging	0.981**
	Total phenolics and reducing power	0.996**
	DPPH and Reducing power	0.971**
CCR	Total phenolics and DPPH$^\bullet$ scavenging	0.936**
	Total phenolics and reducing power	0.986**
	DPPH and Reducing power	0.866*

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

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[43]. Various classes of polyphenolics were identified in hot and cold chloroform extracts of *R.emodi* using the reported retention time as a reference for the analytical characteristics (λ_{max}, retention time, determining λ, slope and limit calibration) of more than 100 phenolic standards[32]. Table 4 depicts the polyphenolics identified in the extracts of *R.emodi*. Many of the compounds in *R. emodi* are reported for the first time in this study. Altogether, twenty two known compounds were identified in both

the extracts and were also found to contain few unknown compounds as apparent from the HPLC chromatograms whose isolation and identification is in prospect.

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

Polyphenols	λ[a] (nm)	Et$_{R}$[b] (min)		Rt$_{R}$[c] (min)
		CHR	CCR	
Benzoic acids				
o-hydroxybenzoic acid	250	33.87	-	**34.3**
p-hydroxybenzoic acid	250	13.15	-	**13.8**
Vanillic acid	250	-	15.75	**16.3**
Gallic acid	250	-	4.92	**5.8**
Cinnamic acids				
Chlorogenisc acid (caffeoylquinic acid)	320	14.29	-	**14.1**
Flavones				
7,4'-dihydroxyflavone	320	-	75.71	**75.7**
Chrysin	320	-	89.58	**88.8**
Genkwanin	320	90.14	-	**90.5**
Luteolin	320	79.03	-	**78.9**
Luteolin-3',7-di-*O*-glucoside	320	31.54	-	**31.6**
Diosmetin-7-*O*-rhamnoside (diosmin)	320	61.56	-	**61.4**
Sinensetin	320	87.22	-	**86.3**
Flavonols				
Flavonol	370	-	91.64	**91.5**
Galangin	370	90.66	89.58	**89.9**
Isoflavones				
Daidzein-8-*C*-glucoside (puerarin)	250	20.50	-	**20.1**
Genistein-7-*O*-glucoside (genistin)	250	-	32.53	**31.9**
Catechins				
(-)-Gallocatechin	280	-	7.93	**8.1**
(-)-Epigallocatechin	280	13.13	-	**13.1**
Theaflavins				
Theaflavine	280	-	80.88	**80.8**
Chalcones				
Isoliquiritigenin	320	-	85.12	**84.2**
Butein	320	79.03	-	**79.2**
Anthraquinones				
Alizarin	250	84.37	-	**84.5**

[a] Wavelength for determination.
[b] Experimental retention times.
[c] Reference retention times[32].

In both extraction methods, the compounds eluted are different. Consequently, hot and cold extraction methods have its own advantages of eluting compounds of interest. Soxhlet extraction generally results in a high yield of compounds, especially from plant sources[17]. The compounds present in each of the extracts were different except for one galangin which appeared to be present in both CHR and CCR. Typically, it depends on the compound, its chemical nature, polarity, structure, and solubility in particular solvent polarity, at particular temperature which makes a compound to be extracted at that specific chemical environment[44]. The composition of the extracts studied by total phenolics and HPLC assay explicates that the hot extracts are better in its phenolic contents and in its reported polyphenol composition.

From this study, it can be incurred that both CHR and CCR exhibited effectual antioxidant potentials, cytotoxicity and apoptosis inductivity. In all these test systems, CHR had a significant (P<0.05) upper-hand in its activity over CCR except in DPPH assay. Our study also gives a firsthand report on the anti-metastatic potential of *R. emodi*, where CHR was exceptionally effective in inhibiting migration of MDA-MB-231 cells. Ultimately, Chloroform extracts of *R. emodi* proves to be effective in its anticancer activities, and CHR especially is efficient in its anti-metastatic properties. The extracts thereby could be considered as a source, which harbors phytochemicals to possibly annihilate cancer cells and lucratively harness metastatic

progression of breast cancer cells upon further explicit metabolic investigation.

ACKNOWLEDGEMENT

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