Ruta graveolens Extract Induces DNA Damage Pathways and Blocks Akt Activation to Inhibit Cancer Cell Proliferation and Survival

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Abstract. *Background: Ruta graveolens is a medicinal herb that has been used for centuries against various ailments. This study examined the anticancer properties of the herb using cancer cell lines. Materials and Methods: Methanolic extract of R. graveolens was tested on colon, breast and prostate cancer cells. Viability, cell cycle profiles, clonogenicity and capase activation were measured. Induction and subcellular localizations of p53, 53BP1 and γ-H2AX proteins were examined. Results: The extract dose-dependently decreased the viability and the clonogenicity of treated cells and induced G_2/M arrest, aberrant mitoses, and caspase-3 activation. It also induced the p53 pathway and focal concentration of the DNA damage response proteins 53BP1 and γ-H2AX. Moreover, the levels of phospho-Akt and cyclin B1 were reduced by treatment, whereas only cyclin B1 was reduced in normal dermal fibroblasts. Conclusion: R. graveolens extract contains bioactive compounds which, independently of known photoactivatable mechanisms, potently inhibit cancer cell proliferation and survival through multiple targets.*

Plants are important sources of medicinal compounds worldwide. A few examples are: aspirin from willow tree, digitalis from foxglove, artemisinin from wormwood, taxol from the pacific yew tree, vinblastine and vincristine from periwinkle, etoposide from mayapple, *etc*. More than 60% of cancer therapeutics on the market or in pre-clinical trials are based on natural products (1, 2). Despite the decline of interest by the pharmaceutical industry in research and development of natural compounds, these unrefined compounds from terrestrial and aquatic sources continue to serve as the chemical foundations from which modified or synthetic versions can be derived.

Ruta graveolens is a medicinal and culinary plant that is native to the Mediterranean region of southern Europe and northern Africa. Widely grown in different parts of the world, this herb has historically been in use since the ancient times (3). Its documented therapeutic uses include the treatment of inflammatory conditions, eczema, ulcers, arthritis, fibromyalgia, antidote for venoms, insect repellent, and as an abortifacient (4-6). The plant has also been commonly used to season some food items such as soup, cheese, butter, coffee, and tea, and in medicinal preparations such as rue oil and infusions that are used as antispasmodics and emmenagogues (7).

Chemical compounds so far known to be present in *R. graveolens* include furanocumarins, carotenoids, chlorophyll, and furanoquinolones (4, 8). Psoralens, a family of furanocumarins in *R. graveolens*, have been widely studied for their DNA interstrand cross-linking activity when exposed to ultra violet (UV) light. This photoactivation property of psoralens has been utilized in the treatment of various skin malignancies including psoriasis, vitiligo and cutaneous lymphoma as a psoralen plus UV-A or UV-B (PUVA or PUVB) regimen (9-11).

Although some bioactive components in the herbal extracts and infusions made from *R. graveolens* have been suggested to be responsible for the plant's beneficial or phototoxic effects, molecular studies that extensively decipher the activities of most of its bioactive ingredients are scarce. While the phytophototoxicity caused by *R. graveolens* has been known for a long time, the bioactivities of *R. graveolens* extracts and its various preparations against tumor cells or pathogenic microbes have attracted attention only recently (12-17).

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This study examined the potency of an extract from *R. graveolens* on cancer cell lines. This study shows that this extract has potent anticancer activity, exhibited through strong anti-proliferative and anti-survival effects on cancer cells.

Materials and Methods

Cell culture and treatments. The human colorectal cancer cell line HCT116 was a generous donation from Dr. Bert Vogelstein (Johns Hopkins University, MD, USA). The cell line was maintained in McCoy's medium (Lonza, Walkersville, MD, USA) supplemented with 10% fetal bovine serum (FBS) and 10,000 U/ml penicillin/10mg/ml streptomycin. The MCF7 cell line was a gift from Dr. Leslie Wilson (University of California at Santa Barbara, CA, USA). RKO (purchased from ATCC, Manassas, VA, USA) and MCF7 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen Corp, Carlsbad, CA, USA) supplemented with FBS and penicillin/streptomycin. Prostate cancer cell lines PC3 and DU-145 cells, purchased from ATCC were cultured in T-medium (Invitrogen Corp, Carlsbad, CA, USA), supplemented with 10% FBS and penicillin/streptomycin. For experimental treatments, cells were seeded in dishes with 96 wells (for viability assays), 12 wells (for colony formation assays), 6 wells (for flow cytometry and clonogenicity assays), 6 cm culture plates (for cell lysate preparations), or 4-well chamber slides (for immunofluorescence staining). All cell cultures were incubated at 37˚C and 5% CO_2 in a humidified incubator.

Extract preparation. Fresh leaves of *R. graveolens* were minced finely in a food processor and extracted in 80% methanol for 24 hours. Particulate matters was removed by two rounds of centrifugations at 1000× *g* and 10,000× *g* for 5 and 10 minutes, respectively. The soluble fraction was desiccated in a rotary evaporator, and the evaporated solids were resuspended in DMSO at a final concentration of 60 mg/ml.

Antibodies. Anti-p53, -β-actin, -phospho-γ-H2AX ser139, -53BP1, -Akt, -phospho-Akt, -cyclin B1 and -CDK1 antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-$p21^{WAF1}$ was purchased from Invitrogen Corp.). Monoclonal antibody to α-tubulin (clone DM1A) was purchased from Sigma (St Louis, MO, USA).

Clonogenicity assays. Clonogenicity assay was performed as described elsewhere (18). The clonogenic potential of untreated and treated cells was determined by seeding approximately 150 cells per well of a 6-well dish. Cells were allowed to adhere for approximately 24 hours and then treated with varying concentrations (0-300 μg/ml) of the *R. graveolens* extract in culture medium. Colony formation was examined daily by light microscopy. The assay was terminated by fixing the cells when the control treated cells formed visible discrete colonies. Formed colonies were stained with 10% crystal violet in methanol for 15-20 minutes, washed to remove excess dye, and air-dried. Colonies were counted using AlphaImager (AlphaInnotech, Santa Clara, CA, USA) in colony counting mode. The relative clonogenicity of the treated cells was computed as percentage of the number of colonies that formed in the control DMSO treated wells.

Flow cytometry. Cells were harvested and prepared for flow cytometry as described elsewhere with some modifications (19). Cells were harvested by trypsinization using 0.25% trypsin-EDTA (Invitrogen Corp.) and centrifuged. Pellets were resuspended in 300 μl phosphate-buffered saline (PBS; Invitrogen Corp.), fixed by addition of 700 μl 100% ethanol while vortexing, and stored at –20˚C for a minimum of 12 hours. Fixed cells were centrifuged, and stained in FACS staining solution (320 mg/ml RNase A, 0.4 mg/ml propidium iodide) in PBS without calcium and magnesium for 15 minutes at 37˚C. Stained cells were filtered through a 70 μm pore size filter and analyzed by flow cytometry on C6 Accuri® flow cytometer (Accuri Cytometers, Ann Arbor, MI, USA). Data was analyzed and histograms were prepared using CFlow™ software (Accuri Cytometers).

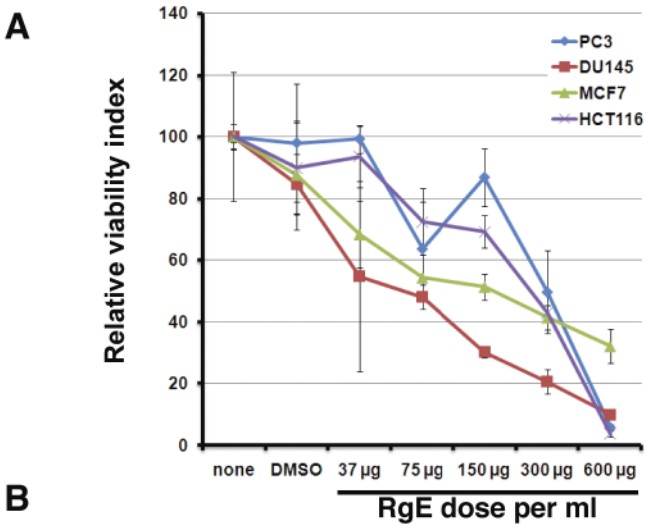
Immunofluorescent staining and microscopy. Phase contrast images of cells were taken at ×20 and ×40 magnification objectives (and ×10 eyepiece) using an Olympus I×71 inverted microscope (Olympus America Inc, Melville, NY, USA) fitted with digital image capture cameras (Digital Microscopy Lab, College of Veterinary Medicine, Nursing and Allied Health, Tuskegee University). HCT116 cells for immunofluorescent staining were grown in 4-well chamber slides. Staining was performed as described previously (19). Confocal images were taken using an Olympus DSU spinning disk confocal microscope (Olympus America Inc) at the Research Center at Minority Institutions (RCMI) core-facility using a ×40 dry objective. Images were captured using Metamorph Premium software (Strategic Triangle Inc, Toronto, Canada), stored in TIFF format and further processed in Adobe Photoshop.

Immunoblotting. Cell lysates were prepared in NP-40 lysis buffer (20 mM Tris-Cl pH 7.5, 150 mM NaCl, 10% glycerol and 0.2% NP-40 plus protease inhibitor cocktail) and protein concentrations were determined using DC detergent compatible protein assay (BioRad Laboratories Inc, Hercules, CA, USA). Samples containing equivalent protein concentrations were mixed with Laemmli buffer, and boiled for 5 minutes. Proteins were resolved by SDS-PAGE, transferred to PVDF membranes (GE Healthcare Life Sciences, Piscataway, NJ, USA) and blocked in 5% non-fat dry milk. Primary antibodies were used at 1:1000 dilutions. Peroxidase-conjugated anti-rabbit and anti-mouse IgG secondary antibodies were purchased from GE Healthcare Life Sciences and used at a 1:5000 dilution. Chemiluminescent detections were performed using Chemiluminescent HRP Substrate (GE Healthcare Life Sciences).

Caspase-3 activation assay. The assay was performed using a colorimetric caspase 3 activation assay kit (caspase-3-C; Sigma). Briefly, treated and control cells were lysed in 1× lysis buffer. Thirty micrograms of total protein were used to perform the assay according to the manufacturer's recommendations. Recombinant caspase 3 was added to untreated (control) cell lysates and used as a positive control for the assay. A separate set of cells were incubated with 1 μM staurosporine for 3 hours to activate endogenous Caspase 3 as another control. Absorbance was read in a kinetic mode every 1.5 minutes at 405 nm using PowerWave XS plate reader and Gen5 software (Biotek Instruments Inc, Winooski, VT, USA).

Results

Dose-dependent inhibition of cell viability by R. graveolens extract. To determine the bioactivity of a methanolic extract of *R. graveolens* against cancer cells, HCT116 (colorectal cancer), MCF7 (breast cancer), DU-145 and PC3 (both prostate cancer) cancer cells were treated with varying



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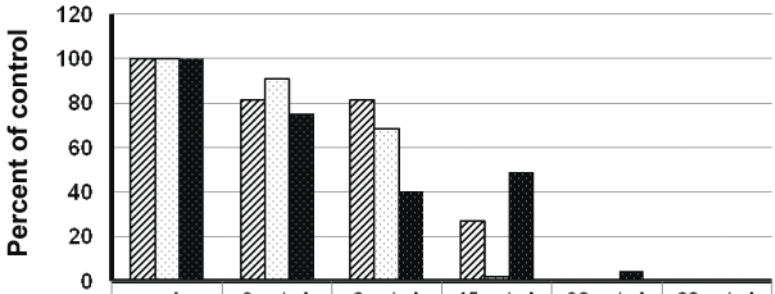

	carrier	3µg/ml	6µg/ml	15µg/ml	30µg/ml	60µg/ml
▨HCT116	100	81.6	81.6	27.2	1.0	0
□RKO	100	91.1	68.7	2.0	0.3	0.8
■DU145	100	75.5	40.6	49.1	4.7	0.9

Figure 1. *A: PC3 (prostate), DU-145 (prostate), HCT116 (colorectal) and MCF7 (breast) cancer cells were exposed to varying concentrations of R. graveolens extract. Viability of control (untreated or DMSO) or treated (R. graveolens extract 37 µg/ml to 600 µg/ml) cells was assessed after 24 hours (MTS cell viability assay, Promega Corp., Madison, WI, USA) Absorbance was read at 490 nm using PowerWave XS (BioTek, Winooski, VT, USA) microplate reader. B: Clonogenicity assay in HCT116, RKO and DU-145 cells treated with R. graveolens extract. The cancer cell lines were treated with the indicated concentrations (µg/ml culture medium) of R. graveolens extract, and their clonogenicities were assessed as described in the Materials and Methods.*

concentrations of the extract and the viability of treated *versus* control cells examined by MTS assay after 24 hours.

As shown in Figure 1A, *R. graveolens* extract reduced the viability of treated cancer cells in a dose-dependent manner. Based on the cell viability measured after only 24 hours, the approximate 50% inhibitory concentration (IC_{50}) of *R. graveolens* extract was approximately 75 µg/ml (PC3 cells), 150 µg/ml (MCF7), 200 µg/ml (HCT116), 300 µg/ml (DU-145 cells), with PC3 cells being the most sensitive. However, as described below, colony formation by the cell lines was completely inhibited by even smaller doses of the extract, suggesting even lower effective growth inhibitory concentration of the extract.

R. graveolens extract inhibits the proliferation of cancer cells. Unlike normal cells, cancer cells have the capacity to proliferate and form cell colonies even when they are seeded as individual cells after being separated from other cancer or stromal cells. It was assessed by colony formation assay whether *R. graveolens* extract would inhibit the ability of HCT116, RKO or DU-145 cancer cells to establish cell colonies from individual cells. As shown in Figure 1B, the colony-forming ability of all tested cancer cells was inhibited by *R. graveolens* extract. Colony formation was nearly 100% inhibited in all cell lines at the 60 µg/ml dose. The inability of the cells to form colonies indicates the presence of very potent anti-proliferative compounds in the extract.

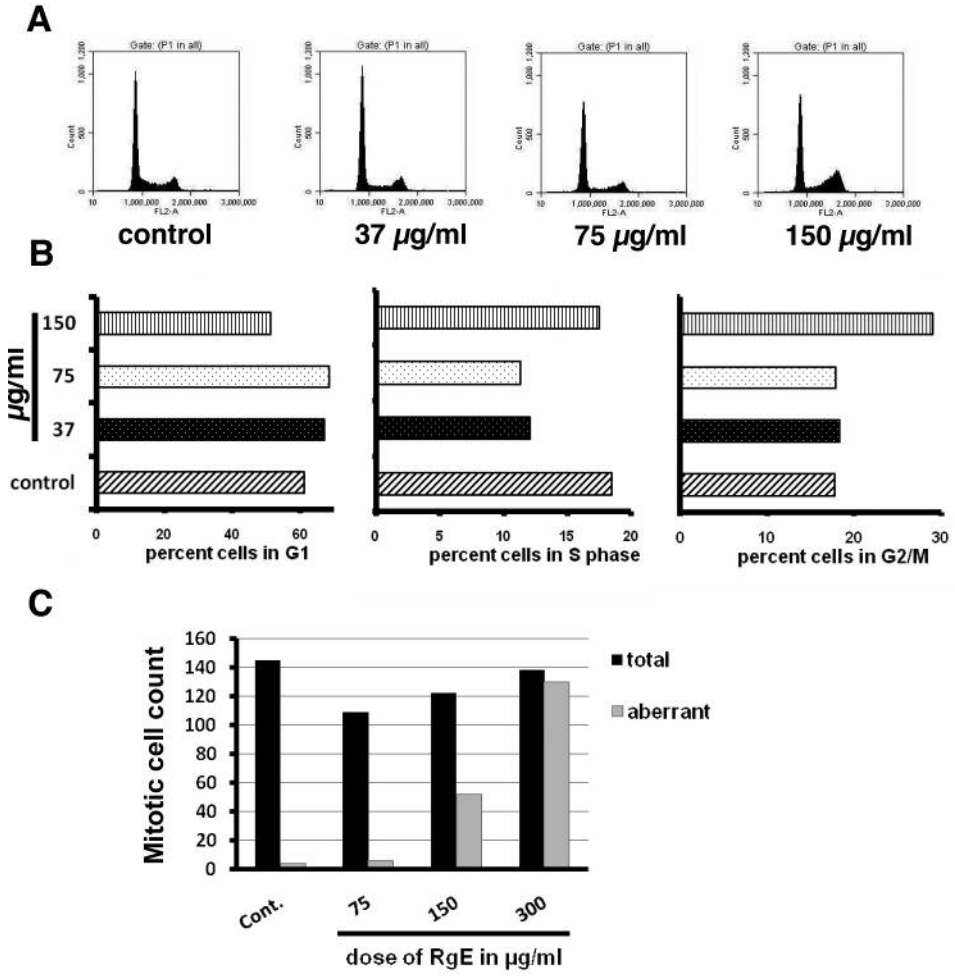

Figure 2. *Cell cycle arrest and aberrant cell division phenotypes induced by R. graveolens extract. A and B: Cell cycle arrest in MCF7 cells. C: Bar graph showing the proportion of aberrant mitoses in control and treated HCT116 cells.*

Cell cycle arrest is one of the mechanisms by which cell proliferation is blocked. Stressed cells, which possess functional cell cycle checkpoints, activate cell cycle arrest, thus allowing the cells to recover from the stress before the cell division. To examine the effect of *R. graveolens* extract on the cell cycle, MCF7 breast cancer cells were treated with vehicle (control), 37 μg/ml, 75 μg/ml, or 150 μg/ml of extract for 24 hours and the cell cycle profile of the cells was analyzed by flow cytometry. As shown in Figure 2A and B, cells treated with *R. graveolens* extract arrested mostly in the late S to G_2/M phases of the cell cycle. HCT116 and DU-145 cells also showed similar cell cycle arrest (data not shown). In addition to the cell cycle arrest, it was also observed that cells treated with *R. graveolens* extract displayed increased number of aberrant mitoses and post-mitotic nuclear integrity, particularly when exposed to higher doses of the extract. The proportion of observed aberrant mitoses for HCT116 cells is shown in Figure 2C. Similarly, MCF7 and other treated cells also showed a higher rate of aberrant mitosis at similar doses. These aberrant phenotypes included tripolar or multipolar spindles, short or disarrayed spindle fibers, lagging chromosomes at metaphase-anaphase transition and micronuclei formation. These data suggests cell cycle arrest and disruption of mitosis are two of the mechanisms by which compounds in *R. graveolens* extract inhibit cell proliferation.

R. graveolens extract activates the p53 pathway. Various cellular stressors induce the stabilization and activation of the p53 tumor suppressor protein. The p53 protein regulates critical cellular mechanisms that modulate the cell cycle, apoptosis, senescence, and various signaling pathways. Therefore, it was examined whether the p53 protein is activated after the treatment of cells with *R. graveolens* extract. HCT116 colorectal cancer cells, which have an intact

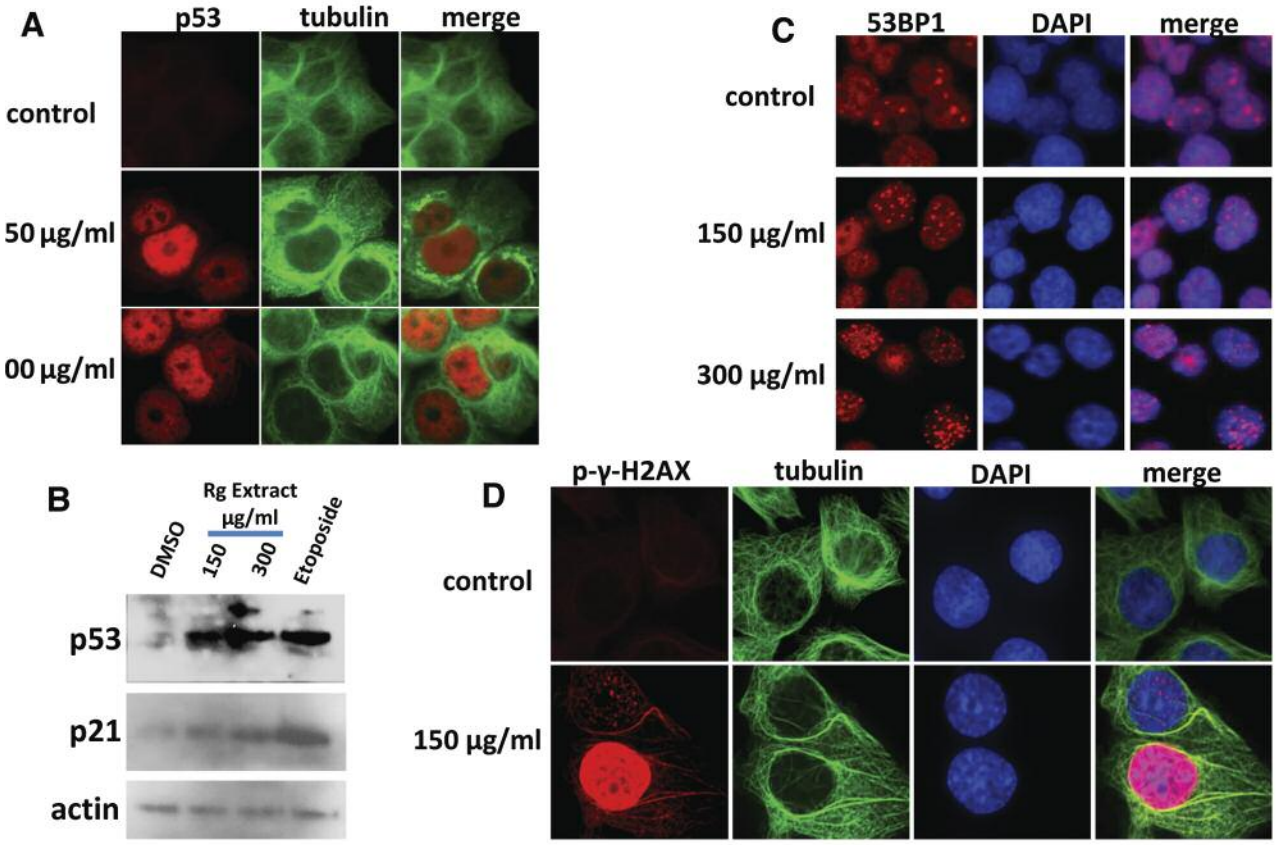

Figure 3. *A: Induction of the p53 tumor suppressor protein by R. graveolens extract. HCT116 cells were treated with carrier control (DMSO) or the indicated concentrations of the R. graveolens extract in culture medium for 24 hours. Nuclear p53 was visualized by immunofluorescent staining using anti-p53 antibodies and Alexa-fluor conjugated secondary antibodies (red). Cells were counterstained with anti-tubulin antibody (green). The far right panels show merged red and green images. B: Immunoblotting to show the induction of p53 protein and its transcriptional target p21. Etoposide was used as a positive control for p53 the induction in HCT116 cells. C: Induction of DNA damage response (DDR) foci in R. graveolens-treated cells. HCT116 cells were exposed to control (carrier DMSO) or R. graveolens extract for 24 hours and the nuclear distribution of 53BP1 protein was analyzed by immunofluorescent staining using anti-53BP1 antibodies and Alexa-fluor conjugated anti rabbit secondary antibodies (red). Nuclei were counterstained using DAPI. The far right panels show merged red and blue images. D: Induction of γ-H2AX phosphorylation by R. graveolens extract. HCT116 cells were treated as shown and the nuclear distribution of 53BP1 protein was examined by immunofluorescent staining.*

p53 pathway, were treated with the extract and the induction of p53 was examined by immunofluorescence staining. As shown in Figures 3A and B, exposure of HCT116 cells to *R. graveolens* extract, but not the vehicle DMSO, led to the accumulation of nuclear p53. The induction of *p53* was also accompanied by the induction of the cell cycle regulator CDK inhibitor protein p21 (Figure 3B), indicating the activation of p53-mediated transcription. This transcriptional activity suggests that compounds in *R. graveolens* extract activate p53-regulated pathways, which include cell cycle arrest that can be mediated by the p21 protein.

R. graveolens extract induces the DNA-damage response pathway. The tumor suppressor protein p53 can be activated by signals originating from activated DNA damage response (DDR) (20, 21). ATM, Chk1, Chk2, p53, 53BP1, MRN complex and other proteins are components of the DDR signaling network. 53BP1 is a large (450 kDa) protein that was initially identified by its ability to bind to p53. Subsequent studies have shown that 53BP1 protein is part of the DDR protein complex, and co-localizes with the DNA repair complex proteins such as Mre11-Rad50-NBS1 (MRN) at distinct nuclear foci (22-26).

To examine if the activation of p53 pathway was the result of DDR signaling, the nuclear distributions of 53BP1 and γ-H2AX proteins were analyzed by immunofluorescence staining of control or *R. graveolens* treated HCT116 cells 24 hours after exposure to the extract. As shown in Figure 3C and D, treatment with *R. graveolens* extract induced the activation and the recruitment of 53BP1 and γ-H2AX proteins

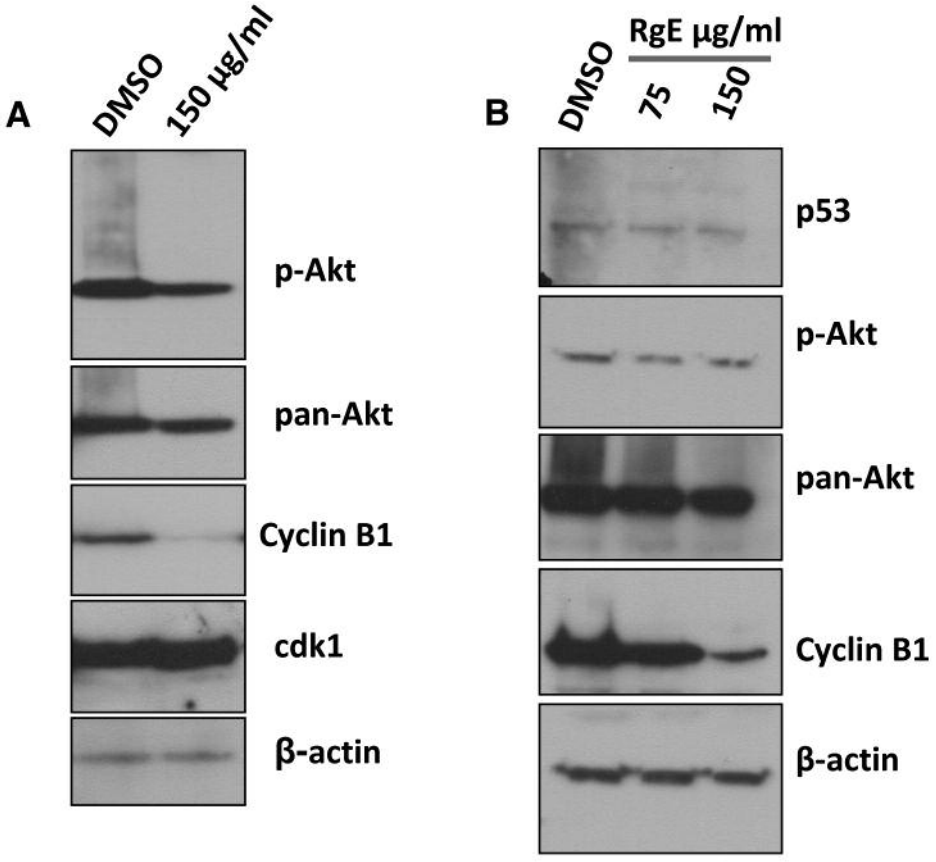

Figure 4. *A: R. graveolens extract suppresses Akt activation and cyclin-B1 levels. DU145 prostate cancer cells were treated with carrier only or 150 µg/ml R. graveolens extract for 48 hours. The expression of the indicated proteins was analyzed by immunoblotting. B: Normal human dermal fibroblasts were treated with the indicated concentrations of R. graveolens extract for 48 hours. The panels indicate the expression of the indicated proteins as assessed by immunoblotting.*

to distinct nuclear foci, typically evident in cellular response to DNA-damage. The re-localization of both 53BP1 and γ-H2AX proteins into sub-nuclear foci indicates that compounds in *R. graveolens* have the capacity to induce DNA damage foci, to which damage-repair proteins are recruited.

The Akt/PKB protein is a pro-survival signaling molecule constitutively activated in a number of solid tumors, and it cross-talks with the p53 pathway (27). To examine if *R. graveolens* extract modulates this critical signaling pathway, the phosphorylation of the Akt protein as a marker of Akt activation in DU145 prostate cancer cells was examined. As shown in Figure 4A, treatment of the cells with *R. graveolens* extract reduced the levels of phospho-Akt, which suggests that the activity of the Akt signaling pathway may be inhibited by the extract. In contrast to the phosphorylated Akt, the total cellular levels of the Akt protein were not affected by the treatment. Additionally, the level of the mitotic cyclin B1 was significantly reduced by *R. graveolens* extract, further indicating the inhibitory effect of the extract on cell cycle transition. To examine the effect of *R. graveolens* extract on untransformed cells, normal human dermal fibroblasts were treated with vehicle or the extract. Interestingly, in contrast to cancer cell lines, no major changes were observed in the levels of phospho-Akt and p53 in fibroblasts exposed to up to 150µg/ml of the extract (Figure 4B). However, the levels of cyclin B1 were markedly reduced in treated fibroblasts, similarly to the cancer cells. Doses of the extract were also higher than 150 µg/ml non-specifically cytotoxic to the fibroblasts.

R. graveolens extract induces caspase activation. During the experiments, it was observed that reduction in cell viability or induction of cell death was associated with an increased number of cells with membrane blebbing, which is a characteristic microscopic phenotype of cells undergoing apoptosis. This was especially evident in cells exposed to *R. graveolens* extract at doses between 150 µg/ml to 300 µg/ml for 24 hours. Figure 5A shows the microscopic appearance of HCT116 cells treated with DMSO (control) or 300 µg/ml *R. graveolens* extract. To examine if the membrane blebbing phenotype was also

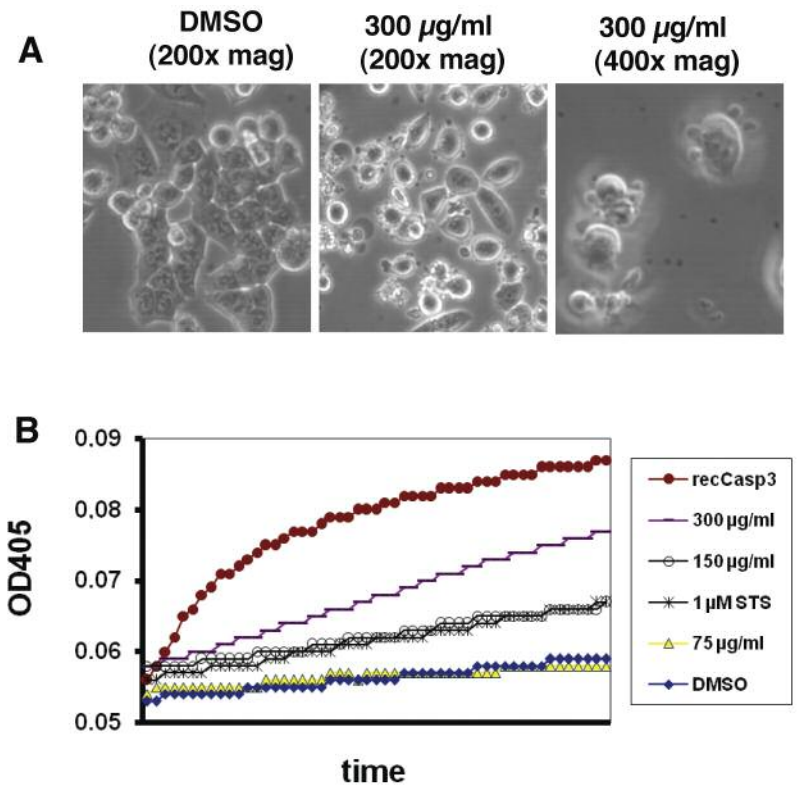

Figure 5. *Activation of caspase 3 and induction of apoptosis by R. graveolens extract. A: HCT116 cells were treated for 24 hours with the carrier DMSO or 300 µg/ml R. graveolens extract in culture medium. Phase-contrast micrography of treated cells were taken at ×200 or ×400 magnifications. B: HCT116 cells were treated with carrier DMSO, R. graveolens extract (75 µg/ml, 150 µg/ml, or 300 µg/ml), or staurosporine (STS, 1 µM, 3 hours). Caspase-3 activation assay was performed as described in the Materials and Methods. Recombinant caspase-3 (recCasp3) was added to untreated cell extract and assayed in parallel, as an assay control. Caspase activity kinetics was recorded as absorbance at 405 nm.*

associated with the activation of intracellular caspases, the activity of caspase-3 in the lysates of HCT116 cells treated with DMSO, or 75 µg/ml, 150 µg/ml, or 300 µg/ml *R. graveolens* extract was assayed. In parallel, HCT116 cells were treated with 1 µM staurosporine for 3 hours as a positive control to induce endogenous caspase 3. Recombinant caspase-3 was used as an assay control. As shown in Figure 5B, the activity of caspase-3 in the lysates of cells treated with 150-300 µg/ml *R. graveolens* extract was markedly enhanced compared to control cells. This result confirmed that the membrane blebbing phenotype observed in cells treated with *R. graveolens* extract was coincidental to the execution of apoptosis, and the induction of apoptotic phenotype correlated with enhanced intracellular caspase activity.

Discussion

This study showed that the medicinal and culinary herb *Ruta graveolens* contains bioactive compounds that inhibit cell proliferation, reduce cell viability, and induce DNA damage response and apoptosis. This is particularly interesting since this herb has been used for centuries as a spicing ingredient for food and to treat various illnesses. *R. graveolens* preparations can be readily commercially obtained as infusions and oils for medicinal use.

These data suggest that *R. graveolens* extract contains candidate compounds that deserve not only to be further evaluated for their mechanisms of action, but for their chemotherapeutic or adjuvant therapy potentials as well. Importantly, treatment with the extract induced nuclear foci formation that is characteristic of the typical DNA-damaging agents used in cancer therapy, such as irradiation and platinum compounds. Moreover, even the smaller doses that were tested still inhibited cell proliferation in colony formation assay, suggesting the presence of long-acting components that inhibit cancer cell proliferation at low doses of the extract. At such low doses, aberrant mitoses were not significantly evident, suggesting a potentially different mechanism of activity at low doses compared to high doses, where DNA damage and aberrant mitoses were evident. Therefore, investigating the therapeutic (high dose) or chemopreventive (low dose) potentials of the extract and its

components is warranted. It is currently unknown, however, if the same pathways involved in DDR are also responsible for the anti-proliferative activity of the extract observed at lower doses. It is possible that other survival pathways may be inhibited at lower doses without inducing a strong DDR. The components that inhibit the long-term survival of treated cancer cells need to be examined in detail, since low doses of *R. graveolens* compounds may be beneficial as chemopreventive agents in high-risk cancer patients.

The induction of DDR by *R. graveolens* extract raises a question about the role of DDR as a checkpoint in the current context, i.e. if compounds in *R. graveolens* would be suitable for chemoprevention, or as sensitizer to chemotherapy. Activated DDR serves as a checkpoint to allow damaged cells to repair their DNA, or to induce apoptosis or senescence, as a primary line of defense against neoplastic transformation of cells (28). Although initiation of DDR is suggested to be a checkpoint to prevent carcinogenesis, persistent DDR may have undesirable outcomes through chronic inflammatory signaling (29) that may in turn promote carcinogenic transformation. To address these issues, it is necessary to identify the bioactive components in *R. graveolens* extract in order to delineate those with DDR-inducing activities, and those with potential effects on other survival signaling pathways. On the other hand, the DDR-inducing property of the *R. graveolens* extract might be expected to sensitize cancer cells to chemotherapeutic drugs that target survival-signaling pathways. Recently, the inhibition of CYP3A by compounds in grapefruits was used to reduce the therapeutic dose of the anticancer drug rapamycin (30, 31), illustrating the potential use of natural compounds as adjuvants or supplements to conventional therapeutic drugs. On the other hand, the DDR-inducing property of *R. graveolens* extract should also raise caution about the potential risks of prolonged use of high doses of medicinal preparations from the herb.

Ruta species and many other plants such as grapefruit, parsley, celery, and parsnip contain a group of phytochemicals called furanocoumarins (furocoumarins) that have been associated with phytophotodermatitis (4, 8, 32), or the grapefruit juice effect (33, 34). The phototoxic effects of furanocoumarin compounds have been associated with topical application of products containing the compounds followed by exposure to sources of UV light (4, 5, 35). However, photoactivation-independent bioactivities of these compounds, including p53 induction and *in vivo* CYP450 inhibition, have been reported (14, 36). Therefore, further studies are needed to identify the individual molecules, the molecular targets for each of the bioactive compounds in *R. graveolens*, and to suggest the rational conditions under which the plant products or the isolated compounds might be used beneficially. Since treated cells were not exposed to known sources of UV light in this study, it is proposed that the DDR and anti-proliferative effects that are reported here are independent of the UV-inducible type of photophytotoxicity. A similar result has been reported for bergapten, one of the photoactivatable furanocoumarins, where the compound induced the p53 pathway and apoptosis of breast cancer cells independent of photoactivation (14). In conclusion, the current data suggest that extracts from the medicinal and culinary herb *R. graveolens* contain bioactive compounds that activate specific molecular signaling pathways and significantly interfere with the survival and proliferation of cancer cells, warranting further investigations.

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References

1 Brower V: Back to nature: extinction of medicinal plants threatens drug discovery. J Natl Cancer Inst *100*: 838-839, 2008.
2 Li JW and Vederas JC: Drug discovery and natural products: end of an era or an endless frontier? Science *325*: 161-165, 2009.
3 Pollio A, De Nauale A, Appetiti E, Aliotta G and Touwaide A: Continuity and change in the Mediterranean medical tradition: *Ruta* spp. (*rutaceae*) in Hippocratic medicine and present practices. J Ethnopharmacol *116*: 469-482, 2008.
4 Eickhorst K, DeLeo V and Csaposs J: Rue the herb: *Ruta graveolens*-associated phytophototoxicity. Dermatitis *18*: 52-55, 2007.
5 Wessner D, Hofmann H and Ring J: Phytophotodermatitis due to *Ruta graveolens* applied as protection against evil spells. Contact Dermatitis *41*: 232, 1999.
6 Oliva A, Meepagala KM, Wedge DE, Harries D, Hale AL, Aliotta G and Duke SO: Natural fungicides from *Ruta graveolens* L. leaves, including a new quinolone alkaloid. J Agric Food Chem *51*: 890-896, 2003.
7 Pronczuk J: International Program on Chemical Safety, Chemical Safety Information from Intergovernmental Organizations (www.inchem.org). http://www.inchem.org/, 1989.
8 Hale AL, Meepagala KM, Oliva A, Aliotta G and Duke SO: Phytotoxins from the leaves of *Ruta graveolens*. J Agric Food Chem *52*: 3345-3349, 2004.
9 De Rie MA, Van Eendenburg JP, Versnick AC, Stolk LM, Bos JD and Westerhof W: A new psoralen-containing gel for topical PUVA therapy: development, and treatment results in patients with palmoplantar and plaque-type psoriasis, and hyperkeratotic eczema. Br J Dermatol *132*: 964-969, 1995.
10 Halder RM, Battle EF and Smith EM: Cutaneous malignancies in patients treated with psoralen photochemotherapy (PUVA) for vitiligo. Arch Dermatol *131*: 734-735, 1995.

11 Vogelsang GB, Wolff D, Altomonte V, Farmer E, Morison WL, Corio R and Horn T: Treatment of chronic graft-versus-host disease with ultraviolet irradiation and psoralen (PUVA). Bone Marrow Transplant *17*: 1061-1067, 1996.
12 Varamini P, Soltani M and Ghaderi A: Cell cycle analysis and cytotoxic potential of *Ruta graveolens* against human tumor cell lines. Neoplasma *56*: 490-493, 2009.
13 Ratheesh M, Shyni GL and Helen A: Methanolic extract of *Ruta graveolens* L. inhibits inflammation and oxidative stress in adjuvant induced model of arthritis in rats. Inflammopharmacology *17*: 100-105, 2009.
14 Panno ML, Giordano F, Palma MG, Bartella V, Rago V, Maggiolini M, Sisci D, Lanzino M, De Amicis F and Ando S: Evidence that bergapten, independently of its photoactivation, enhances *p53* gene expression and induces apoptosis in human breast cancer cells. Curr Cancer Drug Targets *9*: 469-481, 2009.
15 Pathak S, Multani AS and Banerji P: Ruta 6 selectively induces cell death in brain cancer cells but proliferation in normal peripheral blood lymphocytes: A novel treatment for human brain cancer. Int J Oncol *23*: 975-982, 2003.
16 Sunila ES, Kuttan R, Preethi KC and Kuttan G: Dynamized preparations in cell culture. Evid Based Complement Alternat Med *6*: 257-263, 2009.
17 Kumar KB, Sunila ES, Kuttan G, Preethi KC, Venugopal CN and Kuttan R: Inhibition of chemically induced carcinogenesis by drugs used in homeopathic medicine. Asian Pac J Cancer Prev *8*: 98-102, 2007.
18 Franken NA, Rodermond HM, Stap J, Haveman J and van Bree C: Clonogenic assay of cells *in vitro*. Nat Protoc *1*: 2315-2319, 2006.
19 Samuel T, Okada K, Hyer M, Welsh K, Zapata JM and Reed JC: cIAP1 Localizes to the nuclear compartment and modulates the cell cycle. Cancer Res *65*: 210-218, 2005.
20 Vousden KH and Lane DP: p53 in health and disease. Nat Rev Mol Cell Biol *8*: 275-283, 2007.
21 Toledo F and Wahl GM: Regulating the p53 pathway: *in vitro* hypotheses, *in vivo* veritas. Nat Rev Cancer *6*: 909-923, 2006.
22 Rappold I, Iwabuchi K, Date T and Chen J: Tumor suppressor p53-binding protein 1 (53BP1) is involved in DNA damage-signaling pathways. J Cell Biol *153*: 613-620, 2001.
23 DiTullio RA Jr., Mochan TA, Venere M, Bartkova J, Sehested M, Bartek J and Halazonetis TD: 53BP1 functions in an ATM-dependent checkpoint pathway that is constitutively activated in human cancer. Nat Cell Biol *4*: 998-1002, 2002.
24 Fernandez-Capetillo O, Chen HT, Celeste A, Ward I, Romanienko PJ, Morales JC, Naka K, Xia Z, Camerini-Otero RD, Motoyama N, Carpenter PB, Bonner WM, Chen J and Nussenzweig A: DNA damage-induced G_2-M checkpoint activation by histone H2AX and 53BP1. Nat Cell Biol *4*: 993-997, 2002.
25 Zgheib O, Huyen Y, DiTullio RA Jr., Snyder A, Venere M, Stavridi ES and Halazonetis TD: ATM signaling and 53BP1. Radiother Oncol *76*: 119-122, 2005.
26 Mochan TA, Venere M, DiTullio RA Jr. and Halazonetis TD: 53BP1 and NFBD1/MDC1-Nbs1 function in parallel interacting pathways activating ataxia-telangiectasia mutated (ATM) in response to DNA damage. Cancer Res *63*: 8586-8591, 2003.
27 Gottlieb TM, Leal JF, Seger R, Taya Y and Oren M: Cross-talk between Akt, p53 and Mdm2: possible implications for the regulation of apoptosis. Oncogene *21*: 1299-1303, 2002.
28 Bartkova J, Rezaei N, Liontos M, Karakaidos P, Kletsas D, Issaeva N, Vassiliou LV, Kolettas E, Niforou K, Zoumpourlis VC, Takaoka M, Nakagawa H, Tort F, Fugger K, Johansson F, Sehested M, Andersen CL, Dyrskjot L, Ornтoft T, Lukas J, Kittas C, Helleday T, Halazonetis TD, Bartek J and Gorgoulis VG: Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints. Nature *444*: 633-637, 2006.
29 Rodier F, Coppe JP, Patil CK, Hoeijmakers WA, Munoz DP, Raza SR, Freund A, Campeau E, Davalos AR and Campisi J: Persistent DNA damage signalling triggers senescence-associated inflammatory cytokine secretion. Nat Cell Biol *11*: 973-979, 2009.
30 Cohen EE: mTOR: the mammalian target of replication. J Clin Oncol *26*: 348-349, 2008.
31 Cohen EEW, Hartford C, Maitland CH, Fox-Kay K, Vogel P, Wu K and Ratain MJ: Grapefruit juice significnatly increases bioavailability of weekly rapamycin (sirolimus) – evidence of safety and activity in a phase 1 study. AACR 100th Annual Meeting Abstract #LB-135. Denver, CO, USA. American Association for Cancer Research, 2009.
32 Lutchman L, Inyang V and Hodgkinson D: Phytophotodermatitis associated with parsnip picking. J Accid Emerg Med *16*: 453-454, 1999.
33 Kakar SM, Paine MF, Stewart PW and Watkins PB: 6'7'-Dihydroxybergamottin contributes to the grapefruit juice effect. Clin Pharmacol Ther *75*: 569-579, 2004.
34 Paine MF, Widmer WW, Hart HL, Pusek SN, Beavers KL, Criss AB, Brown SS, Thomas BF and Watkins PB: A furanocoumarin-free grapefruit juice establishes furanocoumarins as the mediators of the grapefruit juice-felodipine interaction. Am J Clin Nutr *83*: 1097-1105, 2006.
35 Arias-Santiago SA, Fernandez-Pugnaire MA, Almazan-Fernandez FM, Serrano-Falcon C and Serrano-Ortega S: Phytophotodermatitis due to *Ruta graveolens* prescribed for fibromyalgia. Rheumatology (Oxford), 2009.
36 Girennavar B, Jayaprakasha GK and Patil BS: Potent inhibition of human cytochrome P450 3A4, 2D6, and 2C9 isoenzymes by grapefruit juice and its furocoumarins. J Food Sc *72*: C417-C421, 2007.

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