Research in Pharmaceutical Sciences 2 (2006) 66-72
Received: May 2006
Accepted: Nov 2006

School of Pharmacy & Pharmaceutical Sciences
Isfahan University of Medical Sciences and Health Services

Original Article

Evaluation of some Iranian conifers extracts cytotoxicity, using *Sacharomyces cerevisiae*, RS 322N and RS 188N

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Abstract

Exploration of natural products with antitumoral effects such as etoposide, tenioposide, camptothecin and paclitaxel from conifers provoked us to do some research on anti-tumoral activity of Iranian medicinal plants. Ten different species of Iranian conifers aerial parts were collected across the country. The relative potency of methanolic extract were investigated on the inhibition of topoisomerase I on yeasts (*Sacharomyces cerevisiae*, RS 322N and *S. cerevisiae,* RS 188N, as wild type) using agar diffusion, turbidimetry and agar dilution procedures. Briefly, 0.1 ml of extracts (20 mg/ml) were added to the yeast culture and the obtained zone of inhibition were evaluated compare to the control (camptothecin, 2 μg/ml). Zones of inhibition >12 mm on mutant type and no inhibition effect on wild type were considered as positive results or cytotoxicity. For agar dilution assay, different dilutions of extracts (5, 10, 20, 40, 80 mg/ml) were applied and inhibition of yeast growth was considered as positive result. In turbidimetric assay no turbidity was evaluated as cytotoxicity. Minimum inhibitory concentration was determined by agar dilution and turbidimetric procedures. To determine minimum biocidal concentration, samples were transferred from turbidimetric cells to solid media. According to the agar diffusion results, none of the extracts showed specific cytotoxicity on mutant yeast. The results obtained from growth inhibition of *Platycladus orientalis* extracts were shown to have some good cytotoxic effects on both mutant and wild type. In conclusion, agar dilution and turbidimetry results were similar according to the high concentration of extracts and this means that the cytotoxic effects of these plants on yeasts are not specific.

Keywords: Iranian Conifers; *Sacharomyces cerevisiae*; Turbidimetry; Cytotoxicity

INTRODUCTION

Several drugs with antineoplastic activities have been isolated from plants, including taxoids and vinca alkaloids. The taxoids, one of the most clinically relevant groups of drugs, were isolated originally from the roots, stems and needles of *Taxus* species (Taxaceae) (1,2). Paclitaxel (Taxol), which was isolated from *Taxus brevifolia*, has a unique mode of action and is used as an antitumor agent for treating breast and ovarian cancer (3,4). Consequently, since the discovery of taxol, much efforts have been devoted to isolate new taxane diterpenes from various *Taxus* species or conifers (5). Recently, several nontaxol-type taxane diterpenes were found to increase cellular accumulation of vincristine in multidrug resistant tumor cells (5). Buhagiar et al. (6) showed that essential oil extract from the conifer

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Tetraclinis articulata induces apoptosis in human melanoma, breast and ovarian cancer cell lines; also Lopez et al. (7) in their study indicated that *Cupressus lusitanica* (cupressaceae) leaf extract induces apoptosis in a panel of cancer cell lines. Shinozaki et al. (5) demonstrated that multidrug resistant cancer cells are susceptible to cytotoxic taxane diterpenes extracted from *Taxus yannanensis* and *Taxus chinensis*. Similar results reported by Fukushima and colleagues indicated that non-alkaloidal taxane diterpenes from *Taxus chinensis* are cytotoxic against a paclitaxel-resistant cell line (8). However, few isolated non taxol-type compounds have been examined as potential antitumor agents. Many flavonoids and biflavonoids have also been isolated from various plants and examined as cytotoxic agents (9,10). According to these findings and in our continuing efforts to discover new antitumor agents from higher plants, we have investigated the cytotoxic effects of crude ethanolic extract of Iranian conifers, as a group of higher plants similar to taxus species, in a panel of *Sacharomyces cerevisiae*. *Sacharomyces cerevisiae*, also known as baker's yeast, a eukaryotic cell model which is used to assess cytotoxicity and genotoxicity of potential anticancer compounds (11). It exists in two strains; mutant and wild types. *Sacharomyces cerevisiae* is one of the most intensively studied organisms in molecular and cellular biology much like E. coli as a model of prokaryote. It is useful in the cell cycle studying because it is easy to culture and shares about 23% of its genome with that of humans. Its structure is similar to human cell.

MATERIALS AND METHODS

Chemicals

Methanol, dimethysulfoxide (DMSO), glucose, glycerin and NaCl were purchased from Merck Company (Germany).
Agar (Biomeriux, France), yeast extract (Oxoid, UK), peptone (Biolife, Italy) and camptothecin (Aldrich, USA) were used.
Sacchromyces cerevisiae, mutant RS322N13 (rad 52) and *Sacharmyces cerevisiae* wild type RS188N (rad 52+) were a gift of Professor GH Shahidi, Bahonar University, Kerman, Iran.

Plant samples

Terminal branchlets, bark and fruits of male and female trees of 10 Iranian conifers species of were collected from different parts of the country in summer 1998 as following: *Cupressus sempervirens* cv. *stricta* (Tehran, botanical garden), *Cupressus sempervirens* cv. *cereiformis* (Fars province), *Juniperus foetidissma* (Azarbayejan province), *Juniperus excelsa, Juniperus sabina, Juniperus oblonga, Juniperus communis, Platycladus orientalis, Cupressus sempervirens* var. *horizontalis* and *Taxus baccata* all were collected from Mazandaran province (North of Iran). Plants were identified by the Department of Botany, Tehran University, Tehran, Iran. Voucher specimens (No. 1411-1419) of the plants were deposited in the herbarium of the Faculty of Pharmacy, Isfahan University of Medical Sciences, Isfahan, Iran. The plant materials were kept at -20 ºC before use.

Extraction and sample preparation

Two-hundred and fifty grams of each plant parts was chopped, powdered and soaked in 500 ml of methanol (80% v/v) overnight and then percolated (5 h, 30 drops/min).
Diluted extracts were then concentrated by rotary evaporator to a final volume of 10 ml and then dried in an oven (40 ºC) over 48 h to obtain 1-2 g of semi-solid samples.

Cytotoxicity assay **(12)**

In order to perform cytotoxic evaluation of plant extracts, 20 mg (agar diffusion), 160 mg (agar dilution) or 800 mg (turbidimetric assay) were weighed and

transferred to screw cap test tubes (10-20 ml) and dissolved in 1 ml of DMSO/methanol (1:1 v/v) or in 4 ml of this solvent for turbidimetric assay. To avoid microbial cross contamination gentamycine sulfate (5 µg/ml) was added to the media.

Preparation of YPD-agar media

Yeast peptone dextrose (YPD) media was prepared using following components: agar (1.5%), peptone (2%), yeast extract (1%) and dextrose (2%). All above components were dissolved in 500 ml of distilled water and heated until a clear solution was obtained and sterilized (15 min, 121 ºC and 15 psi). In order to perform agar diffusion and agar dilution assays solid sterile media and for turbidimetric assay broth sterile media was used.

Preparation of yeast suspension

Yeasts were transferred from stock to petri dishes (5 cm ID) containing solid agarose media using a loop and incubated at 30 ºC for 24 h or 30 h in the case of mutant species. A few colonies of yeasts were then transferred to screw cap test tubes containing 10 ml of normal saline and shaked to get homogenous suspension with an approximate concentration of 10^8 cells/ml, in a laminar flow cabinet. This suspension was used for seeding yeast in media containing plant extracts.

Cytotoxicity evaluation using agar diffusion method

Two ml of yeast suspension (10^8 cells/ml) were seeded under sterile condition on the surface of a petri dish (12 cm ID) containing 50 ml of YPD agar media. Ten wells were made in the agar, using a sterile stainless steel tubes and each well was supplied with 100 µl of plant extracts (20 mg/ml). Petri dishes were then incubated for 48-72 h at 30 ºC and finally the zones of inhibition were measured. In this study camptothecin (2 µg/ml) was used as positive control while 100 µl of solvent was applied as negative control. This assay was done in triplicate.

Determination of minimum inhibition concentration (MIC), using agar dilution

Each ml of sample (160 mg/ml) was diluted with 1 ml of warm (~50 ºC) YPD-agar media and dilution was continued so that the final concentrations were 80, 40, 20, 10 and 5 mg/ml. In the next step 1 ml of each concentration was diluted with the same amount of solvent (negative control) and transferred into a 24-multiwell plate. Yeast suspension (10^8 cells/ml) was spread over the wells and incubated at 30 ºC for 48-72 h. Finally the growth of yeasts in the presence of extracts in each well was evaluated.

Determination of minimum inhibition concentration (MIC), using turbidimetric assay

Four ml of each sample (200 mg/ml) and 6 ml of broth-YPD media were added to a test tube to obtain stock solution with a concentration of 80 mg/ml. Then it was diluted with media to obtain other required concentrations (40, 20, 10, 5 mg/ml). 0.1 ml of the yeast suspension was then added to each test tube and incubated for 48-72 h. Finally the growth of yeasts in these test tubes was observed.

Determination of minimum Biocidal concentration (MBC)

0.1 ml of precipitated materials from tubes which showed no growth in the turbidimetric assay was spread in the surface of agar-YPD media, using a 5 cm Petri dish. These dishes were then kept at 30 ºC for 48-72 h. No yeast growth in each Petri dish means that the extract was toxic. All experiments were in triplicate and performed against both mutant and wild type yeasts.

Statistical analysis

SIGMASTAT™ (Jandel Software, San

Raphael, CA) was used to perform statistical tests. Analyze-of-variance followed by Dunkan test was used to see the differences among groups. Significance was assumed at the 5% level.

RESULTS

Evaluation of extracts cytotoxicity, using agar diffusion method

Cytotoxicity of Iranian conifers extract against mutant and wild type of *Sacharomyces cerevisiae* is shown in Table 1. In this experiment no growth of wild and mutant type of yeasts was observed, applying different concentrations of extracts (80, 40, 20, 10, 5 mg/ml). Results are summarized in Table 2.

yeast, using turbidimetric procedure Determination of MIC of extract of Iranian conifers on mutant and wild

Results showed that Minimum biocidal concentrations for all tested extracts are 80 mg/ml or higher. Obtained data are summarized in Table 3 along with MIC.

Table 1: Cytotoxicity of Iranian conifers extract (20 mg/ml) against mutant *Sacharomyces cerevisiae*, using agar diffusion method.

Species	Part used	Zone of inhibition (mm), wild type		Zone of inhibition(mm), mutant type	
		Average (n = 3)	SD	Average (n = 3)	SD
T. baccata	bark	0.3	0.57	2.6	1.52
	F/branchlet	0	0	0.3	0.57
	M/branchlet	0	0	0	0
C. sempervirens cv. stricta	fruits	0	0	0.3	0.57
	branchlet	0.3	0.57	0.3	0.57
C. sempervirens cv. cereiformis	fruits	0.3	0.57	1.6	1.52
	branchlet	1.3	0.27	2.3	1.52
C. sempervirens var. horizontalis	fruits	0.3	0.57	0.3	0.57
	branchlet	0.3	0.57	1.6	1.52
P. orientalis	fruits	8.6	1.52	11	1
	branchlet	11.3	0.57	14.3	0.57
J. communis	fruits	8	1.41	8.3	1.15
	F/branchlet	0.3	0.57	1.6	1.52
	M/branchlet	1	1	2.3	1.52
J. oblonga	fruits	2	1	7.5	2.1
	F/branchlet	5.3	0.57	9	0
	M/branchlet	1.3	0.57	3.6	1.15
J. sabina	fruits	1.6	1.52	2.3	0.57
	F/branchlet	0.6	1.52	2.6	2.08
	M/branchlet	2.6	0.57	3.6	1.15
J. excelsa	fruits	0.6	0.57	5	0
	branchlet	0.6	0.57	1.6	0.57
J. foetidissma	fruits	1	1	1.6	0.57
	F/branchlet	1	0	1.6	0.57
	M/branchlet	1.6	1.52	2.6	0.57
+ control		0	0	18	0
- control		0	0	0	0

F: female; M: male (n = 4)

DISCUSSION

In general there are 6 different ways to choose plants for cytotoxic evaluations.1) Taxonomy, 2) Data from folklore medicine, 3) Phytochemical evaluations, 4) Random sampling, 5) Previous reports and 6) By chance. This study was performed according to taxonomy of plants and also the results from previous works. Many years ago, Kelly and Hartwell (13) showed that extracts from different parts of *Juniperus sabina* had cytotoxic effects.

Jacort et al. (14) and Ma et al. (15) reported the cytotoxic and antitumor properties of taxoids obtained from *Taxus baccata*. Isolated podophyllotoxine from *Juniperus communis* have been tested for its cytotoxic and antiherpetic effects by Markkanen (16,17); also lignans from *Platyclaus orientalis* were evaluated for their cytotoxicity by Kosuge et al. (18). In continuation to work on conifers, we decided to study the cytotoxic activity of above mentioned species of Iranian conifers against yeasts. Between different in vitro

Table 2: MIC of Iranian conifers extract (20 mg/ml) against mutant (RS322N) and wild type (RS188N) of *Sacharomyces cerevisiae*, using agar dilution method.

Species	Part used	Plant extract used against RS322N (mg/ml)					Plant extract used against RS188N (mg/ml)				
		80	40	20	10	5	80	40	20	10	5
T. baccata	bark	-	-	2+	3+	3+	-	+	3+	3+	3+
	F/branchlet	-	-	2+	3+	3+	-	-	3+	3+	3+
	M/branchlet	-	-	3+	3+	3+	-	3+	3+	3+	3+
C. sempervirens cv. stricta	fruits	-	2+	3+	3+	3+	-	3+	3+	3+	3+
	branchlet	-	2+	3+	3+	3+	-	+	3+	3+	3+
C. sempervirens cv. cereiformis	fruits	-	-	2+	3+	3+	-	-	3+	3+	3+
	branchlet	-	-	+	3+	3+	-	-	2+	3+	3+
C. sempervirens ar. horizontalis	fruits	-	2+	3+	3+	3+	-	3+	3+	3+	3+
	branchlet	-	+	3+	3+	3+	-	+	3+	3+	3+
P. orientalis	fruits	-	-	-	2+	3+	-	-	-	2+	3+
	branchlet	-	-	-	-	3+	-	-	-	+	3+
J. communis	fruits	-	-	-	2+	3+	-	-	-	3+	3+
	F/branchlet	-	-	2+	3+	3+	-	-	3+	3+	3+
	M/branchlet	-	-	+	3+	3+	-	-	+	3+	3+
J. oblonga	fruits	-	-	-	2+	3+	-	+	3+	3+	3+
	F/branchlet	-	-	-	3+	3+	-	+	3+	3+	3+
	M/branchlet	-	-	-	2+	3+	-	-	2+	3+	3+
J. sabina	fruits	-	-	2+	3+	3+	-	-	2+	3+	3+
	F/branchlet	-	-	-	2+	3+	-	-	3+	3+	3+
	M/branchlet	-	-	-	3+	3+	-	-	+	3+	3+
J. excelsa	fruits	-	-	+	3+	3+	-	-	3+	3+	3+
	branchlet	-	-	3+	3+	3+	-	-	3+	3+	3+
J. foetidissma	fruits	-	-	3+	3+	3+	-	-	3+	3+	3+
	F/branchlet	-	-	3+	3+	3+	-	-	3+	3+	3+
	M/branchlet	-	-	2+	3+	3+	-	-	3+	3+	3+
+ control		-	-	-	-	-	-	-	-	-	-
- control		+	+	+	+	+	+	+	+	+	+

(-) means no colony was seen; (+) means colonies were seen on the media surface; (2+) indicate quite a few colonies were seen and (3+) indicate that too many colonies were seen. (n = 4), F: female; M: male.

evaluation methods for cytotoxicity of natural products, the topoisomerase I procedure was used in this study, previously used by Hann et al. (19) to determine the toxicity of natural products. Presence of topoisomerase I enzyme in these yeasts, allowed us to study the underlying mechanism of extracts cytotxicity. According to results obtained from Table 1 none of the studied extracts showed selective cytotoxicity against yeasts. Although methanolic extracts of fruits and branchlets of *Platycladus orientalis* against mutant yeast showed zone of inhibition in the accepted range, but did not show any effect against wild type, therefore, it would not be considered as a selective cytotoxic product and may be considered as an antifungal agent.

Results obtained from the agar dilution and other methods consistently indicated that all samples inhibited the growth of yeasts in a concentration of 80 mg/ml which is not considered as a cytotoxic concentration.

Finally it should be mentioned that these extracts are not selective products against topoisomerase I and these experiments are unable to show the specific toxicity of extracts against yeasts. On the

Table 3: MIC and MBC* of Iranian conifers extracts against mutant (RS322N) and wild (RS188N) type of *Sacharomyces cerevisiae*, using turbidimetric method.

Species	Part used	Plant extract (mg/ml) used against RS322N (*data for MBC)					Plant extract (mg/ml) used against RS188N (data for MIC)				
		80/*	40/*	20/*	10	5	80	40	20	10	5
T. baccata	bark	-/-	-/+	+/ND	+	+	-	+	+	+	+
	F/branchlet	-/-	-/+	+/ND	+	+	-	+	+	+	+
	M/branchlet	-/-	-/+	+/ND	+	+	-	+	+	+	+
C. sempervirens cv. stricta	fruits	-/-	-/+	+/ND	+	+	-	+	+	+	+
	branchlet	-/-	-/+	+/ND	+	+	-	-	+	+	+
C. sempervirens cv. cereiformis	fruits	-/-	-/+	+/ND	+	+	-	-	+	+	+
	branchlet	-/-	-/+	+/ND	+	+	-	-	+	+	+
C. sempervirens var. horizontalis	fruits	-/-	+/ND	+/ND	+	+	-	+	+	+	+
	branchlet	-/-	+/ND	+/ND	+	+	-	-	+	+	+
P. orientalis	fruits	-/-	-/+	-/+	+	+	-	-	-	+	+
	branchlet	-/-	-/+	-/+	+	+	-	-	-	+	+
J. communis	fruits	-/-	-/+	+/ND	+	+	-	-	+	+	+
	F/branchlet	-/-	-/+	+/ND	+	+	-	-	+	+	+
	M/branchlet	-/-	-/+	+/ND	+	+	-	-	+	+	+
J. oblonga	fruits	-/-	+/ND	+/ND	+	+	-	-	+	+	+
	F/branchlet	-/-	-/+	-/ND	+	+	-	-	-	+	+
	M/branchlet	-/-	+/ND	+/ND	+	+	-	-	+	+	+
J. sabina	fruits	-/-	-/+	+/ND	+	+	-	+	+	+	+
	F/branchlet	-/-	-/+	+/ND	+	+	-	+	+	+	+
	M/branchlet	-/-	-/+	+/ND	+	+	-	-	+	+	+
J. excelsa	fruits	-/-	-/+	+/ND	+	+	-	+	+	+	+
	branchlet	-/-	-/+	+/ND	+	+	-	+	+	+	+
J. foetidissma	fruits	-/-	-/+	+/ND	+	+	-	+	+	+	+
	F/branchlet	-/-	+/ND	+/ND	+	+	-	-	+	+	+
	M/branchlet	-/-	-/+	-/+	+	+	-	-	-	+	+
+ control		-	-	-	-	-	-	-	-	-	-
- control		+	+	+	+	+	+	+	+	+	+

(-) means no colony was seen; (+) means colonies were seen on the media surface. F: female; M: male. *MBC was determined only against mutant type (n = 4). ND: no data.

other hand, based on the inhibition of toposiomerase I, only *Platycladus orientalis* showed some effects and need more experiments to find out the exact mechanism of action.

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