Selectivity of *Pinus sylvestris* extract and essential oil to estrogen-insensitive breast cancer cells *Pinus sylvestris* against cancer cells

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ABSTRACT

Background: So far, the anticancer action of pine tree extracts has mainly been shown for the species distributed widely around the Asian countries. **Objective:** Therefore, this study was performed to examine the potential cytotoxicity of Scots pine (*Pinus sylvestris* L.) native also to the European region and growing widely in Estonia. **Materials and Methods:** The cytotoxic activity of methanol extract and essential oil of Scots pine needles was determined by sulforhodamine B assay in different human cancer cell lines. **Results:** This needle extract was found to suppress the viability of several human cancer cell lines showing some selectivity to estrogen receptor negative breast cancer cells, MDA-MB-231(half maximal inhibitory concentration [IC_{50}] 35 μg/ml) in comparison with estrogen receptor-positive breast cancer cells, MCF-7 (IC_{50} 86 μg/ml). It is the strongest cytotoxic effect at all measured, thus far for the needles and leaves extracts derived from various pine species, and is also the first study comparing the anticancer effects of pine tree extracts on molecularly different human breast cancer cells. The essential oil showed the stronger cytotoxic effect to both negative and positive breast cancer cell lines (both IC_{50} 29 μg/ml) than pine extract (IC_{50} 42 and 80 μg/ml, respectively). **Conclusion:** The data from this report indicate that Scots pine needles extract and essential oil exhibits some potential as chemopreventive or chemotherapeutic agent for mammary tumors unresponsive to endocrine treatment.

Key words: Anticancer effect, mammary adenocarcinoma, pine extract, pine oil, *Pinus sylvestris*

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INTRODUCTION

Breast cancer affects more than 1.3 million women worldwide each year and accounts for about 14% of cancer-related deaths.[1,2] In western countries, the woman's lifetime risk of developing this disease is more than 10%.[3] The incidence has increased in the past decades and is expected to rise substantially in the coming years.[2] Breast cancer is a heterogeneous group of pathologic entities consisting of several molecular subtypes, each with distinct natural histories and biological behaviors requiring also different management approaches.[2,4] Most newly diagnosed breast carcinomas (about 70%) are estrogen-receptor (ER)-positive and can be classified as luminal subtypes. Determining the ER status of tumor samples is a standard practice in clinical oncology as the patients with ER-positive tumors have likely benefit from endocrine therapies, and have generally a better prognosis than those with ER-negative breast cancers for which the therapeutic options are more complicated and prognosis is worse.[1,2,5]

In recent years, interest in natural plant components with potential anticancer effects has continuously grown and considerable attention has focused on identifying chemopreventive phytochemicals to slow, reverse or completely halt the multistage process of carcinogenesis.[6-10] Plants provide an extensive reservoir of natural products with a wide structural diversity and offer novel and exciting chemical entities in modern medicine.[11] The majority of current antitumor drugs have severe side effects accompanying their therapeutic action and, therefore, studies of traditional herbs and identifying novel natural products with high anticancer activity, but low cytotoxicity

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in normal cells are receiving considerable attention in the field of anticancer studies.[10-12]

Pine trees are widely distributed worldwide being with around 105 species the largest genus of conifers.[13-15] Consumption of various parts, including needles, bark, cones, and pollen is believed to promote health and prevent some aging-related chronic diseases.[13] There is growing evidence that pine needles can exert also antioxidant, antimutagenic, and antiproliferative effects on cancer cells.[8] Bark has been used in traditional medicine for over 2000 years as a nutritional supplement and phytochemical remedy.[16] Extracts derived from the bark of *Pinus maritima* Lam. (Pycnogenol and Flavangenol) and *Pinus massoniana* Lamb. may hold promise as anticancer agents and are good candidates for chemoprevention or chemotherapeutics in the future.[6,16] They can strongly inhibit the migration capability of human cervical cancer HeLa cells and induce selectively, the apoptosis of human liver cancer Bel-7402 and HepG2 cells.[9,10,12,16,17] Pycnogenol has been shown to exert antileukemic effects and protective properties against skin carcinogenesis.[6,7,18,19] Moreover, it can selectively induce cell death in human mammary cancer MCF-7 cells, but not in normal human mammary MCF-10 cells.[19,20] However, extracts prepared from needles of different pine species (*Pinus thunbergii* Parl., *Pinus rigida* Mill., *Pinus koraiensis* Siebold and Zucc., *Pinus densiflora* Siebold et Zuccarini) reveal only very limited anticancer effects on breast adenocarcinoma MCF-7 cells with half maximal inhibitory concentration (IC_{50}) values in the range of more than 200 μg/ml.[13,21]

There are no reports available about the potential anticancer action of extracts from Scots pine (*Pinus sylvestris* L.) growing natively in Europe and Asia and being a very common coniferous tree in Estonia.[22] For this reason, we evaluated the effects prepared from needles of *P. sylvestris* on various human cancer cell lines, and performed for the first time the comparative analysis of action of pine extract on estrogen receptor positive and negative breast carcinoma cells.

MATERIALS AND METHODS

Cell culture

Human cancer cell lines including ER-positive breast cancer MCF-7, ER-negative breast cancer MDA-MB-231, prostate cancer LNCaP, gastric carcinoma MKN7, colon adenocarcinoma SW480, oral epidermoid carcinoma KB, lung adenocarcinoma LU-1, liver hepatocellular carcinoma HepG2, and promyelocytic leukemia HL-60 cells were cultured in Dulbecco's Modified Eagle Medium or RPMI-1640 cell culture medium, both supplemented with 10% fetal bovine serum. Cells were cultivated at 37°C in a humidified atmosphere containing 5% carbon dioxide.

Plant material and preparation of extracts

Pine needles collected from Northern Estonia were dried and crushed to a fine powder, and then extracted with methanol for three times (48 h per time) at room temperature (20°C). Next, the methanol extracts were recovered under reduced pressure to obtain crude extracts, which were used in the cytotoxic assays. Extracts were dissolved in dimethyl sulfoxide (DMSO) to prepare 4 mg/ml stock solutions that were later mixed with the cell culture medium to achieve the desired concentrations. The final test concentrations were 0.8, 4, 20, and 100 μg/ml.

In vitro cytotoxic assay

The effects of pine needle extracts on the viability of malignant cells were determined by sulforhodamine B cytotoxic assay.[23] Briefly, cells were grown in 96-well microtiter plates with each well containing 190 μl medium. After 24 h, 10 μl of test samples dissolved in DMSO were added to each well. One plate with no samples served as a day 0 control. The cells were continuously cultured for additional 48 h, fixed with trichloroacetic acid and stained with sulforhodamine B, followed by the determination of optical densities at 515 nm using a Microplate Reader (BioRad). The percentage of growth inhibition was calculated using the following equation:

$$\%\,\text{Growth} = \frac{\left[\begin{matrix}\text{OD}\left(\text{reagent}\right)\\ -\,\text{OD}\left(\text{day 0}\right)\end{matrix}\right]}{\left[\begin{matrix}\text{OD}\left(\text{negative control DMSO 10 \%}\right)\\ -\,\text{OD}\left(\text{day 0}\right)\end{matrix}\right]} \times 100$$

Where, OD is optical density or absorbance values. The potent anticancer agent ellipticine and tamoxifen citrate were used as a positive control.

Isolation of essential oil

The essential oil was isolated from fresh pine needles by the hydrodistillation method described in a previous study.[24] The pine oil used for the cytotoxic assay was also hydrodistilled.

Gas chromatography–mass spectrometry analysis

Gas chromatography-mass spectrometry (GC-MS) analysis was carried out using an Agilent 5975 Series Mass Selective Detectors, Agilent 7890A GC (Agilent Technologies, Inc.) with two detectors (MS and FID) on a fused silica capillary column (30 m × 0.25 mm) with a bonded stationary phase: Poly (5%-diphenyl-95%-dimethyl) siloxane (DB-5). The film thickness of the stationary phase was 0.25 mm. The carrier gas was helium with the split ratio of 1:30 and the flow rate of 1.3 ml/min was applied. The temperature program was from 50°C to 240°C at 2°C/min; the injector temperature was 300°C. The MS detector was operated in

the EI mode of 70 eV, and at a scan rate of 2 scans/s with an acquisition mass range of 20–400 u.

Statistical analysis

Cytotoxic data were calculated and expressed as concentrations, at which 50% of cell growth was inhibited (IC_{50} values ± standard deviation). All experiments were carried out in triplicate and the Table Curve 2Dv4 software was used for calculation of IC_{50} values. $P < 0.01$ were considered significant.

RESULTS AND DISCUSSION

A dose-dependent decrease in viability of human breast cancer cells was observed after 48 h of treatment with 0.8, 4, 20, and 100 μg/ml of pine needles extract. The effect on ER-negative MDA-MB-231 cells was almost three-fold stronger (IC_{50} 35.56 μg/ml) than for ER-positive MCF-7 cells (IC_{50} 86.37 μg/ml) indicating some selectivity of pine needles extract to hormone refractory breast cancer cells [Figure 1].

The half-maximal cytotoxic effects of methanol extract prepared from Scots pine needles on other malignant cell lines studied in this work remained in the concentration range of 50 μg/ml to 80 μg/ml [Table 1], being somewhat stronger on leukemia, colon cancer, lung adenocarcinoma, and hepatocellular carcinoma cell lines compared to oral epidermoid carcinoma KB cells.

Previously, extracts prepared from needles of various pine species have been shown to exert some anticancer effects. However, cytotoxicity expressed to human breast cancer cells has described only at very high concentrations (IC_{50} values certainly more than 200 μg/ml) and for species growing mainly in the Asian region.[13,21] In this work, we demonstrated for the first time that methanol extract derived from needles of Scots pine tree widely and natively distributed throughout Estonia has potential to suppress the viability of human breast cancer MCF-7 cells. These cells are derived from a patient with metastatic breast cancer, and earlier studies have shown that estrogen can directly stimulate the growth of this tumor cell line.[20] Moreover, the same extract revealed almost three-fold stronger cytotoxic activity on MDA-MB-231 human breast cancer cells, which do not express estrogen receptor-α and are, therefore, not responsive to endocrine treatment. These data indicate that Scots pine needles can contain some compounds with the high potential to be developed as candidates for chemoprevention or chemotherapeutic adjuvants for endocrine insensitive breast tumors.

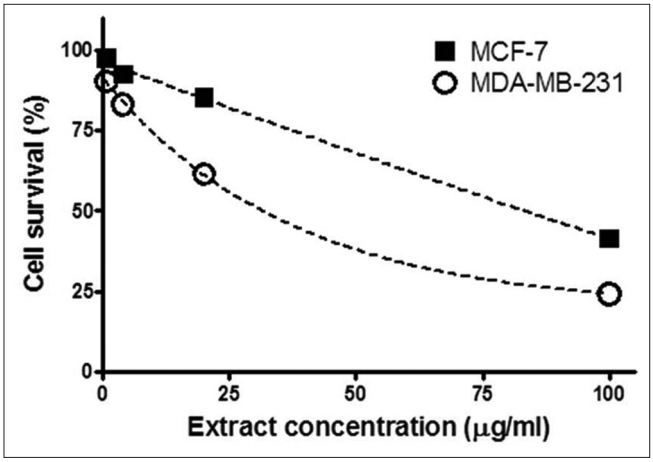

Figure 1: Cytotoxic effect of Scots pine needles extract on human breast cancer cell lines sulforhodamine B assay

Phytochemical analyses of pine needles have found numerous compounds as possible effective components.[13,15] Needles are especially rich in various polyphenols, which may exert different beneficial effects on human health.[13,25] Indeed, three structurally related 6-C-methyl flavonoids were recently isolated from twigs and leaves of *Pinus densata* Mast. showing some antiproliferative activity also on ER-positive human breast cancer ZR-75-30 cells.[26] Polyphenolic compounds are also major ingredients of pine bark extracts that contain more than 40 kinds of natural bioactive components among, which procyanidins are the major substances.[27,28] Pycnogenol as an extract from French maritime pine bark is a mixture composing mainly of procyanidins, flavonoids, and phenolic acids.[6,7,19,20] This extract lacks toxicity, is nonteratogenic and non-mutagenic, and can selectively induce cell death in human mammary cancer MCF-7 cells, but not in normal human mammary MCF-10 cells.[9,10,12,19,20] However, proanthocyanidins from bark extract of *P. massoniana* Lamb expressed only very weak inhibitory effect on the growth of human mammary cancer MDA-MB-231 cells with IC_{50} value essentially higher than 200 μg/ml.[29] Taken together, this can indicate that either the profile of specific polyphenolic compounds

Table 1: Cytotoxic activity of extract prepared from Scots pine (*Pinus sylvestris* L.) needles on various cancer cell lines measured by sulforhodamine B assay

Cancer cell line	IC_{50} (μg/ml)
MDA-MB-231	35.56±2.04
HL-60	52.16±1.24
SW480	52.94±1.54
LU-1	54.62±1.15
HepG2	57.87±0.78
MKN7	69.68±1.42
LNCaP	70.63±1.81
KB	79.22±1,25
MCF-7	86.37±1.60

IC_{50}: Half maximal inhibitory concentration; MDA-MB-231

with anticancer activity varies in distinct parts of pine tree being somewhat different in needles and bark; various pine species contain different bioactive compounds; or there are some nonphenolic substances responsive for the antiproliferative effect of *P. sylvestris* needle extract on ER-negative breast cancer cells.

In addition, the cytotoxic activity of both Scots pine extract and essential oil was compared by us on ER-positive MCF7 and ER-negative MDA-MB-231 using also tamoxifen citrate and ellipticine as a positive control [Table 2]. The data revealed that the Scots pine essential oil exhibited stronger cytotoxic effects on both breast cancer cell lines than the extract: IC_{50} values of oil were 28.67 and 29.23 μg/ml, respectively, but 80.20 and 42.27 μg/ml for the extract studied. Thus, the cytotoxic activity of the essential oil was practically the same on both cell lines.

It was only very recently demonstrated that essential oils from the needle extract of *Pinus roxburghii* Sarg. express a rather strong (about 70%) cytotoxic activity on human breast cancer MCF-7 cells at the dose of 100 μg/ml. This effect was likely related to the high concentrations of terpinen-4-ol, (*E*)-caryophyllene, and α-humulene in the needle essential oil.[15] However, the content of these substances in the essential oil hydrodistilled from fresh Scots pine needles of Estonian origin is only very small (0.1% to 0.3%), and the principal constituents were α-pinene (48.1%) and camphene (10.1) [Table 3]. Low concentrations of terpinen-4-ol and α-humulene (0.05–0.4%), as well as somewhat higher amount of (*E*)-caryophyllene (2.9%) has been determined in Estonian pine oil also previously.[30] It is interesting to compare that the essential oil of juniper (*Juniperus communis* L.) growing in Estonia contained at the mean 0.4% of terpinen-4-ol, 1.1% of (*E*)-caryophyllene, and 0.9% of α-humulene.[24]

This report describes for the first time the anticancer effects of needle extracts and essential oil obtained from *P. sylvestris* on various human malignant cell lines showing a clear cytotoxic selectivity to ER-negative breast cancer cells compared to ER-positive cell line. Although, the exact isolation and structure elucidation of the active compound(s) is needed to perform in the further studies it is likely that Scots pine needle extract and oil may serve as an easily accessible source of potential candidate for the development of novel therapeutic anticancer agents.

CONCLUSION

The Scots pine needles extract and essential oil exhibits some potential as chemopreventive or chemotherapeutic

Table 2: Cytotoxic activity of Scots pine needles extract and essential oil on MCF-7 and MDA-MB-231

Concentration (μg/ml)	50% inhibitory concentration (IC_{50} μg/ml)			
	Extract	Essential oil	Tamoxifen citrate	Ellipticine*
MCF-7				
100	58.41	101.87	98.11	84.99
20	18.83	39.60	84.38	71.94
4	8.75	18.42	30.26	45.66
0.8	2.61	5.24	−8.49	15.58
IC_{50}	80.20	28.67	8.66	0.50
MDA-MB-231				
100	69.39	97.58	101.00	86.06
20	35.72	41.86	99.79	76.07
4	18.01	18.53	17.13	42.50
0.8	3.04	12.67	1.07	11.24
IC_{50}	42.27	29.23	5.57	0.55

*The concentrations of ellipticine in those experiment were 10 μg/ml; 2 μg/ml; 0.4 μg/ml and 0.08 μg/ml. IC_{50}: Half maximal inhibitory concentration; MDA-MB-231

Table 3: Composition of the essential oil hydrodistilled from needles of *Pinus sylvestris*

Compound	RI DB-5	Content (%)
α-thujene	924	0.1
α-pinene	933	48.1
α-fenchene	942	-
Camphene	944	10.1
Thuja-2,4(10)-diene	950	tr
Benzaldehyde	952	0.1
Verbenene	963	-
Sabinene	972	0.7
β-pinene	975	3.5
3-octen-1-ol, propanoate	976	-
β-myrcene	990	3.2
2-carene	1001	0.1
α-phellandrene	1003	0.4
Δ-3-carene	1009	6.6
α-terpinene	1014	0.1
p-cymene	1023	0.3
Limonene	1025	-
β-phellandrene	1027	3.0
(E)-β-ocimene	1044	0.4
Isopentyl butanoate	1054	0.2
γ-terpinene	1056	-
Benzaldehyde, 2-methyl-	1058	0.1
(E)-4-pentenyl butanoate	1062	-
(Z)-sabinene hydrate	1064	-
Benzaldehyde, 4-methyl-	1072	0.1
Terpinolene	1086	1.3
(E)-sabinene hydrate	1095	-
Linalool	1098	0.2
n-nonanal	1100	tr
3-methylbutyl isovalerate	1101	-
α-thujone	1101	-
2-methylbutyl isovalerate	1105	-

Contd...

Table 3: Contd...

Compound	RI DB-5	Content (%)
β-thujone	1113	-
3-methyl-3-butenyl isovalerate	1115	-
1,3,8-menthatriene	1118	tr
α-campholenal	1120	0.1
trans-p-mentha-1(7),8-dien-2-ol	1132	0.1
Camphor	1135	0.1
3-methyl-2-butenyl valeriate	1146	-
Isoborneol	1158	0.3
Terpinen-4-ol	1169	0.2
p-cymen-8-ol	1180	0.1
α-terpineol	1184	0.1
Myrtenal	1190	tr
Myrtenol	1199	0.1
(Z)-verbenone	1211	-
β-citronellol	1224	-
Thymol methyl ether	1230	-
Carvone	1232	-
Isopentyl hexanoate	1245	-
Undecanal	1245	-
Piperitone	1245	-
Methyl citronellate	1258	-
Bornyl acetate	1280	7.6
Verbenyl acetate	1287	-
2-undecanone	1291	0.2
Thujic acid	1318	-
Myrtenyl acetate	1320	-
4-terpinenyl acetate	1330	tr
α-terpinyl acetate	1344	0.4
Citronellyl acetate	1350	-
α-copaene	1372	tr
Trans-myrtanyl acetate	1378	-
β-elemene	1388	0.4
Longifolene	1399	-
(E)-β-caryophyllene	1414	0.3
α-gurjunene	1428	tr
α-humulene	1447	0.1
γ-muurolene	1473	0.1
Germacrene D	1476	0.6
α-amorphene	1489	0.1
β-selinene	1490	0.2
α-muurolene	1494	0.3
γ-cadinene	1507	0.5
Germacrene A	1513	-
δ-cadinene	1517	1.7
Cadina-1,4-diene, trans-	1531	0.1
Germacrene B	1550	0.1
Spathulenol	1569	1.6
Caryophyllene oxide	1575	0.1
NI 3 (selinadiene alcohol?)	1589	-
Ethyl dodecanoate	1590	tr
10-epi-γ-eudesmol	1624	-
τ-cadinol	1635	1.4
δ-cadinol	1639	0.2
T-muurolol	1643	-
epi-α–cadinol	1646	-
α-muurolol	1648	2.0
α-cadinol	1661	-
Juniper camphor	1683	0.1
Nerolidol acetate	1718	-
Sclarene	1923	-
Palmitic acid	1974	0.1
Abietatriene	2049	-
Abietadiene	2071	-
Cembrene C	2095	0.5
Semberviol	2270	-
Abietal, 4-epi-	2278	-
Abietal	2292	-
(E)-totarol	2295	-
Total, %	-	98.4

tr: Traces (<0.05%); -: Not determined, DB-5: Poly (5%-diphenyl-95%-dimethyl) siloxane

agent for mammary tumors unresponsive to endocrine treatment.

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