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Furanocoumarins: Novel topoisomerase I inhibitors from *Ruta graveolens* L.

Renuka Diwan, Nutan Malpathak *

Department of Botany, University of Pune, Pune 411007, Maharashtra, India

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ABSTRACT

Topoisomerase I inhibitors from *Ruta graveolens* are reported for the first time. Potent topoisomerase I inhibitory activity from in vitro culture extracts *R. graveolens* were observed. Stabilization of DNA–topoisomerase covalent complex was observed in all the tested extracts. The mechanism of topoisomerase inhibition was determined by preincubation studies. The irreversible topoisomerase I mediated relaxation of plasmid in enzyme–substrate preincubation study, indicated that the observed inhibitory activity of extract constituents was not mediated through conformational changes in the DNA. Furthermore, the affinity of inhibitors with the enzyme was tested by enzyme–extract preincubation study. Increase in inhibition of topoisomerase activity and promotion of DNA–enzyme complex was observed after enzyme–extract preincubation. The activity could be assigned to furanocoumarins—psoralen, bergapten and xanthotoxin, identifying them as novel, potent topoisomerase I inhibitors.

1. Introduction

DNA topoisomerases are essential cellular enzymes required for cell proliferation and therefore, have recently emerged as important cellular targets for chemical intervention in the development of anti-cancer agents. Topoisomerases can be inhibited by two distinct mechanisms, and are divided into two classes accordingly: class I and II. Class I inhibitors stabilize the enzyme–DNA-covalent complex and block the subsequent rejoining of DNA break, enhance apoptosis through blocking the advancements of replication forks. Class II inhibitors prevent the enzyme and DNA from binding by interacting with either the topoisomerase enzyme or DNA (Kawaik et al. 2007, Yonezawa et al. 2005).

Topoisomerase inhibitors are very rare, the most widely studied and characterized inhibitors being camptothecin, a topo I poison,[3] and etoposide and doxorubicin, topo II poisons.[4–6] Although inhibitors against topoisomerase I have been developed and applied clinically, their severe side effects remain a serious problem. Besides, there are fewer known inhibitors of DNA topoisomerase I.[7] The success of camptothecin and its analogues as anticancer agents has spurred a search for additional agents acting by inhibition of topoisomerase I; to date several new classes of inhibitors have been described, like nitidine, fagaronine, epiberberine[8,9] and coralyne.[10] In the development of new inhibitors, natural products from plant sources can be a valuable source of novel inhibitors and may also serve as a suitable lead for the production of semi-synthetic active agents.

Ruta graveolens L. (Rutaceae) is a medicinal plant which has a long history of use as a domestic remedy. It is extensively used in homeopathic, ayurvedic and unani preparations. *R. graveolens* has been traditionally used in treatment of leucoderma, vitiligo, psoriasis, multiple sclerosis, cutaneous lymphomas, rheumatic arthritis. Recently its extracts were shown to have potent anti-cancer activity.[11,12] Therefore *R. graveolens* was assessed for its topoisomerase I inhibitory potential. Previously established culture lines, selected for their high furanocoumarin productivity, were also evaluated for their topoisomerase inhibitory activity.

To determine the exact mechanism of topoisomerase inhibition the enzyme and DNA were preincubated prior to addition of extract. Also to test the affinity of inhibitors with the enzyme, the enzyme and extract were preincubated before addition of extract. Furanocoumarins (Illustration 1), the major active constituents of the extracts, were isolated and evaluated for their topoisomerase inhibitory potential.

This is the first report on DNA topoisomerase I inhibitors from *R. graveolens* and its in vitro cultures leading to identification of furanocoumarins—psoralen, bergapten and xanthotoxin as novel topoisomerase I inhibitors.

Illustration 1.

* Corresponding author. Tel.: +91 020 25601439; fax: +91 020 25690498.
E-mail address: mpathak@unipune.ernet.in (N. Malpathak).

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2. Experimental

2.1. Plant material

Fruits, shoots and roots were obtained from *R. graveolens* L. (Rutaceae) plants grown in Botanic garden, Department of Botany, University of Pune. A voucher specimen of which, was deposited and authenticated by Botanical Survey of India (BSI), Western Circle, Pune, India.

2.2. In vitro cultures

Cell suspension cultures with varying degree of differentiation were established from leaves of elite *R. graveolens* plant, as described elsewhere.[13] Cell lines with varying degree of differentiation, dispersed cell line RC1, aggregated cell line RC3 and differentiated cell line RC6, were selected for determining their topoisomerase I inhibitory potential.[13] Several shoot lines were established from nodes and internodes of this plant. Shoot line RS2 was selected due to its lower doubling time and high furanocoumarin productivity, as described elsewhere.[14] Several transformed shoot clones of *R. graveolens* were obtained by *Agrobacterium tumefaciens* (LBA 4404) mediated genetic transformation. Clone Ia3 was selected due to its rapid growth and enhanced productivity. Biomass was harvested after three weeks of culture and shade dried (30 °C).

In vivo fruits, shoots and roots were used as reference.

2.3. Preparation of extracts

For topoisomerase inhibitory activity, methanolic extracts of selected culture lines were prepared. Methanolic extracts of in vivo plant material (fruits, stems and roots) were used as reference. Finely pulverized plant material (100 mg) was sonicated (33 MHz) for 20 min at room temperature and cold-extracted in ultra-pure methanol overnight. The extract was centrifuged at 10,000 rpm for 20 min and supernatant was filtered (using a membrane filter 0.45 μM). After filtration, the extract was evaporated to dryness at room temperature. The dried residue was dissolved in appropriate volume of dimethyl sulfoxide (DMSO) to obtained 10 mg/ml concentration and stored at −20 °C until use.

2.4. Compound isolation

Ethanolic extracts of shoot line RS2 were used for isolation of psoralen, bergapten and xanthotoxin. Compounds were separated by preparative thin layer chromatography using methanol/water (70:30) as mobile phase. Purity of compounds was determined by comparing retention time of isolated compounds and standard psoralen, bergapten and xanthotoxin (Sigma) using high performance chromatography (HPLC)[14] and co-chromatography. Purity of compounds was observed to be 99% using HPLC. For topoisomerase I inhibition assay 10, 20, 40 μM of psoralen, bergapten and xanthotoxin were used. Camptothecin (20 μM) was used as a positive control.

2.5. Topoisomerase I inhibitory activity

2.5.1. Inhibition of catalytic activity (relaxation assay)

For the determination of topoisomerase I inhibitory activity Calf thymus topoisomerase I (Takara, Japan) was used. Reactions were carried out as per product instruction, briefly, pUC19 DNA (250 ng) was used as the substrate in a reaction volume of 20 μl containing reaction buffer (10×), 0.1% bovine serum albumin (BSA), autoclaved distilled water. The appropriate inhibitor was added as indicated, and the reaction was initiated by the addition of 1.5 units of topoisomerase I. Reactions were carried out at 37 °C for 60 min. Gel electrophoresis was performed at 4 V/cm for 5 h in TAE (Tris + acetic acid + EDTA) buffer on a 1% agarose gel. Gel was stained with 0.5 mg/ml ethidium bromide. The area representing supercoiled and relaxed plasmid DNA was determined using software (Alpha Ease FC, version V 4.0.0).

Stabilization of DNA–enzyme covalent complex was studied. Relaxation of plasmid was carried out as mentioned earlier. After 60 min incubation at 37 °C, SDS–proteinase K was added. Following 30-min incubation at 37 °C, samples were electrophoresed on a 1% agarose gel. Presence of nicked form of DNA was recorded as a DNA-covalent complex.

2.5.2. Preincubation analysis

Enzyme (topoisomerase I) was preincubated with either inhibitor (extract or isolated compounds) or substrate (pUC19) at 37 °C for 30 min followed by addition of substrate or inhibitor respectively. Reaction products were electrophoresed as mentioned earlier. Intercalating agent, ethidium bromide was used as a control for enzyme–substrate preincubation studies.

3. Results and discussion

3.1. Inhibition of topoisomerase I

Effect of *R. graveolens* extracts on topoisomerase I inhibition was determined by measuring relaxation of supercoiled pUC 19 DNA using camptothecin as a positive control (Fig. 1). Extracts of cell suspension, shoot and transformed shoots showed inhibitory action against topoisomerase I enzyme. The order of potency of topoisomerase I inhibition by tested extract was observed to be differentiated cell suspension > shoot line RS2 > Transformant Ia3 > dispersed cell suspension RC6. Topoisomerase I inhibitory activity for *R. graveolens* has been reported for the first time.

In addition, it is of interest to know whether a novel topoisomerase I inhibitor likely exerts its cytotoxic effect by stabilizing complexes containing strand-cleaved DNA, as does camptothecin.[15] This information can be inferred from the ability of the test compound to stabilize the covalent enzyme–DNA complex. Camptothecin is known to stabilize the covalent enzyme–DNA binary to a greater extent, even at much lower concentration.[7] The ability of extracts to stabilize the topoisomerase I–DNA covalent binary complex was also assessed. The binary complex was converted to nicked, circular form after enzyme denaturation and degradation with SDS and proteinase K. Stabilization of covalent complex was observed in all tested extracts (Fig. 1).

3.2. Preincubation analysis

Topoisomerases can be inhibited by two distinct mechanisms, either by stabilization of the enzyme–DNA covalent complex or

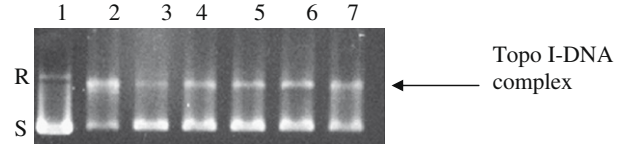

Figure 1. Topoisomerase I inhibition by extracts of *R. graveolens*. Lane 1: Supercolied pUC 19 DNA, lane 2: DNA and topoisomerase I enzyme, lane 3: camptothecin (20 μM), lane 4: dispersed cell suspension (10 μg), lane 5: differentiated cell suspension (10 μg), lane 6: shoot line RS2 (10 μg), lane 7: Transformant Ia3 (10 μg). Position of relaxed (R) and supercoiled (S) plasmids have been shown on the left side.

by preventing the enzyme and DNA from binding by interacting with either the topoisomerase enzyme or DNA.[1,2]

R. graveolens is one of the most promising source of furanocoumarins. The culture lines used in this study were selected for their high furanocoumarin productive potential.[13,14] Figure 2 represents the amount of psoralen, bergapten and xanthotoxin in selected culture lines. The structure of furanocoumarins leads to the suggestion that it might act as a DNA intercalator since they are well-known for their ability to slot between base pairs and so form a complex with DNA. Topoisomerase inhibition seen by in vitro culture extracts therefore, could be due to catalytic inhibition by test compound or indirectly through DNA-intercalation. To determine the exact mechanism of topoisomerase inhibition preincubation studies were conducted.

When pUC 19 DNA was first completely relaxed by topoisomerase I and then incubated with extracts, the relaxed plasmid did not revert back to its supercoiled form, indicating that extract constituents did not inhibit the topoisomerase activity through intercalation into the DNA (Fig. 3). On the contrary, the reaction containing ethidium bromide (positive control) showed slight change in relaxation state of plasmid (Fig. 3, lane 4).

Increase in inhibition of topoisomerase activity was observed after preincubation of enzyme with extract (on the basis of band area of relaxed DNA) (Fig. 4). The formation of DNA–enzyme complex was also promoted due to preincubation. Extract by itself did not relax DNA. To test this, supercoiled plasmid was incubated with extract without addition of enzyme. No relaxation was observed in after incubation of extract with DNA (data not shown).

3.3. Topoisomerase I inhibition by isolated furanocoumarins

In experiments with extracts it was observed that, topoisomerase inhibition was mediated through both ways viz. stabilization of covalent complex and inhibition of catalytic activity of enzyme. Results proposed that there could be several active constituents present in extracts.

Several potential inhibitors may have contributed to the inhibitory activity of *R. graveolens* extracts. The major constituents of the extracts are furanocoumarins—psoralen, bergapten and xanthotoxin. Therefore, enzyme inhibitory potential of psoralen, bergapten and xanthotoxin, isolated from in vitro cultures, was assessed at different concentrations (10, 20, 40 μM). Psoralen at 10 μM was not effective in inhibiting the enzyme but caused 100% inhibition at 20 μM concentration (Fig. 5, lanes 4 and 5). Bergapten at 10 μM caused complete inhibition forming DNA–enzyme complex (Fig. 5 lane 7), compared to camptothecin (20 μM) (Fig. 5, lane 3). Whereas, xanthotoxin could inhibit enzyme activity only at higher (40 μM) concentrations (Fig. 5, lanes 10–12).

Figure 6 represents the inhibition dose–response curves of isolated furanocoumarins against topoisomerase I. All compounds were effective at inhibiting topoisomerase I activity and the inhibition was dose dependent with IC_{50} values for psoralen, bergapten and xanthotoxin observed to be 11 μM, 6.5 μM and 28 μM, respectively. Thus bergapten and psoralen were potent topoisomerase I inhibitors. Furanocoumarins were previously reported to be DNA gyrase inhibitors,[16] however there are no reports of topoisomerase inhibition.

In conclusion topoisomerase I inhibitors have been reported from *R. graveolens* for the first time. Present results demonstrated the effectiveness of *R. graveolens* in vitro cultures as a alternative source of topoisomerase inhibitors. The inhibitory activity could be attributed to furanocoumarins—psoralen, bergapten and xanthotoxin, which were identified as novel topoisomerase I inhibitors.

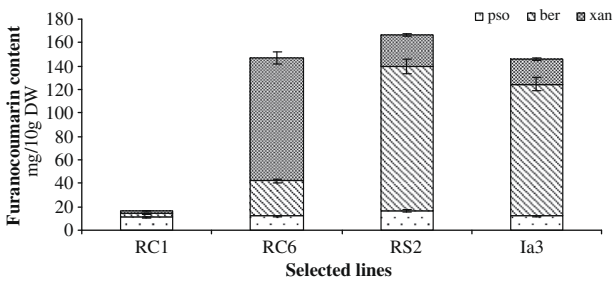

Figure 2. Furanocoumarin content in selected culture lines. Furanocoumarin content varied in selected lines. Maximum amount of furanocoumarins were found in organized cultures.

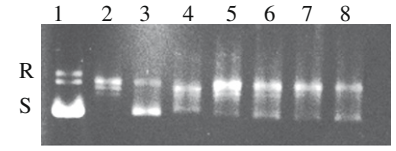

Figure 3. Effect of preincubation of relaxed DNA with extract. Lane 1: Supercolied pUC 19 DNA, lane 2: DNA and topoisomerase I enzyme, lane 3: camptothecin (20 μM), lane 4: ethidium bromide (20 μM), lane 5: dispersed cell suspension (10 μg), lane 6: differentiated cell suspension (10 μg), lane 7: shoot line RS2 (10 μg), lane 8: Transformant Ia3 (10 μg).

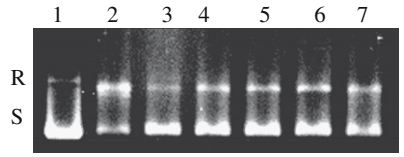

Figure 4. Effect of preincubation of enzyme and extract on inhibition of catalytic activity. Lane 1: Supercolied pUC 19 DNA, lane 2: DNA and topoisomerase I enzyme, lane 3: camptothecin (20 μM), lane 4: dispersed cell suspension (10 μg), lane 5: differentiated cell suspension (10 μg), lane 6: shoot line RS2 (10 μg), lane 7: Transformant Ia3 (10 μg).

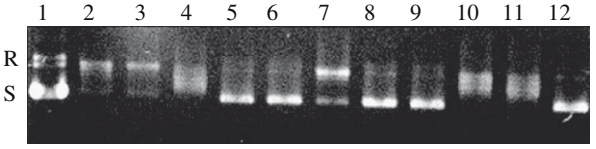

Figure 5. Inhibitory effect of furanocoumarins on topoisomerase I mediated DNA relaxation. Lane 1: Supercolied pUC 19 DNA, lane 2: DNA and topoisomerase I enzyme, lane 3: DNA and topoisomerase I enzyme + camptothecin (20 μM), lanes 4–6: psoralen (10, 20, 40 μM), lanes 7–9: bergapten (10, 20, 40 μM), lanes 10–12: xanthotoxin (10, 20, 40 μM).

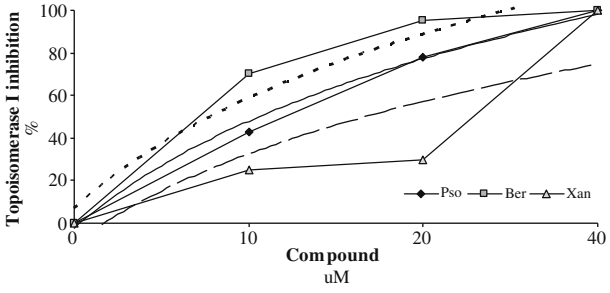

Figure 6. Dose–response curves for topoisomerase I inhibition by isolated furanocoumarins. Dose dependent increase in topoisomerase I inhibition was seen for psoralen, bergapten and xanthotoxin. Bergapten was most potent inhibitor of topoisomerase I with IC50 of 6.5 μM. Xanthotoxin inhibited topoisomerase I only at higher concentrations.

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