Research Article

Evaluation of chemopreventive potentials of ethanolic extract of *Ruta graveolens* against A375 skin melanoma cells *in vitro* and induced skin cancer in mice *in vivo*

Samrat Ghosh, Sourav Sikdar, Avinaba Mukherjee, Anisur Rahman Khuda-Bukhsh

Cytogenetics and Molecular Biology Laboratory, Department of Zoology, University of Kalyani, Kalyani-741235, West Bengal, India

ABSTRACT

**OBJECTIVE:** Chemopreventive approach with natural products, particularly plants and plant-derived ones, is receiving increasing attention for their effective role against cancer without any palpable side effects. In this study, efficacy of ethanolic extract of *Ruta graveolens* (RG) on skin melanoma cells (A375) *in vitro* and on 7,12-dimethylbenz(a)anthracene (DMBA)-induced skin cancer *in vivo* has been tested in Swiss albino mice.

**METHODS:** Studies on cell viability, apoptosis and autophagy induction were conducted *in vitro*. To check apoptosis, assays like alteration in mitochondrial membrane potential, annexin V-fluorescein isothiocyanate/propidium iodide assay and immunoblot were performed. Fluorescence microscopic and immunoblot assays were performed to confirm autophagy induction. The effects of RG were determined by evaluating body weight, tumor incidence, tumor volume and tumor burden in mice. Enzymatic and non-enzymatic antioxidant status was assessed. The role of some relevant signaling proteins was also analyzed.

**RESULTS:** RG caused death of A375 cells through induction of caspase 3-mediated apoptosis and Beclin-1-associated autophagy. Moreover, RG administration (75 mg/kg body weight) which showed no acute or chronic toxicity, showed significant reduction in the skin tumor burden of DMBA-painted mice. RG also demonstrated potent anti-lipid peroxidative and antioxidant functions during the course of skin cancer induction by DMBA.

**CONCLUSION:** Chemopreventive potential of RG was demonstrated from overall results of this study, indicating its possible use in therapeutic formulation of an effective drug to treat skin cancer.

**Keywords:** *Ruta graveolens*; plant extracts; chemoprevention; skin neoplasms; apoptosis; autophagy

**Citation:** Ghosh S, Sikdar S, Mukherjee A, Khuda-Bukhsh AR. Evaluation of chemopreventive potentials of ethanolic extract of *Ruta graveolens* against A375 skin melanoma cells *in vitro* and induced skin cancer in mice *in vivo*. *J Integr Med*. 2015; 13(1): 34–44.
1 Introduction

Unwanted side effects of many currently used drugs make them unsuitable or of limited use in the treatment of cancer. The search for drugs relatively free of side effects leads to the arena of alternative mode of treatment for cancer, which mainly uses certain medicinal plants and plant-derived products for this purpose. *Ruta graveolens* is a medicinal and culinary plant growing in the Mediterranean region of southern Europe and northern Africa. It is used for the treatment of inflammatory conditions, eczema, ulcers, arthritis and fibromyalgia and as an antitode for venoms, insect repellent, and also as an abortifacient[1-3]. Extract of this plant is considered as an effective ingredient in medicinal preparations of rue oil and infusions that are used as antispasmodics and emmenagogues[4].

A major therapeutic goal of recent cancer treatment is to trigger tumor-selective cell death. This target can be achieved by two major ways: by apoptosis (type I programmed cell death; PCD I) and/or by autophagy (type II programmed cell death; PCD II). Drugs having the ability to induce apoptosis are preferred in medical oncology. In order to survive, cancer cells many a time develop intricate mechanisms that give them the ability to escape apoptosis and to develop resistance against anticancer drugs[5]. For this reason, in many cases, anticancer drugs fail to curb cancer spread after initial success. Therefore, drugs that can destroy cancer cells by inducing both apoptosis and autophagy are considered to have added advantage, in view of the fact that cancer cells can be killed by autophagy even if they escape apoptosis[5]. Thus, study to determine the efficacy of some medicinal plants and plant-derived products to stop proliferation of cancer cells or to annihilate them by apoptotic, necrotic, or autophagic means has achieved considerable importance. Plants and plant-derived products with chemopreventive activities are gaining importance in the field of anticancer drug research. Certain chemopreventive agents are known to act through their anti-lipid-peroxidative and antioxidant properties[6]. For experimental induction of skin cancer in Swiss albino mice, 7,12-dimethylbenz(a)anthracene (DMBA) is generally used as a site- and organ-specific carcinogen, either as an initiator or as a promoter[8,9].

In this study, the hypotheses tested were: if ethanolic extract of *Ruta graveolens* (RG) could annihilate skin melanoma cells A375 *in vitro*, if RG could combat DMBA-induced skin cancer in Swiss albino mice *in vivo*, and if it could delineate the possible mechanism of action.

2 Materials and methods

2.1 Drug used

RG was obtained from Boiron Laboratory, Lyon, France. For the preparation of dried extract, the ethanol content was first evaporated under reduced pressure in a rotary evaporator; a concentrated ingredient that looked dark brownish in color and semisolid in nature, was obtained. Then a solution of the extract was made by adding distilled water and gently stirring. The solution was kept in 4 °C for further use in experiments as the ‘stock solution’ of the drug in case of *in vitro* experiments; the same was also used for feeding the experimental mice *in vivo*.

2.2 In vitro experiments

2.2.1 Cell line and cell culture

Skin melanoma (A375) and skin keratinocyte (HaCaT) cell lines were procured from National Centre for Cell Science (Pune, India); peripheral blood mononuclear cells (PBMCs) were isolated from human blood sample by the standard Ficoll Paque density gradient method[10]. Cell-culture method (routine adherent cell culture method) followed has been described elsewhere[11].

2.2.2 Cell viability assay

Cell viability assay was made following 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as per the technique described previously[11]. The dose of RG that reduced the viability of cells at 48 h to about 50% was determined. The viability of A375 cells exposed to 3-methyladenine (3-MA; autophagy inhibitor, 10 mmol/L, Santa Cruz Biotechnology Inc., CA, USA) for 2 h before RG administration was also assessed.

2.2.3 Alteration in mitochondrial membrane potential

Flow cytrometric analysis was done to estimate quantitatively alteration in mitochondrial membrane potential (MMP) in the RG-exposed A375 cells. At first, cells were exposed to 44.80 µg/mL of RG for different periods of time, starting from 0 h through 12, 24, 36 and 48 h. Then fixation of the treated cells was done in 70% ethanol. After that fixed cells were incubated with 10 µmol/L rhodamine 123 for 30 min at 4 °C in the dark and the fluorescence intensity was measured by flow cytometer using FL-1H filter. Cylogic software (CyFlo Ltd, Turku, Finland) was used for analyzing the data.

2.2.4 Annexin V-fluorescein isothiocyanate/propidium iodide dual-stain assay

A375 cells were exposed to RG of three specified doses, one being the IC50 dose, one below and one above the IC50 dose (D1 = 35 µg/mL, D2 = 44.80 µg/mL, and D3 = 50 µg/mL) for 48 h of time interval. The cells were then processed for the dual stain assay[12]. Briefly, at 4 °C in the dark, 15-minute incubation was done after adding 10 µmol/L annexin V-fluorescein isothiocyanate (FITC, Santa Cruz Biotechnology Inc., CA, USA) and 5 µmol/L of propidium iodide (PI) to the cell suspensions. A flow cytometer (BD FACS Calibur, USA) with FL-1 (530 nm) and FL-2 (585 nm) filters was used to determine the fluorescence intensities and the distribution frequencies of...
live and dead cells were analyzed with Cylogic software (CyFlo Ltd, Turku, Finland).

2.2.5 Expression study of signaling proteins by immunoblot analysis

Anti-Bcl-2 (mouse IgG₂), anti-BAX (mouse IgG₁), anti-cleaved poly ADP ribose polymerase (PARP) (rabbit IgG), anti-cytochrome c (mouse IgG₁), anti-Akt (mouse IgG₁), anti-caspase 3 (mouse IgG₂), anti-pAkt (rabbit IgG), anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (mouse IgG₁), anti-voltage-dependent anion-selective channel protein 1 (VDAC1) (anti-goat IgG), anti-mechanistic target of rapamycin (mTOR) (rabbit IgG), anti-Atg5 (rabbit IgG), anti-Beclin-1 (rabbit IgG), anti-LC3 (rabbit IgG) and anti-Ag7 (rabbit IgG) (Cell Signaling Technology, Santa Cruz Biotechnology Inc., CA, USA), anti-LC3 (rabbit IgG), anti-Atg5 (rabbit IgG), anti-Beclin-1 (rabbit IgG), and anti-Ag7 (rabbit IgG) (Cell Signaling Technology, Inc.) antibodies were used for immunoblot study[13]. For cytosolic and mitochondrial protein expression analysis, GAPDH and VDAC1 served the purpose of loading control, respectively.

2.2.6 Detection of acidic vesicular organelles

The vital staining method with acridine orange (AO) was used to detect the acidic vesicular organelles (AVOs) in RG-treated cells (both in presence or absence of 3-MA). The cytoplasm and the acidic compartments fluoresce as bright green and bright red objects, respectively presented in AO-stained cells[14]. Chilled ethanol (70%) was used to fix the RG-exposed cells. Then 5 mmol/L AO was used for the staining of fixed cells. After that fluorescence of the stained cells was measured with a fluorescence microscope (Axioskop plus 2, Zeiss).

2.3 In vivo experiments

2.3.1 Animals

Six/eight weeks old healthy inbred strains of Swiss albino mice (Mus musculus) weighing about 25 g were kept for at least 14 d in an environmentally controlled room (temperature (24±2) ℃; humidity 55%±5%, 12-hour light/dark cycle), and were allowed to take in food and water ad libitum. All experiments were conducted as per the guidelines cleared by the Animal Ethics Committee of the University of Kalyani, India (Registration number-892/OC/05/CPCSEA) and under the supervision of the Animal Welfare Committee (Department of Zoology, University of Kalyani, India). Randomized mice of both sexes were used for various enzymatic assays. Spectrophotometric analysis of catalase (CAT)[17], superoxide dismutase (SOD)[18], reduced glutathione (GSH)[19], lipid peroxidase (LPO)[20], glutathione S-transferase (GST)[21] and glutathione peroxidase (GPX)[22] was undertaken with skin tissue homogenates whereas spectrophotometric analysis of GSH and GST was performed with liver tissue homogenates according to the standard protocols.

2.3.2 Acute toxicity study of RG

Normal mice were force fed with different doses of RG (75, 100, 125, and 150 mg/kg body weight) through gavage to check acute toxicity of RG, if any, produced by any of these doses. Any change in behavior pattern or mortality in mice over the next 24 h was considered as indicative sign of acute toxicity. However, none of experimental doses of RG showed acute toxicity; of these, the lowest dose (75 mg/kg body weight) that did not show slightest of toxicity has been chosen for treatment for the longer periods.

2.3.3 Experimental design

Thirty animals used in the present study were divided into 5 groups, each consisting of 6 mice. Development of skin papilloma was induced by following the standard practice of chemical carcinogenesis[15]. Hair of skin was first removed by applying depilatory cream over the surface of back of all experimental and control mice. Acetone (0.1 mL/mouse) was rubbed on the depilated back of the group II mice twice a week for 8 weeks (vehicle-treated control). DMBA (25 μg in 0.1 mL acetone per mouse) was applied on depilated back of the groups III and IV mice, twice a week for 8 weeks. While no treatment was given to the group III mice, the group IV mice were force fed RG at a dose of 75 mg/kg body weight for 1 week before they were subjected to the DMBA treatment continuously for the next 25 weeks, 3 times a week on alternate days. To maintain another group of control, the group V mice were orally administered with RG only at the same dose of the experimental mice (75 mg/kg body weight) throughout the time course of the experimental study. To maintain a negative control, back of mice of group I was painted with neither acetone nor DMBA and this group of mice was not fed RG. Through cervical dislocation all mice were sacrificed at the end of experiment.

2.3.4 Body weight, tumor incidence, tumor volume and measurement of tumor burden

The body weight of all the experimental mice was measured before and after the time period of RG administration.

The tumor volume was determined by using the following formula: \(4\pi/3 \times (D_1/2) \times (D_2/2) \times (D_3/2)\), where \(D_1\), \(D_2\) and \(D_3\) represented the three diameters (in mm) of the tumors.

Tumor burden was evaluated through multiplication of the value of tumor volume with the number of tumors per animal.

2.3.5 Skin and liver tissue homogenates preparation

From experimental mice, the skin and liver tissues were collected and homogenized in lysis buffer and then centrifuged at 12 000×g for 30 min at 4 ℃. The supernatants were collected and used further. The total protein was estimated by the method of Bradford[26]. Skin and liver tissue homogenates were used for various enzymatic assays. Spectrophotometric analysis of catalase (CAT)[17], superoxide dismutase (SOD)[18], reduced glutathione (GSH)[19], lipid peroxidase (LPO)[20], glutathione S-transferase (GST)[21] and glutathione peroxidase (GPX)[22] was undertaken with skin tissue homogenates whereas spectrophotometric analysis of GSH and GST was performed with liver tissue homogenates according to the standard protocols.

2.3.6 Immunoblot assay

Immunoblot assay was done as described in the “In vitro experiments” section.
2.4 Statistical analysis
Data are represented as mean ± standard error of mean. Significance of the differences between the mean values was determined by one-way analysis of variance with Dunnett’s t post-hoc test, using SPSS 14.0 software. \( P < 0.05 \) was considered as statistically significant.

3 Results

3.1 In vitro experiments
3.1.1 RG reduced A375 cell viability
RG was non-cytotoxic to normal PBMCs and HaCaT cells whereas it reduced A375 cell viability significantly. Upon exposure to \((44.80±0.81)\ \mu\text{g/mL}\) of RG for 48 h, the cell viability was reduced to about 50% (Figure 1).

3.1.2 RG altered MMP
The histogram of flow cytometric data revealed shifting of fluorescence peak towards left (Y-axis) upon RG administration, indicating marked decreases in fluorescence intensity. Histogram implied the MMP alteration between 12 and 24 h of RG administration (Figure 2).

3.1.3 RG induced externalization of phosphatidyl serine
Annexin V and PI stain assay showed that cell population shifted from lower left co-ordinates to the right side co-ordinates (annexin V+) upon RG exposure (Figure 3), revealing the fact of externalization of phosphatidyl serine. Annexin V does not bind with the normal cells because it can only bind with phosphatidyl serine which is available in the inner membrane of the cell. When the cells become apoptotic, the phosphatidyl serine comes out to the external membrane surface (externalization of phosphatidyl serine) and readily binds with the annexin V, resulting in the shift of cell population towards the right side quadrants. Thus this result (Table 1) indicates the ability of RG to induce apoptosis in the cells.

3.1.4 RG modulated expression of certain proteins related to apoptosis
Significant up-regulation of BAX, cytochrome \(c\) (cytosolic fraction), cleaved caspase 3 and cleaved PARP expressions was observed from immunoblot study (Figure 4). Down-regulated expressions of Bel-2 and cytochrome \(c\) (mitochondrial fraction) were observed upon RG administration (Table 2).

3.1.5 RG induced autophagy
Autolysosomes (fusion of autophagosomes with lysosomes) are forms of AVOs. During autophagosome formation, conversion of LC3 (microtubule-associated protein light chain 3) and activation of Beclin-1 take place.

The presence of AVOs in RG-treated cells was observed from AO stain distribution (orange-red fluorescence) (Figure 5), and there was very much reduction in fluorescence intensity upon administration of 3-MA (autophagy inhibitor) with RG (Figure 5).

Conversion of LC3-I (18 kD) to LC3-II (16 kD) upon RG administration (Figure 6) was confirmed from result of immunoblot assay. During the time period 18–24 h, expression of LC3-I was reduced and that of LC3-II increased, indicating the autophagic activation upon RG administration.

Upon RG administration up-regulated expression of Atg7, Beclin-1, and Atg5 and down-regulated expression...
Table 1  Cell population percentages in specific quadrants obtained from annexin V-FITC/PI stain assay

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Live (LL)</th>
<th>Early apoptotic (LR)</th>
<th>Late apoptotic (UR)</th>
<th>Dead (UL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>98.75</td>
<td>1.08</td>
<td>0.01</td>
<td>0.16</td>
</tr>
<tr>
<td>D1</td>
<td>80.50</td>
<td>19.35</td>
<td>0.14</td>
<td>0.01</td>
</tr>
<tr>
<td>D2</td>
<td>53.91</td>
<td>41.88</td>
<td>4.05</td>
<td>0.16</td>
</tr>
<tr>
<td>D3</td>
<td>40.56</td>
<td>44.89</td>
<td>9.93</td>
<td>4.62</td>
</tr>
</tbody>
</table>

Data represent percentages of cell population in specific quadrants. LL: lower left; LR: lower right; UR: upper right; UL: upper left; D1: 35 µg/mL dose of *Ruta graveolens*; D2: 44.80 µg/mL dose of *Ruta graveolens*; D3: 50 µg/mL dose of *Ruta graveolens*.

Figure 3  Annexin V-FITC/PI stain assay
Externalization of phosphatidyl serine was assessed by conducting FACS analysis (FL1 and FL2 filters). Cells were treated for 48 h with D1 (35 µg/mL), D2 (44.80 µg/mL) and D3 (50 µg/mL) doses of *Ruta graveolens*. LL-lower left (Annexin V-/PI-), UL-upper left (Annexin V-/PI+), LR-lower right (Annexin V+/PI-), and UR-upper right (annexin V+/PI+) represent the specific quadrants. Annexin V and PI stain assay showed that cell population shifted from lower left coordinates to the right side coordinates (annexin V+) upon *Ruta graveolens* extract exposure, revealing the fact of externalization of phosphatidyl serine.
FITC: fluorescein isothiocyanate; PI: propidium iodide.

Figure 4  Results of immunoblotting
A375 cells were treated with D1 (35 µg/mL), D2 (44.80 µg/mL) and D3 (50 µg/mL) doses of *Ruta graveolens* for 48 h. For cytosolic and mitochondrial protein expression analysis, GAPDH and VDAC1 respectively served the purpose of equal loading protein.
VDAC1: voltage-dependent anion-selective channel protein 1; PARP: poly ADP ribose polymerase; mTOR: mechanistic target of rapamycin; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

of mTOR and Bcl-2 occurred, as revealed from immunoblots (Figure 4 and Table 2).

3.1.6  RG-induced cell death involved autophagy
To find out whether RG-induced autophagy was involved in A375 cell death, cell viability was assessed by exposing the cells to both RG and 3-MA (autophagy inhibitor). The cell viability increased to 70.58% upon administration of IC_{50} dose (44.80 µg/mL) of RG in presence of 3-MA, specifying thereby autophagy induction led the cells to death (Figure 7).

3.2  In vivo experimental results
3.2.1  Effect on body weight
The body weight of mice of the group III (DMBA-treated) was significantly decreased as compared to the control ones (group I) (Table 3), whereas RG administration significantly prevented loss of body weight in the DMBA-treated mice.
**Table 2** Relative band intensities of corresponding immunoblots of Figure 4

<table>
<thead>
<tr>
<th>Name of the proteins</th>
<th>Control</th>
<th>D1</th>
<th>D2</th>
<th>D3</th>
</tr>
</thead>
<tbody>
<tr>
<td>VDAC1</td>
<td>100</td>
<td>98.329±0.483</td>
<td>99.488±0.360</td>
<td>102.603±0.275</td>
</tr>
<tr>
<td>Cytochrome c (mitochondrial fraction)</td>
<td>100</td>
<td>37.967±0.097*</td>
<td>34.408±0.122*</td>
<td>27.729±0.057*</td>
</tr>
<tr>
<td>Cytochrome c (cytosolic fraction)</td>
<td>100</td>
<td>110.499±0.575*</td>
<td>115.589±3.459*</td>
<td>143.190±0.895*</td>
</tr>
<tr>
<td>Cleaved PARP</td>
<td>100</td>
<td>129.752±0.602*</td>
<td>172.766±0.970*</td>
<td>177.100±0.309*</td>
</tr>
<tr>
<td>Cleaved caspase 3</td>
<td>100</td>
<td>136.599±0.960*</td>
<td>143.998±0.653*</td>
<td>188.298±0.157*</td>
</tr>
<tr>
<td>BAX</td>
<td>100</td>
<td>158.640±0.265*</td>
<td>178.426±0.787*</td>
<td>214.164±0.251*</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>100</td>
<td>59.932±0.221*</td>
<td>41.879±0.295*</td>
<td>38.956±0.345*</td>
</tr>
<tr>
<td>mTOR</td>
<td>100</td>
<td>78.067±0.668*</td>
<td>73.451±0.036*</td>
<td>52.875±0.310*</td>
</tr>
<tr>
<td>Beclin-1</td>
<td>100</td>
<td>128.316±0.876*</td>
<td>135.266±0.577*</td>
<td>150.618±0.086*</td>
</tr>
<tr>
<td>Atg5</td>
<td>100</td>
<td>135.362±0.796*</td>
<td>156.723±0.671*</td>
<td>170.345±0.443*</td>
</tr>
<tr>
<td>Atg7</td>
<td>100</td>
<td>196.600±0.219*</td>
<td>246.660±0.041*</td>
<td>290.086±0.115*</td>
</tr>
<tr>
<td>GAPDH</td>
<td>100</td>
<td>101.706±0.576</td>
<td>98.878±0.260</td>
<td>99.376±0.264</td>
</tr>
</tbody>
</table>

Band intensity was measured by ImageJ software. Taking the value of untreated control as 100%, percentages of other groups were calculated. Data are expressed as mean ± standard error of mean, n=6; *P<0.05, vs control group.

VDAC1: voltage-dependent anion-selective channel protein 1; PARP: poly ADP ribose polymerase; mTOR: mechanistic target of rapamycin; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

**Figure 5** Fluorescence microscopic study

Cells were treated for 48 h with or without 3-MA (10 mmol/L) in addition of 44.80 µg/mL of RG. Acridine orange (5 mmol/L) was used for cell staining. Photographs were captured with fluorescence microscope (400×). Orange-red fluorescence indicates presence of acidic vesicular organelles. 3-MA: 3-methyladenine; RG: Ruta graveolens.

**Figure 6** Immunoblotting of LC3

A375 cells were treated with RG (44.80 µg/mL) for various time intervals (0–48 h). GAPDH served as the loading control. The densitometic plot represents the expression of LC3-I and LC3-II. Band intensity was measured by ImageJ software. The values are represented as mean ± standard error of mean, n=3; *P<0.05, vs GAPDH.

RG: Ruta graveolens; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.
(group IV). However, in mice treated with only RG (group V), the body weights were not significantly different from the control (group I).

3.2.2 Effect on tumor formation

One-hundred percent tumor formation with mean tumor volume (150.59±0.47 mm$^3$) and tumor burden (412.16±23.87 mm$^3$) was recorded (Table 4) in only DMBA-treated (group IV) mice. The actual pictures showing gross appearance of skin tumors in DMBA- and DMBA+RG-treated mice are presented in Figure 8. Oral administration of RG appeared to lower tumor incidence in DMBA-painted mice (group IV).

3.2.3 Biochemical analysis of skin and liver tissue homogenates

The activity levels of GSH, SOD, CAT, GPx and GST of skin tissues were significantly decreased whereas that of LPO was increased in skin tissues of the group III mice as compared to the control (group I) (Table 5).

The drug administration to DMBA-treated mice (group IV) significantly increased the enzymatic and non-enzymatic antioxidant levels as compared to the DMBA-treated (group III) only. The enzymatic and non-enzymatic antioxidant status of mice receiving RG only (group V) was unchanged as compared to the negative control group (group I).

GSH and GST (phase II detoxification agents) levels were significantly decreased in the liver of only DMBA-treated mice (group III) as compared to the control (Table 5). Oral administration of RG to DMBA-treated mice (group IV) had significantly increased GSH and GST levels. The group V mice treated with RG alone showed no significant difference in phase II detoxification enzyme status as compared to the control (group I).

On the other hand, all activities related to enzymatic

Table 3

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Body weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Initial</td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>24.66±1.05</td>
</tr>
<tr>
<td>Acetone (vehicle)</td>
<td>6</td>
<td>24.83±1.04</td>
</tr>
<tr>
<td>DMBA</td>
<td>6</td>
<td>25.16±0.60</td>
</tr>
<tr>
<td>DMBA + RG</td>
<td>6</td>
<td>25.16±0.54</td>
</tr>
<tr>
<td>RG</td>
<td>6</td>
<td>25.16±0.83</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± standard error of mean. ▲ P<0.05, vs initial body weight.

RG: Ruta graveolens; DMBA: 7,12-dimethylbenz(a)anthracene.

Table 4

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Tumor incidence (%)</th>
<th>Total number of tumors (n)</th>
<th>Tumor volume (mean ± standard error of mean, mm$^3$)</th>
<th>Tumor burden (mean ± standard error of mean, mm$^3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Acetone (vehicle)</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DMBA</td>
<td>6</td>
<td>100% (6/6)</td>
<td>14 (6)</td>
<td>150.59±0.47</td>
<td>412.16±23.87</td>
</tr>
<tr>
<td>DMBA + RG</td>
<td>6</td>
<td>16.6% (1/6)</td>
<td>1 (1)</td>
<td>8.23±0.12</td>
<td>8.23±0.12</td>
</tr>
<tr>
<td>RG</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

▲ P<0.05, vs DMBA group.

RG: Ruta graveolens; DMBA: 7,12-dimethylbenz(a)anthracene.

Figure 7

MTT assay in presence of 3-MA

Figure 8

Captured photograph of appearance of skin of group III and group IV mice

DMBA: 7,12-dimethylbenz(a)anthracene; RG: Ruta graveolens.
and non-enzymatic activities remained unchanged in the acetone-painted group II (vehicle control). The same was true for body weights in this group as compared to the control.

3.2.4 Effect of RG on expression of constitutive and activated Akt

Significant down-regulation of expression of both constitutive and active forms of Akt was noted after RG administration to DMBA-painted mice (group IV) as compared to the DMBA-treated group III (Figure 9).

Relative band intensities of the immunoblots are shown in Table 6.

4 Discussion

For effective control and management of the deadly disease cancer, chemopreventive drugs, particularly derived from the plant kingdom, are being harnessed throughout the world, and some amount of success has also been achieved with systematic research. Results of the present

Table 5 Status of enzymatic and non-enzymatic antioxidants in skin and liver tissues

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>GSH (mg/mL)</th>
<th>SOD (μmol/(L·min·mg))</th>
<th>CAT (μmol/(L·min·mg))</th>
<th>LPO (μmol/(L·min·mg))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Acetone</td>
<td>6</td>
<td>99.293±0.490</td>
<td>100.730±0.249</td>
<td>100.447±0.573</td>
<td>92.920±0.135</td>
</tr>
<tr>
<td>DMBA</td>
<td>6</td>
<td>52.966±0.740</td>
<td>60.330±0.460</td>
<td>53.395±0.984</td>
<td>161.371±0.470</td>
</tr>
<tr>
<td>DMBA+RG</td>
<td>6</td>
<td>81.355±0.556</td>
<td>77.092±0.708</td>
<td>73.706±0.697</td>
<td>125.093±0.144</td>
</tr>
<tr>
<td>RG</td>
<td>6</td>
<td>100.423±0.441</td>
<td>98.030±0.351</td>
<td>91.493±0.388</td>
<td>105.790±0.068</td>
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<td>105.790±0.068</td>
</tr>
</tbody>
</table>

Table 6 Relative band intensities of corresponding immunoblots of Figure 9

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Akt</th>
<th>pAkt</th>
<th>GAPDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Acetone</td>
<td>6</td>
<td>100.927±0.337</td>
<td>98.960±0.764</td>
<td>99.443±0.515</td>
</tr>
<tr>
<td>DMBA</td>
<td>6</td>
<td>252.145±0.638*</td>
<td>151.990±0.241*</td>
<td>98.831±0.596</td>
</tr>
<tr>
<td>DMBA+RG</td>
<td>6</td>
<td>122.305±0.019a</td>
<td>110.331±0.766a</td>
<td>100.008±0.898</td>
</tr>
<tr>
<td>RG</td>
<td>6</td>
<td>100.870±0.145</td>
<td>98.759±0.807</td>
<td>99.419±0.485</td>
</tr>
</tbody>
</table>

Taking the value of untreated control as 100%, percentages of other groups were calculated. Values are expressed as mean ± standard error of mean, n=6; *P<0.05, vs control group; aP<0.05, vs DMBA group.

RG: Ruta graveolens; DMBA: 7,12-dimethylbenz(a)anthracene; GSH: reduced glutathione; SOD: superoxide dismutase; CAT: catalase; LPO: lipid peroxidase; GPx: glutathione peroxidase; GST: glutathione S-transferase.
study reveals that RG was able to cause cell death of skin melanoma (A375) cells in vitro through induction of apoptosis and autophagy to a significant extent and also plays a protective role through modulation of enzymatic and non-enzymatic antioxidants against skin papilloma formation in mice in vivo.

Present research directions are mostly aimed at innovative targets at the molecular level, so that more specific drugs capable of hitting these targets can be developed. In view of the characteristic immortality attained by cancer cells, one major target of drug discovery involves the ability of the drug to annihilate cancer cells through induction of programmed cell death like apoptosis or autophagy. The findings of the present study are encouraging as RG demonstrated its profound ability of killing cancer cells by inducing apoptosis or and by triggering molecular mechanism of autophagy. The ability of a drug to generate cytotoxicity in cancer cells, but not much in the normal cells is an important criterion in control and management of cancer. Most orthodox drugs lack in this property. Most of these drugs can effectively kill cancer cells by producing cytotoxicity, but the normal cells are not spared in this process, precluding their use to a large extent. Therefore, the results showing considerable ability of RG to produce preferential cytotoxicity in cancer cells over normal cells are significant findings.

Most anticancer drugs act through induction of apoptosis/necrosis in cancer cells as a major means. However, many recent report suggest autophagy to have role in cell killing as well[23,24]; although autophagy is generally known as a cell-survival mechanism induced by stresses. Therefore, drugs having both apoptotic and autophagic effects are of added advantage, because even after apoptosis is blocked by cancer cells, autophagy can play the cell death process. Therefore, in recent years molecular mechanisms of induction of autophagy through autolysosomes (forms of AVOs) pathway have received considerable attention[25–25]. In this investigation, existence of AVOs was confirmed from the result of fluorescence microscopic study with AO stain. Presence of cellular autophagosome punctae containing LC3-II is another confirmation of activation of autophagy[26]. In this study, conversion of LC3-I to LC3-II in RG-exposed cells was confirmed from the immunoblot study. Result of the present study showed upon exposure to RG, expression of mTOR, which prevents induction of autophagy[27], was down-regulated. Up-regulated expression of Atg5 and Atg7, two crucial molecules of the autophagic vacuole[27], was revealed from immunoblot assay. Findings in this study revealed down-regulated expression of Beclin-2 that inhibits both apoptosis and autophagy[28] in RG-treated cells. Becl-1 plays a crucial role in the process of autophagy induction[29]. Up-regulation of Beclin-1 expression was found in RG-exposed cells. All these outcomes collectively confirmed that there was induction of autophagy in RG-exposed A375 cells.

To find out whether this RG-mediated autophagy induction was to conduct cell death, cell viability was assessed after treating cells with RG and 3-MA, an autophagy inhibitor. A noticeable increase in viability was evident from the result which confirmed that RG-induced autophagy led the cells to the cell death pathway.

The cancer cells develop the property to evade apoptotic signals or signals that can trigger autophagy. On the other hand, they tend to show hyperactivity in response to all signals that promote cell divisional activities, causing them to divide and re-divide relentlessly propagating thereby a rapid growth. Thus agents that can target molecular mechanism leading to blockage of divisional activity, or antagonize all kinds of growth-promoting signals, or promote apoptotic signals, have considerable role to play in formulation of anticancer drugs. Apoptosis is mainly characterized by depolarized MMP, externalization of phosphatidyl serine (PS) from mitochondrial inner membrane to outer membrane, release of cytochrome c from mitochondria and massive caspase activation[30]. BAX, a Bcl-2 family protein, once activated by upstream pro-apoptotic signals[31], relocates from the cytosol to the surface of mitochondria and forms pores in the mitochondrial outer membrane[32]. This causes terminal release of pro-apoptotic factors, particularly cytochrome c[33], from the mitochondrial inner-membrane space. Released cytochrome c in cytosol now activates the caspase cascade and the activated caspase 3 in turn cleaves PARP, as a result of which fragmentation of DNA and ultimately induction of apoptosis ensues. In the present study, flow cytometry assay with rhodamine 123 staining indicated depolarization of MMP, and results of annexin V/PI dual stain assay confirmed the externalization of PS in RG-exposed cells. Immunoblot assay suggested BAX activation, cytochrome c release and activation of caspase 3. Increased expression of cleaved PARP (85 kD) was also found in RG-treated A375 cells. All these findings collectively suggest that RG induces mitochondria-dependent caspase 3-mediated apoptosis in A375 cells.

To test efficacy of RG in a mammalian model in vivo, skin cancer was induced in mice (Mus musculus). RG administration alone (75 mg/kg body weight) did not show any acute or chronic toxicity in normal mice, but showed significant reduction in the skin tumor burden in DMBA-painted mice. While 100% tumor incidence was noted in RG-untreated DMBA-painted mice, much less cancer burden was observed when they were also administered with RG. Our results thus suggest that RG had the ability to actively interfere with skin cell proliferation induced by DMBA.

Akt, an important regulator of proliferation and differ-
entiation\textsuperscript{34}, is thought to be involved in DMBA-induced skin cancer. Results of the present study showed Akt expression was modulated upon RG administration. RG treatment down-regulated both constitutive and activated (pAkt) forms of Akt as compared to the DMBA treatment alone, indicative of its potential for use in anticancer drug formulation.

Liver is an organ where detoxification process takes place, but many orthodox chemotherapeutic drugs are known to deposit their byproducts in liver, resulting in further toxicity. Thus a critical study of toxicity biomarkers in liver was considered important for determining their status during the process of carcinogenesis. In this study therefore the multiple biochemical mechanisms including phase-II detoxification enzyme induction and antioxidant activities\textsuperscript{35,36} were considered. Studies reported earlier that chemopreventive agents convert DNA damaging entities through the induction of detoxification agents such as GST\textsuperscript{37}. In this study, enzyme activities of GSH and GST were significantly decreased in the liver of tumor-bearing animals (group III), but improved considerably upon RG administration in DMBA-painted animals (group IV) as compared to the control.

Over production of reactive oxygen species in the cells is one of the major reasons for DNA damage that is also implicated in carcinogenesis. Decreased activities of enzymatic antioxidants and drop in non-enzymatic antioxidant levels were well documented in skin cancer\textsuperscript{38}. In this study it was revealed that RG administration at a dose of 75 mg/kg body weight to DMBA-painted mice significantly improved the status of lipid peroxidation and antioxidants, further confirming its anticancer role.

5 Conclusion

Overall results would suggest that RG is a strong candidate for being used in formulation of anticancer drugs, particularly against skin cancer.

6 Acknowledgements

The authors are sincerely thankful to Boiron Laboratories, Lyon, France for financially (partial) supporting the work.

7 Competing interests

The authors declare that they have no competing interests.

REFERENCES

16 Bradford MM. A rapid and sensitive method for the quantitation


