ISSN: 2321-3132

Research Article

International Journal of Chemistry and Pharmaceutical Sciences

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Antiproliferative and Antioxidant activities of *Picea smithiana* (Wall) Boiss oil

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Abstract

Identificaton and investigation of *Picea smithiana* (Wall) Boiss oil for its antiproliferative and antioxidant activities. *Picea smithiana* (Wall) Boiss oil was extracted from aerial parts of the plant material by hydrodistillation method (0.06%). The oil was then subjected to GC-MS for identification of oil constituents. Oil (12.5, 25, 50, 100µg/ml) was also evaluated for its antiproliferative activity by *in-vitro* MTT {**3-(4,5-**Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide} assay against a panel of human cancer cell lines like human monocyte (THP-1), lung carcinoma (A-549), liver adenocarcinoma (HEP-1) and ovarian carcinoma (IGR-OV-1), while antioxidant activity was evaluated by *in-vitro* DPPH assay method. GC-MS of *Picea smithiana* oil indicated the presences 36-constituents were identified by mass spectroscopy which was the first time report from this plant, with delta-3-carene (26.49%), limonene (25.88%), and beta pinene (14.91%), α-pinene (6.62%), Camphene (4.84%), Alpha-terpinolene (2.48%), P-cymene (1.40%) and beta phelledrene (0.98%) as major constituents. *Picea smithiana* oil (12.5, 25, 50, 100µg/ml) showed significant antiproliferative activity in MTT assay against a panel of human cancer cell lines like human monocyte (THP-1), lung carcinoma (A-549), liver adenocarcinoma (HEP-1) and ovarian carcinoma (IGR-OV-1). The antioxidant activity was also found in the oil in significant manner. *Picea smithiana* oil was first time evaluated for its constituents and we found that it contains 32 constituents; some of these were in major quantity. *Picea smithiana* oil showed the antiproliferative activity in human monocyte (THP-1), lung carcinoma (A-549), liver adenocarcinoma (HEP-1) and ovarian carcinoma (IGR-OV-1) which may lead to treat lung, liver, ovarian and other cancers. Antioxidant activity also supports its antiproliferative action.

Keywords: *Picea smithiana* oil, Antiproliferative activity, Antioxidant activity, Gas-chromatography with Mass spectroscopy

Contents

Received 12 December 2013
Accepted 15 January 2014
Available Online 27 January 2014

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Manuscript ID: PRL2014-IJCPS1928

PAPER-QR CODE

1. Introduction

Picea smithiana is commonly known as *Morinda Spruce* belonging to family pinaceae. It is a spruce native to the western Himalaya and adjacent mountains, from northeast Afghanistan east to central Nepal. It grows at altitudes of 2,400-3,600 m in forests together with Deodar Cedar, Blue Pineand Pindrow Fir. Morinda Spruce is a popular ornamental tree in large gardens in western Europe for its attractive pendulous branchlets. It is also grown to a small extent in forestry for timber and paper production, though its slower growth compared to Norway Spruce reduces its importance outside of its native range. The name *morinda* derives from the tree's name in Nepalese. It is also found in Kashmir to Kumaon in india. Its essential oil used in bath salts, room sprays, deodorants and as antiseptic [1]. Plants from tropical regions are considered to be one of the potential sources for the screening of anticancer agents. Several essential oils have been exported as fragrances and flavors, for example, Citronella (Cymbopogon nadus L.) oil, Holy Basil (Ocimum sanctum L.) oil and Turmeric (Curcuma longa L.) oil. Some plant extracts have potential to be developed as drugs and neutraceuticals [2]. Essential oils are natural extracts of aromatic plants used in many fields like agriculture, aromatherapy and nutrition etc. The constituents of essential oils contribute the overall activity of each essential oil against target organisms, some of them have been used in cancer treatment. However, standard cancer chemotherapy is frequently compromised by the development of drug resistance and unwanted partly life-threatening side effects. Therefore, there is an urgent need for novel treatment options with improved features. Many plant-derived compounds, e.g., Paclitaxel, ACH-1, Vinblastine, or Vincristine, and semi-synthetic derivatives of natural products, such as Etoposide and Teniposide, are used as anti-cancer drugs. As pointed out recently, natural products from medicinal plants represent a fertile ground for the development of novel anticancer agents [3-5].

2. Materials and Methods

Plant material

The aerial parts of *Picea smithiana* were collected from Gulmarg region J&K, India. The plant material was identified by Dr. A.R. Naqshi, Head centre of plant taxonomy, University of Kashmir. Voucher specimen (2065) was deposited at KASH herbarium in Centre of Plant Taxonomy University of Kashmir, Hazratbal Srinagar for further references.

***Picea smithiana* oil extraction**

Each 100 g fresh aerial parts (needles) of plant material were subjected to hydrodistillation with 1 L of water using a Clevenger-type apparatus for 3 hours. Distillation was carried out twice for each plant, and the oils obtained for each were pooled, dried over anhydrous sodium sulphate and stored at 4°C in amber glass vials until analysis.

Gas chromatography-mass spectrometry analysis of the *Picea smithiana* oil

About 1 µL of aliquot of each oil sample, appropriately diluted in hexane, was subjected to gas chromatography-mass spectrometry (GC-MS) analysis. The GC-MS analysis was performed using a Varian GC-MS series 3800 with a VF-5MS column (60 cm × 0.25 mm; film thickness, 0.25 µm). The column temperature was kept at 60°C for 3 minutes then programmed to 280°C at a rate of 3°C/min and kept constant at 250°C for 1 minute. Flow rate of helium as a carrier gas was 1 mL/min. Each sample was analyzed twice.

In vitro assay for antiproliferative activity

The cell lines under investigation ware human monocyte (THP-1), lung carcinoma (A-549), liver adenocarcinoma (HEP-1) and ovarian carcinoma (IGR-OV-1). The cells were cultured in Cancer Pharmacology Division, Indian Institute of Integrative Medicine (IIIM) 1640 medium supplement with 10% heated fetal bovine serum, 1% of 2 mmol/L l-glutamine, 50 IU/mL penicillin, and 50 µg/mL streptomycin.

Antiproliferative assay

Assay was carried out by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) protocol to evaluate the antiproliferative effect. For this purpose, a sufficient number of exponentially growing cells were used to avoid confluence of the culture during the treatment. Cells (A-549, HEP-1, IGR-OV-1) were seeded at 10^4 cells/well and allowed to adhere for 12 hours. THP-1 cells were seeded at 2×10^3 cells/well and media was replaced with 200µl of fresh medium before treatment with oil. In order to evaluate the optimal concentration at which the oil inhibit the cell proliferation in all the four cell lines, cells were treated with concentrations ranging from 100µg - 10µg/ml. DMSO was used as a solvent for dilution of oil which was a control. Mytomycin-C and Paclitaxel were used as positive controls at a concentration of 61µg/ml and 45µg/ml respectively. After 48 h of treatment, cell growth was evaluated using a MTT assay [6-7]. MTT solution of 50 µl (5 mg/ml of PBS) was added to each well, and the plates were incubated for 3 hours at 37°C in dark. The media was aspirated and 150 µl of MTT solvent (4 mM HCl, 0.1 % Nondet P-40, all in isopropanol) was added to each well to solubilize the formazan crystals. The absorbance of the plates was measured on ELISA reader (Benchmark, BioRad) at a wavelength of 570 nm. Each sample was performed in triplicate, and the entire experiment was repeated thrice.

In vitro antioxidant activity by DPPH Assay

The capacity to scavenge the DPPH free radical was monitored according to a method previously reported. Various concentrations of sample extracts (0.3 mL) were mixed with 2.7 mL of methanolic solution containing DPPH radicals (6 x 10 x 5 M). The mixture was shaken vigorously and left to stand in the dark until stable absorption values were obtained. The reduction of the DPPH radical was measured by monitoring continuously the decrease of

absorption at 517 nm. DPPH scavenging effect was calculated as percentage of DPPH discolouration using the equation:

Antiradical activity=100 × (1-absorbance of sample/absorbance of reference)

Experiments were performed in triplicate [8-9].

Statistical analysis

All the values are expressed as means ± S.D. The results were analyzed statistically by Analysis of Variance (ANOVA) followed by by Dunnett's test. *p<0.05, **p<0.01.

3. Results and Discussion

***Picea smithiana* oil composition**

Picea smithiana oil contains 32-constituents but only the 30 constituents were identified by mass spectroscopy which was the first time report from this plant. Delta-3-carene (26.49%), limonene (25.88%), and beta pinene (14.91%), α-pinene (6.62%), Camphene (4.84%), Alpha-terpinolene (2.48%), P-cymene (1.40%) and beta phelledrene (0.98%) are major constituents. All important constituents major as well as minor are depicted in Table 1.

Table1: Chemical composition of the essential oil of *Thuja Orientalis*

S. No	Compound	% Peak Area	Methods of identification
1	α- Thugene	0.3	MS, RI, Std
2	**α- pinene**	6.6	MS, RI,
3	**Camphene**	**4.8**	MS, RI, std
4	sabinene	0.6	MS, RI,
5	β-pinene	14.0	MS, RI, std
6	α-Phellandrene	tr	MS, RI
7	**δ-3-Carene**	**26.0**	MS, RI, Std
8	α-Terpinene	0.2	MS, RI
9	P-Cymene	1.4	MS, RI
10	**Limonene**	**25.0**	MS, RI
11	β-Phellandrene	0.9	MS, RI
12	1,8-cineole	tr	MS, RI
13	γ-Terpinene	0.6	MS, RI
14	α-Terpinolene	2.4	MS, RI
15	Dehydro-p-cymene	0.1	MS, RI
16	Terpinen-4-ol	0.6	MS, RI
17	α-terpinol	0.2	MS, RI, std
18	α-Citronellol	0.2	MS, RI
19	Methyl thymol ether	0.1	MS, RI
20	Borneol -acetate	0.5	MS,RI
21	E-pinocarvyl acetate	0.4	MS, RI
22	Geranyl acetate	0.1	MS,RI
23	β- Bourbonene	0.2	MS,RI
24	Trans-Caryophyllene	0.8	MS,RI
25	α-Humulene	0.5	MS,RI
26	γ-Murolene	0.1	MS,RI
27	Germacrene-D	2.4	MS,RI
28	α-Muurolene	0.3	MS,RI
29	δ-Cadiene	1.4	MS,RI
30	Cis-Calamenene	0.1	MS,RI
31	Germacrene-D-4-ol	0.1	MS,RI
32	Spathulenol	0.1	MS,RI
33	Caryophyllene	0.3	MS,RI
34	Leden-oxide	0.2	MS,RI
35	α-Muurol	0.5	MS,RI, std
36	α-cadinol	1.1	MS,RI, std
Total (%)		**96.4**	
Monoterpene hydrocarbons		**85.5**	
Oxygenated monoterpenes		**2.2**	
Sesquiterpene hydrocarbons		**5.8**	
Oxygenated sesquiterpenes		**2.3**	

Table 2: Antiproliferative effects of volatile oils in human monocyte (THP-1), lung carcinoma (A-549), liver adenocarcinoma (HEP-1) and ovarian carcinoma (IGR-OV-1)

Materials	**Percentage growth inhibition (%)**			
Concentration of oil (µg/ml)	**THP-1 Lukemia**	**Lung (A-549)**	**Liver (HEP-2)**	**Overay IGR-OV-1**
DMSO	4 ± 0.31	3 ± 0.02	3 ± 0.02	1 ± 0.04
PS-12.5	91 ± 1.04**	71 ± 3.04**	52 ± 1.42**	48 ± 2.04**
PS-25	95 ± 2.43**	86 ± 4.02**	58 ± 1.56**	76 ± 3.01**
PS-50	95 ± 1.78**	87 ± 1.78**	69 ± 2.04**	78 ± 2.86**
PS-100	96 ± 2.08**	90 ± 1.04**	86 ± 3.05**	84 ± 3.89**
Pacilitaxel-45	23 ± 0.32**	87 ± 1.43**	34 ±1.03**	82 ± 4.56**
Mitomycin-C- 61	32 ± 0.42**	56 ± 2.81**	90 ±2.88**	29 ± 1.99**

Where, n=3, Values are expressed as Mean ± S.E.M. DMSO = Dimethylsalphoxide control sample, PS- 12.5µg/ml = Picea smithiana oil concentration of 12.5µg/ml, PS- 25µg/ml = Picea smithiana oil concentration of 25µg/ml, PS-50µg/ml = Picea smithiana oil concentration of 50µg/ml, PS- 100µg/ml = Picea smithiana oil concentration of 100µg/ml, Pacilitaxel- 45µg/ml = Pacilitaxel concentration of 45µg/ml, Mitomycin-C- 61µg/ml = Mitomycin-C concentration of 61µg/ml. The results were expressed as Mean ± SEM. Test and standard groups (Pacilitaxel, mitomycin-C) were compared with control group (DMSO), statistically analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's test. **P< 0.01.

Table 2: Antiradical activity of *Picea smithiana* oil

Materials	**DPPH RSA (%) (**antiradical activity)
***Picea smithiana* oil** (20µg/ml)	7.77%
***Picea smithiana* oil** (50µg/ml)	21.22%
***Picea smithiana* oil** (100µg/ml)	35.08%
α-tocopherol (20µg/ml)	64.67%
α-tocopherol (50µg/ml)	73.47%
α-tocopherol (100µg/ml)	78

Antiproliferative activity

Resulst showed maximum growth inhibition like 96, 90, 86, 84% when treated with 100 µg/ml oil at concentration of 100µg in MTT assay against a panel of human cancer cell lines like human monocyte (THP-1), lung carcinoma (A-549), liver adenocarcinoma (HEP-1) and ovarian carcinoma (IGR-OV-1) respectively. The standard drug paclitaxel at concentration 45µg/ml showed 23, 87, 34, 82% and mitomycin at concentration 61µg/ml showed 32, 56, 90, 29% in THP-1, lung carcinoma (A-549), liver adenocarcinoma (HEP-1) and ovarian carcinoma (IGR-OV-1) respectively Table 2.

Antioxidant activity:

The antioxidant potential of essential oils from *Picea smithiana* was determined by DPPH test. The reduction of DPPH absorption is indicative of the capacity of the oils to scavenge free radicals, independently of any enzymatic activity. The scavenging effects of essential oils on DPPH were examined at 100µg/ml concentrations. The absorbance decreases as a result of a colour change from purple to yellow as the radical is scavenged by antioxidants. All essential oils were able to reduce the stable free radical DPPH to the yellow-coloured 1,1-diphenyl-2-picrylhydrazyl**.** *Picea smithiana* oil (100µg/ml) showed 35% and standard α-tocopherol (100µg/ml) showed 78% antiradical activity Table 3.

Discussion

Currently chemotherapy is regarded as one of the most efficient cancer treatment approach. Although chemotherapy significantly improves symptoms and the quality of life of patients with cancer, only modest increase in survival rate can be achieved. Faced with palliative care, many cancer patients use alternative medicines, including herbal therapies [10]. Numerous cancer research studies have been conducted using traditional medicinal plants in the form of specific herbal extracts, and combinations to treat specific diseases including cancer, in an effort to discover new therapeutic agents that lack the toxic side effects associated with current chemotherapeutic agents [11]. Worldwide, breast cancer is the second most common type of cancer after lung cancer and the fifth most common cause of cancer death. Lukemia is one of the major disease in children and liver cancer is also growing because of day to day life style [12]. Hence it was decided to study anticancer effect of *Picea smithiana* oil on human monocyte (THP-1), lung carcinoma (A-549), liver adenocarcinoma (HEP-1) and ovarian carcinoma (IGR-OV-1) cell lines. ***Picea smithiana*** oil was first time evaluated for its constituents and was found to contain 32 constituents; some of these were in major quantity. *Picea smithiana* oil showed the antiproliferative activity in human monocyte (THP-1), lung carcinoma (A-549), liver adenocarcinoma (HEP-1) and ovarian carcinoma (IGR-OV-1). Mitomycin-C and Pacilitaxel were used as standards which inhibits the cancer by different mechanisms. Mitomycin C is a potent DNA

cross linker. A single crosslink per genome has shown to be effective in killing bacteria. This is accomplished by reductive activation followed by two N-alkylations. Both alkylations are sequence specific for a guanine nucleoside in the sequence 5'-CpG-3'. Potential bis-alkylating heterocylic quinones were synthetised in order to explore their antitumoral activities by bioreductive alkylation. **Paclitaxel**-treated cells have defects in mitotic spindle assembly, chromosome segregation, and cell division. Unlike other tubulin targeted drugs such as colchicine that inhibit microtubule assembly, however, paclitaxel stabilizes the microtubule polymer and protects it from disassembly. The inability of the chromosomes to achieve a metaphase spindle configuration leads to a mitotic block in which there is prolonged activation of the mitotic checkpoint with the subsequent triggering of apoptosis or slippage back into the G_1-phase of the cell cycle without cell division. The ability of paclitaxel to inhibit spindle function is generally attributed to its suppression of microtubule dynamics; but recent studies have demonstrated that suppression of dynamics occurs at concentrations lower than those needed to block mitosis. At the higher antimitotic concentrations, paclitaxel appears to act by suppressing microtubule detachment from centrosomes, a process normally activated during mitosis. The binding site for paclitaxel has been shown to reside on the beta-tubulin subunit *Picea smithiana* oil in different concentrations showed more inhibitions as compared to Mitomycin-C and Pacilitaxel. The *Picea smithiana* oil may follow both mechanisms. The mechanism of action of oil was not clear yet due to presence of number of constituents. *Picea smithiana* oil also showed antiradical activity in respect of **α**-tocopherol.

4. Conclusion

The results clearly show that ***Picea smithiana* oil** analysed in our study present strong cytotoxic activity against the four human tumour cell lines tested and antioxidant activity in DPPH assay. ***Picea smithiana* oil** could be regarded as promising drugs for cancer therapy, but the mechanisms of their anti-cancer activity and their toxicity should be further addressed. Further investigations are needed to isolate and identify active components of the studied oil and to confirm the pharmacological activity of these oils in more detail in animal models. The plant is rich in our biodiversity especially in the form of forest. The arial parts/leaves of plant are wasted every year in tons in the forest. This study may lead to utilize the plant in national as well as world economy. ***Picea smithiana* oil may** lead to treat lung, liver, ovarian and other cancers. Antioxidant activity also supports its antiproliferative action.

5. Acknowledgment

We are deeply grateful to Dr. A. R. Naqshi, centre of plant taxonomy, university of Kashmir, Hazratbal Srinagar for botanical identification of plant material

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