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Research Article

RUBIA CORDIFOLIA LINN MITIGATES EXPERIMENTAL HEPATOCARCINOGENESIS BY REGULATING MMP-2 AND SPARC

PN SHILPA[1,2*], SN DEVARAJ[1] AND V SIVARAMAKRISHNAN[3*]

[1]Department of Biochemistry, University of Madras, Guindy campus, Chennai 600025, Tamilnadu, India, [2]Virtis Bio Labs, Salem 636008,Tamilnadu, India, [3]Department of Bioinformatics, School of Chemical and Biotechnology (SCBT), SASTRA University, Thanjavur 613401, Tamilnadu, India. Email: sivaramakrishnan@scbt.sastra.edu

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ABSTRACT

Objective: *Rubia cordifolia* (RC) is an important medicinal plant which is used for treatment of various ailments in ayurvedic system of medicine. However its cellular and molecular mechanisms involved in abrogation of liver tumor metastasis has not been proved experimentally. The purpose of this study was to investigate the anti-metastatic effect of RC in N-Nitrosodiethylamine (DEN) induced hepatocarcinogenesis.

Methods: In this study, DEN (0.02% in drinking water) induced rats were co-treated with RC extract (500 mg/kg body weight) for 90 days to mitigate the tumor invasion potential of the carcinogen. Serum and plasma was collected and subjected to biochemical estimation of hydroxyproline, glycoprotein (hexose, hexosamines and sialic acid) and plasma lysosomal enzymes (β-Galactosidase and N-acetyl -β-D-glucosaminidase). Expression of MMP-2 (matrix metalloproteinase-2) and SPARC (secreted protein, acidic and rich in cysteine) in liver and kidney tissue was confirmed by zymography, western blotting and immunohistochemistry.

Results: The serum levels of sugar components of glycoproteins and plasma level lysosomal enzymes were found to be increased upon administration of DEN to rats, but significantly lowered on oral supplementation of RC extract (500 mg/kg). Western blots of MMP-2 and SPARC in liver and kidney confirmed their up-regulation in DEN-induced animals when compared to control. Expression levels of both proteins were down-regulated in case of RC supplemented animals, when compared to DEN-induced animals favoring attenuation of tumor invasion.

Conclusion: Our findings suggest that RC extract possessed anti-cancer properties favoring attenuation of DEN-induced tumor invasion.

Keywords: *Rubia cordifolia*, Diethylnitrosoamine, Hepatocarcinogenesis, Antimetastatic activity, Matrix metalloproteinase-2, SPARC.

INTRODUCTION

Hepatocellular cancer (HCC) is the fifth most common cancer with more than 1 million deaths annually worldwide [1]. The major etiological risk factors for HCC include viral hepatitis B and C infections, excessive alcohol consumption and aflatoxin exposure [2]. During HCC invasion and metastasis involves extracellular matrix (ECM), particularly basement membrane (collagen type IV and laminin) degradation [3]. Basement membrane (BM) degradation is facilitated by the family of zinc dependent proteases known as matrix metalloproteinases (MMPs). In particular, several studies have suggested gelatinase A (MMP-2) to be categorically associated with HCC invasiveness [4].

Recently, SPARC also known as osteonectin / LF-53 has been found associated with tissues undergoing cell proliferation, migration and extracellular matrix remodeling [5]. It has also been shown to take part in proteolytic pathways by increasing the expression of collagenase and thereby activating MMP-2 [6]. SPARC is frequently over-expressed in numerous cancers and its expression correlates with their invasion *in vitro* and *in* vivo [7]. Both SPARC and MMP-2 could be considered as diagnostic markers of metastasis and as attractive targets for intervention. New therapeutics blocking the formation of tumor invasion and metastasis will provide greater chances of survival for the HCC patient. The highly invasive heterogeneous phenotype of malignant HCC means that patients have a poor prognosis, even using multidisciplinary treatment strategies including surgery, radiotherapy and chemotherapy. Therefore, a better therapeutic strategy for malignant HCC is needed urgently. The increasing understanding of molecular mechanisms underlying these events has provided excellent opportunity to target tumor invasion and metastasis especially through the usage of nontoxic and biologically effective herbs.

Rubia cordifolia (RC) also known as Manjishtha (Rubiaceae) is one such well-known ayurvedic herb. Various parts of the plant have been employed in the indigenous traditional systems of medicine in India and China for various diseases [8]. Many ethnobotanical and pharmacological activities of RC have been reported such as: antimicrobial, antifungal, antioxidant, analgesic, anti-leukemic, antioxidant, anti-stress, anti-inflammatory, anti-cancer and immunomodulatory actions [9-16]. Apart from its medicinal value, this plant has also been used as natural food colorant.

The presence of bio-active principles like anthraquinone, pentacyclic triterpenes, quinine, and cyclic hexapeptides and diethyl esters have been reported from the RC root extract. In our earlier study, we found significant ameliorative effect of RC extract against DEN-induced experimental carcinogenesis model [17]. Albeit RC extract as an anticancer agent has been studied, its effect on abrogation of tumor invasion and metastasis is not yet documented. Hence the present study was undertaken.

MATERIALS AND METHODS

Source of chemicals and the plant

DEN, BSA (Bovine serum albumin), and Anti-MMP-2 antibody were purchased from Sigma Aldrich (St.Louis, M.O, USA). The Anti-SPARC antibody was purchased from R&D Systems (Minneapolis, MN, USA). Non-fat dry milk powder, DAB (3, 3-diamino benzidine tetra hydrochloride) and IgG-HRP conjugated secondary antibodies (anti-rabbit and anti-goat) was purchased from Genei (Bangalore, India). All other chemicals used were of analytical grade and purchased from SRL Chemicals (Mumbai, India). The dried roots of RC were purchased from Sami Labs (Bangalore, India).

Experimental hepatocarcinogenesis model using DEN

The experimental hepatocarcinogenesis model was initiated by using DEN. DEN is the most important environmental carcinogen among nitrosamines and primarily induces tumors of liver and later metastases to other parts such as kidney and lung [17]. The presence of nitrosamines and their precursors in human environment, together with the possibility of their endogenous formation in human body from ingested secondary amines and nitrites have led to the suggestions of their potential involvement in HCC. It is now widely used as a standard experimental model for HCC.

Preparation of plant extract

Roots of RC (1 kg) were treated with 2.5 L of petroleum ether at room temperature in closed container and repeated till 7 days without allowing it to dry. The plant material was removed by vacuum filtration. The plant extract obtained from the above step was subjected to cold percolation using methanol (95%, v/v) as solvent and suitably concentrated by rotary pump vacuum evaporator and dried in a vacuum desiccator and stored at room temperature. It was dissolved in water and administered orally to rats.

Experimental animals

Male Wistar albino rats (120–150 g) were procured from TANUVAS (Tamil Nadu University of Veterinary Animal Sciences), India and fed with standard rat chow (Amrut Laboratory Animal Feed, Bangalore, India; containing protein 22.06%, oil 4.28%, fibre 3.02%, ash 7.8%, sand (silica) 1.37% w/w) and water, *ad libitum*. The rats were barrier housed two per cage in a temperature (25±2°C) and light controlled environment with a 12:12 h light–dark cycle. Animal experiments were carried out in strict accordance with the guidelines set by the institutional ethical committee for the use of small animals in biomedical research at University of Madras, Chennai, India (IAEC No. 01/004/02).

Experimental design

Animals were divided into four groups, for a total experimental period of ninety days and studied as follows. Group I (control) rats received normal diet and drinking water. Group II (induced) rats were treated with DEN (0.02%) alone orally in drinking water [18]. In our preliminary study of RC root extract, among the various doses (250, 500 and 750 mg/kg body weight) studied, the best efficient dosage for its hepatoprotective efficacy was found to be 500 mg/kg body weight of experimental rats [18]. Hence 500 mg/kg body weight was further chosen as the dosage for the present study [18]. Group III (co-treated) rats were provided DEN (0.02%) orally in drinking water; along with the RC root extract (500 mg/kg body weight respectively) orally by gauge. Group IV (drug-control) rats were supplemented with RC alone (500 mg/kg/body weight) orally by gauge. After 90 days, experimental rats (n=6 per group) were anaesthetized with sodium pentothal after overnight fasting and euthanized. Serum and plasma were separated from blood of the experimental rats. The liver and kidney tissues were excised with ice-cold saline. One part of the tissue was stored at -80°C for immunoblot analysis and the remaining tissue was used for preparing paraffin embedded tissue slides for immunohistochemistry analysis.

Biochemical Analysis

The serum collected was subjected to the estimation of hydroxyproline, hexose, hexosamines and sialic acid [19-22]. The estimation of plasma lysosomal enzymes, β-Galactosidase and N-acetyl -β-D-glucosaminidase was also performed [23, 24].

Gelatin zymography

A portion of the liver tissue (100 mg) and kidney tissue (100 mg) was homogenized independently in 0.1M Tris buffer, pH 7.4 to prepare a 10% homogenate and centrifuged at 3000×*g* for 10min at 4°C. The supernatant was subjected to gelatin zymography for MMP-2 activity [25].

Immunohistochemistry

A portion of the liver and kidney tissue was fixed in 10% buffered neutral formalin solution for histological studies. After fixation, tissues were embedded in paraffin wax and solid sections were cut at 5µm. The sections were deparaffinised in xylene, hydrated using graded series of alcohol and blocked using 3% BSA in phosphate buffered saline (PBS) for 1 h at room temperature. The sections were then incubated overnight at 4 °C with specific primary antibody for anti-MMP-2 (1:100 dilution) and anti-SPARC (1:100 dilution) independently. The respective slides were washed with PBS for 5 min, after which, the slides were incubated with secondary antibody IgG-HRP (1:500 dilution) in PBS and 0.05% Tween 20 for 1 h at room temperature. The slides were developed with DAB solution containing 0.05% DAB, H_2O_2 in PBS in a dark room for 10 min and counter stained with hematoxylin followed by dehydration and mounted with DPX (distyrene plasticizer and xylene). The slides were visualized and quantitative analysis was made manually under a light microscope.

Immunoblotting

Briefly, 60 µg of the tissue (liver and kidney) homogenate was resolved in 12% SDS-PAGE gel. After the run, the gel was transferred to nitrocellulose membranes and blocked in 5% non-fat dry milk powder in blocking buffer (TBS containing 0.1% Tween20). The membranes were incubated with anti-MMP-2 rabbit polyclonal antibody and anti-SPARC goat polyclonal antibody, diluted in blocking buffer, overnight at 4 °C, with gentle shaking. The membranes were washed and subsequently incubated with their corresponding secondary antibodies (anti-rabbit and anti-goat) conjugated to horseradish peroxidase and the membranes were developed using DAB as the chromogen. Intensity of the bands was digitized with a gel scanner.

Statistical analysis

All the grouped data were evaluated with SPSS/10 software. Hypothesis testing includes one way analysis of variance (ANOVA) followed by Posthoc Dunnet's test for multiple comparisons. *p* values of less than 0.001 were considered to indicate statistical significance. All the results were expressed as mean ± S.D. for six animals in each group.

RESULTS

Figure 1 shows the levels of hexose, hexosamine, sialic acid and fucose in the serum of control and experimental groups of animals. In DEN induced (Group II) animals, there was a significant (p<0.001) increase in the levels of hexose, hexosamine, sialic acid and fucose when compared with control (Group I) animals. Whereas in RC extract co-treated (Group III) animals there was a significant decrease in the levels of hexose (p<0.001), hexosamine (p<0.001), sialic acid (p<0.001) and fucose (p<0.001) when compared with tumor bearing (Group II) animals.

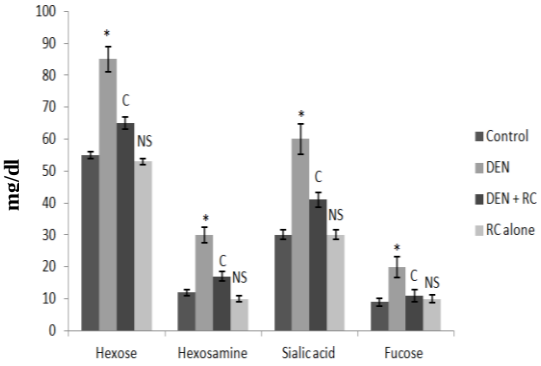

Fig. 1: Effect of RC Linn extract on the levels of hexose, hexoseamine, sialic acid and fucose in the serum of control and experimental animals.

Values are expressed as mean±S.D. (n = 6);Statistical significance represented as *p<0.001. Comparisons are made between Control Vs DEN; RC alone Vs Control; DEN Vs DEN+RC; DEN+RC Vs RC alone; NS represents Non-Significant. The glycoprotein levels are expressed as mg/dl.

Figure 2 shows the plasma levels of lysosomal enzymes. β-N-acetylglucosaminidase, β-D-Galactosidase and β-D-Glucuronidase was significantly (p<0.001) increased in DEN induced (Group II) rats when compared to control (Group I) rats. After supplementation of RC extract to co-treated (Group III) animals, there was significant (p<0.001) reduction in the level of lysosomal enzymes were observed when compared to DEN induced (Group II) animals.

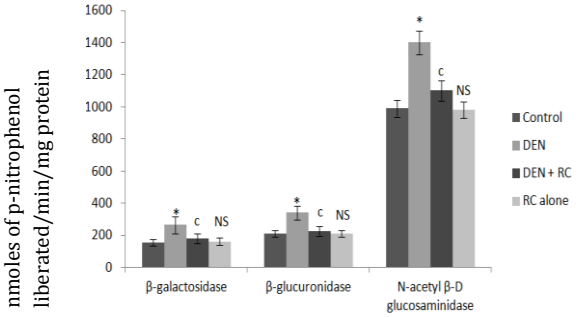

Fig. 2: Effect of RC Linn extract on the levels of β-galactosidase, β-glucuronidase, N-acetyl β-D glucosaminidase in the plasma of control and experimental rats.

Values are expressed as mean±S.D. (n = 6); Statistical significance represented as *p<0.001; Comparisons are performed between Control Vs DEN; RC alone Vs Control; DEN Vs DEN+RC; DEN+RC Vs RC alone; NS represents Non-Significant; Lysosomal enzymes are expressed as nmoles of p-nitrophenol liberated/min/mg protein. Figure 3 shows the effect of RC on liver hydroxyproline levels. Administration of DEN to (Group II) induced animals significantly (p<0.001) increased liver hydroxylproline levels when compared to control (Group I) rats. On administration of RC, decreased hydroxyl proline level significantly (p<0.001) when compared to (Group II) DEN induced rats.

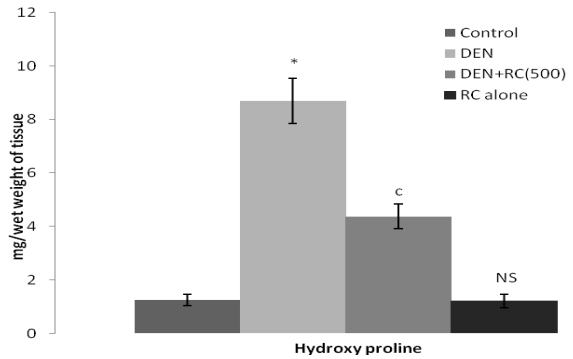

Fig. 3: Effect of RC Linn extract on the levels of hydroxyproline in control and experimental rats.

Values are expressed as mean±S.D. (n = 6); Statistical significance represented as *p<0.001. Comparisons are made between Control Vs DEN; RC alone Vs Control; DEN Vs DEN+RC; DEN+RC Vs RC alone. NS represents Non-Significant. Hydroxy proline levels are expressed as mg/wet weight of tissue. In order to characterize the proteolytic activity of RC, rat liver homogenate was examined for zymography by incorporating various protease inhibitors in gel substrate buffer. Qualitative data indicate that RC inhibited the proteolytic activity in presence of certain metalloproteinase inhibitors. Since the presence of divalent ions such as calcium is considered essential for activation of metalloproteinase [26]. EDTA or DTT (Chelators of divalent ions) was added to substrate buffer to visualize inhibition of MMP activity. Table 1 confirms there was complete inhibition of proteolysis activity upon incubation with EDTA or DTT in Group III, suggesting that the proteinases regulated by RC are metalloproteinases.

Table 1: Qualitative analysis of RC inhibition of proteolytic activity in presence of certain metallo proteinase inhibitors

Treatments	SB	SB + EDTA	SB + DTT
Control	+	-	-
DEN	+++	-	-
DEN + RC	+	-	-
RC alone	+	-	-

DEN when treated with RC and without RC and separated on SDS – PAGE. The gels were subsequently incubated with substrate buffer (SB) or SB containing EDTA (10 mM) or DTT (5mM). The proteins were fixed, stained and visualized. The extent of activity is expressed qualitatively as positive (+) and the absence of proteolytic activity is expressed as Negative (**-**).

Figure 4 shows the gelatin zymographic analysis of MMP-2 in liver and kidney. Administration of DEN to (Group II) induced animal's significantly up-regulated MMP-2 activity when compared to control (Group I) rats. On supplementation of RC, down-regulated MMP-2 activity was observed when compared to (Group II) DEN induced rats.

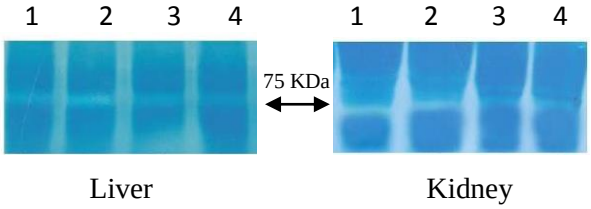

Fig. 4: Gelatin Zymographic analysis of MMP-2 in the liver and kidney of control and experimental group of animals.

Lane 1 represents Control; Lane 2 represents DEN induced; Lane 3 represents DEN+RC; Lane 4 represents RC alone.

Figure 5 shows the effect of RC on MMP-2 and SPARC (LF-23) protein in liver and kidney. To determine whether the above inhibition of tumor invasion is related to direct inhibition of MMP-2/ LF-23 enzyme activity or related to inhibition of MMP-2/ LF-23 protein expression, western blot analysis was carried out. Administration of DEN significantly up-regulated both MMP-2 and LF-23 expression when compared to control (Group I) rats. On supplementation of RC, down-regulation of MMP-2 and LF-23 expression was observed when compared to (Group II) DEN induced rats.

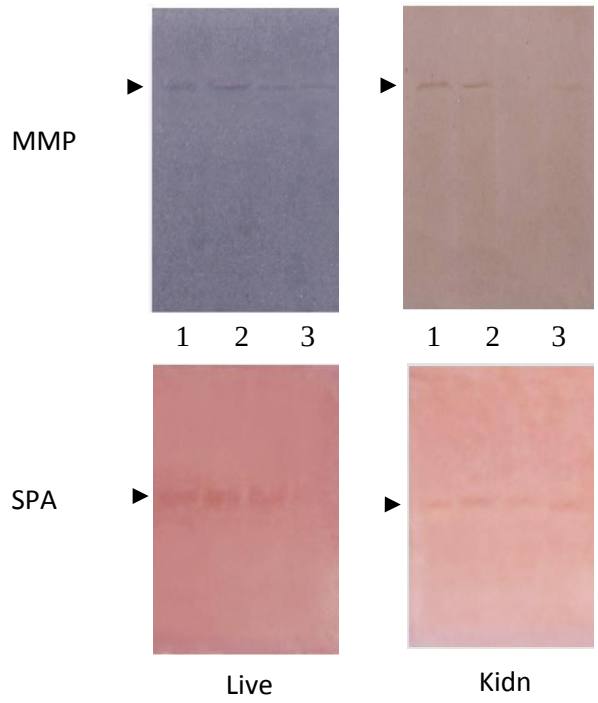

Fig. 5: Western blotting analysis of MMP-2 and SPARC expression in liver and kidney of control and experimental animals

Lane 1 represents control; Lane 2 represents DEN induced; Lane 3 represents DEN+RC; Lane 4 represents RC alone.

Figure 6 and 7 respectively shows immunohistochemical staining of MMP2 in the liver and kidney of control and experimental group of animals. Administration of DEN to (Group II) induced animals

showed significant increase in the number of MMP-2 positive cells when compared to control (Group I) rats, while RC treatment significantly decreased the number of MMP-2 positive cells when compared to (Group II) DEN induced rats.

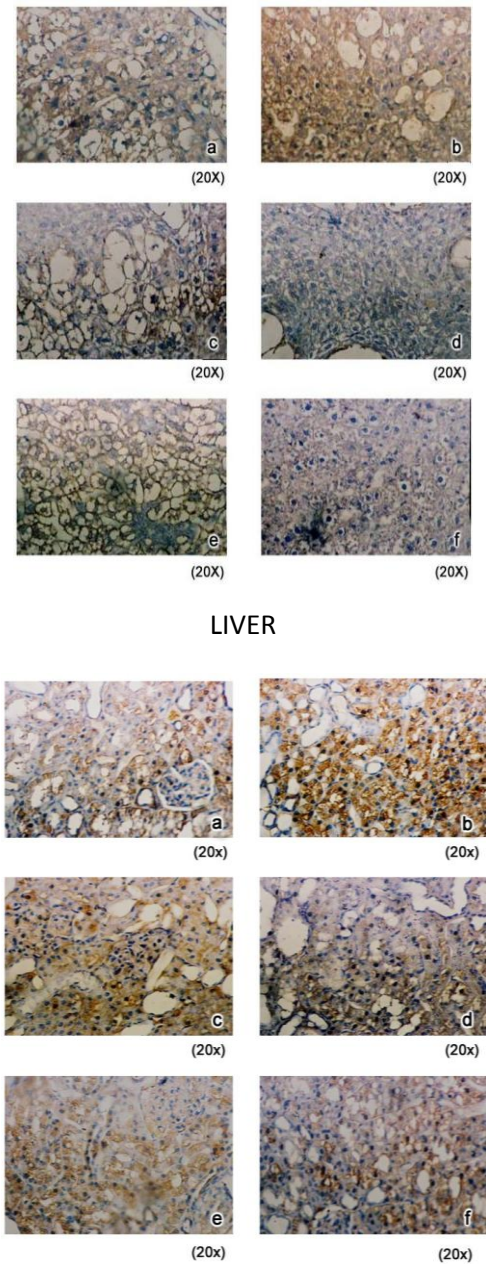

Fig. 6 & 7: Immunohistochemical analysis of MMP-2 in the liver and kidney of control and experimental animals respectively.

(a) Control rats showing absence of MMP-2 expression; (b) DEN induced rats showing mild expression of MMP-2; (c, d, e) DEN+RC showing mild expression of MMP-2; (f) RC extract alone treated experimental animals showing complete absence of MMP-2 expression.

Figure 8 and 9 respectively show immunohistochemical staining of LF-23 in the liver and kidney of control and experimental group of animals. Administration of DEN to (Group II) induced animals showed significant increase in the number of LF-23 positive nuclei when compared to control (Group I) rats, while RC treatment significantly decreased the number of LF-23 positive nuclei when compared to (Group II) DEN induced rats.

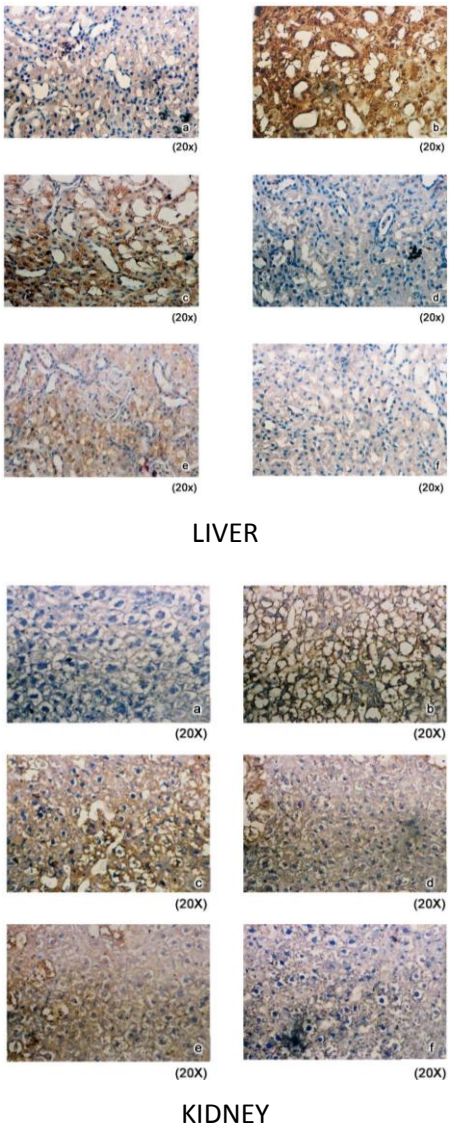

Fig. 8 & 9: Immunohistochemical analysis of SPARC in liver and kidney of control and experimental animals.

(a) Control rats showing absence of SPARC expression; (b) DEN induced rats showing mild expression of SPARC; (c, d, e) DEN+RC showing mild expression of SPARC; (f) RC extract alone treated experimental animals showing complete absence of SPARC expression.

For all the above parameters studied (Figure 1 to Figure 9 inclusive of Table 1), RC alone drug-control animals (Group IV) did not show any significant changes when compared with control (Group I) animals.

DISCUSSION

Current chemotherapeutic drugs kill cancerous cells predominantly by inducing apoptosis. However, they become ineffective once cancer cell metastasize, hence result in poor prognosis and high mortality rate. Therefore, the present study was undertaken to evaluate the anti-metastatic activity of RC and its mechanism of action. DEN induced HCC is well studied and known to increase occurrence of metastasis [26]. The appearance of glycocomponents of glycoproteins in sera of tumor bearing animals and humans has diagnostic and prognostic value and are important determinants in the pathophysiology of cancer [27]. Significant increase in hexose and hexosamine content of serum glycoprotein is found to be associated with neoplastic disease [28]. Sialic acid, group of acylated

neuraminic acid, imparts a net negative charge to cell membrane and has an important role in cell-cell and cell-matrix interactions. Sialic acid concentration on the surface of malignant cell can be used to correlate directly with its ability to metastasize [29]. Elevated levels of total sialic acid and lipid bound sialic acid were reported in several cancers [29-32]. Elevated plasma sialic acid concentration is probably either due to shedding or secretion of sialic acid from tumor cell surfaces or due to membrane degradation [32]. The observed increase in plasma lipid bound sialic acid in our study is probably due to shedding of aberrant sialic acid rich glycolipids into circulation. An affirmative association between serum fucose level and cancer progression has been reported [32]. The observed increase in plasma total sialic acid and fucose in tumor bearing rats could be either due to increased turnover of malignant cells or shedding from tumor tissues and membranes into circulation. The increase in serum glycocomponents might be due to elevated activities of glycosyltransferases, the enzyme responsible for glycoprotein synthesis. Among all the four components of glycoproteins, more pronounced changes were observed with sialic acid. The increased levels of these glycocomponents of glycoprotein were reduced significantly on RC treatment.

Lysosomal enzymes are able to degrade cell organelles and digest cell material and modified ground substrate of connective tissues, thereby favoring growth or migration of both normal and malignant cells. β-glucuronidase is shown to be a sensitive marker of lysosomal integrity. Increased serum glycosidase levels found in cancerous condition may thus be associated with structural changes in the enzymes which make the liver cell unable to recognize them, thereby preventing their clearance from the blood [33]. The levels of glycan moieties and the activities of glycosidases can be used as diagnostic markers to assess the stage of cancer can be prognostic marker during therapy. The decreased enzyme activities observed upon RC treatment could be an evidence for its ability to significantly reduce the leakage of enzymes, stabilizing the membrane texture.

Accumulation of extracellular matrix in the liver induces high metastatic potential of hepatocellular carcinoma [34].The elevated levels of hydroxyproline indicate the fibrosis of liver due to high collagen levels. It was observed independently by another group that collagen content of liver tissue samples from HCC patients was significantly lower than normal subjects [35]. This was due to increased collagenase expression, which degrades the collagen matrix in HCC nodules and its surrounding liver tissue. Other studies suggested that tumor cells stimulate the expression of MMP-2 and 9, which contribute to decreased collagen deposition as a response to chemical hepatocarcinogenesis [36].

Invasion and metastasis of firm tumors requires the action of its associated proteases (MMPs) that promote the disintegration of the surrounding matrix and the basal membrane [37]. In this study, we showed that RC decreased levels of MMP-2. RC is believed to lead to suppression of MMP-2 expression and secretion, which would lead to degradation of the ECM and BM, thereby summoning growth factors that would promote cancer cell survival, cell migration, and invasion. Our results demonstrated that RC significantly inhibited the invasion of HCC cells, at least in part, by down-regulating the expression of MMP-2. It has been demonstrated previously that SPARC over-expression is correlated with an increase in invasiveness and survival of cancer cells [38]. The expression of SPARC and MMP-2 was positively correlated in liver cancer and might have close relation in the tumorigenesis, malignant transformation and invasiveness similar to meningiomas [39]. The expression of SPARC and MMP-2 was positively related in invasive liver cancer, there may be a mutual synergy, which could enhance the hepatomas invasive ability. In addition, our results correlate with a previous study showing MMPs as potential cofactors of SPARC, since SPARC was cleaved by various MMPs and SPARC expression was associated with expression of MMP-1 and MMP-2 [40, 41]. Thus, our findings imply that inhibition of expression of SPARC and MMP-2 by RC administration in turn inhibits tumor progression. In conclusion, we demonstrated that RC was able to inhibit metastasis of liver cancer by decreasing the components of glycoprotein, hydroxyproline, lysosomal enzymes and down regulating MMP-2 and SPARC expression. Thus, above *in vivo* experimental evidences clearly underlines the anti-metastatic potential of RC on experimentally induced metastasis of HCC.

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CONFLICT OF INTEREST

There exists no conflict of interest between the authors.

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