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Posted Date: 8 May 2024

doi: 10.20944/preprints202405.0419.v1

Keywords: *Betula pendula*; *Betula pubescens*; birch; cancer, cell lines; chaga; Estonia; glucans; Inonotus obliquus; triterpenoids

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Chemical Content and Cytotoxic Activity on Various Cancer Cell Lines of Chaga (*Inonotus obliquus*) Growing on *Betula pendula* and *Betula pubescens*

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Abstract: Chaga (*Inonotus obliquus*) is a pathogenic fungus that grows mostly on birch species (*Betula pendula* and *B. pubescens*) and has traditionally been used as an anti-cancer medicine. The study aimed to compare the chemical composition and cytotoxic activity of chagas growing on both *Betula* spp. on various cancer cell lines. The freeze-dried extracts contained triterpenes inotodiol, lanosterol betulin and betulinic acid typical to conks growing on *Betula* species. The cytotoxic activity of chaga growing on *Betula pendula* and *B. pubescens* 80% ethanolic extracts against 31 human cancer cell lines has been evaluated by a sulforhodamine B assay. The strongest inhibitions were observed with chaga (growing on *B. pendula*) extract on the HepG2 and CAL-62 cell line, and with chaga (from *B. pubescens*) extract on HepG2 cell line, with IC_{50} values of 37.71, 43.30, and 49.99 µg/mL, respectively. The chaga extracts from *B. pendula* exert somewhat stronger effects on most cancer cell lines studied than *B. pubescens* extracts, which can be attributed to higher content of inotodiol in *B. pendula* extracts. This study highlight the potential of chaga as a source of bioactive compounds with selective anti-cancer properties, encouraging further exploration of its therapeutic mechanisms and potential applications in cancer treatment. To the best of our knowledge, this study is the first investigation of the chemical composition of *I. obliquus* parasitizing on *B. pubescens*.

Keywords: silver birch; downy birch; Estonia; glucans; triterpenoids; inotodiol; betulin; betulinic acid

1. Introduction

A basidiomycete fungus chaga (*Inonotus obliquus*) typically occurs on the trunks of trees growing in the Northern Hemisphere [1]. This pathogenic fungus grows mostly on birch species (*Betula* spp.) and is less known on other trees [2,3]. As a real parasite, chaga kills the host organism to fulfil its lifecycle [4]. Due to being found mainly on birch trunks, the chaga drug is known in pharmacy as *Fungus betulinus*, not to be confused with another species, e.g., *Piptoporus betulinus*.

 doi:10.20944/preprints202405.0419.v1

As a natural source of biologically active substances, the chaga has been traditionally used as an anti-tomor, anti-inflammatory, antibacterial, hepatoprotective, and antioxidant natural remedy [5]. Chaga has been recognized for its medicinal uses since the sixteenth century [6]. Owing to containing a range of pharmaceutical and nutraceutical value, including polyphenols, triterpenoids, polysaccharides, and lignin, chaga has demonstrated diverse therapeutic effects. These encompass antioxidant, anti-inflammatory, antibacterial, antiviral, hepatoprotective, antidiabetic, anti-obesity, renoprotective, hepatoprotective, immunomodulatory, anti-tomor and anti-fatigue activities. Significantly, chaga has shown efficacy in anticancer activities [6–8].

The conks of *I. obliquus* have been the best known and most popular anti-cancer remedy in Estonian ethnomedicine among other 43 natural drugs [9]. Nowadays, chaga is rather well studied and primarily known as a promising natural material against cancer and for immunotherapy [10–14]. The anticancer activity of chaga has been studied by several researchers [11,15–20].

The aim of the study was to compare 1) chemical composition, and 2) cytotoxic activity of chagas growing on *Betula pendula* and *B. pubescens* on various cancer cell lines.

To the best of our knowledge, except for cytotoxic activity against HepG2 cell lines, the investigation of cytotoxicities against the tested cell lines of chaga parasitizing on *B. pendula* and *B. pubescens* for the first time.

2. Results

2.1. Phytochemical Study of Chaga

2.1.1. Extracts and Content of Biochemical Compounds

Freeze-dried ethanol extracts of chaga growing on two species, *Betula pendula* and *B. pubescens*, were prepared and analysed for triterpenoid composition and antioxidant activity (Table 1).

Table 1. The average content of triterpenoids and antioxidant activity of freeze dried extracts of chaga conks growing on *Betula pendula* and *B. pubescens*.

Antioxidant activity	Triterpenoids					Species
Aox GA eq [1] mg/g	Sitosterol mg/g	Lanosterol mg/g	Betulin mg/g	Betulinic acid mg/g	Inotodiol mg/g	
21.5±0.065	0.780±0.149	8.89±$^{0.497a}$	7.22±$^{0.104a}$	2.77±0.165	169±6.60^{a}	*B. pendula* dry extract
21.4±0.045	0.770±0.0964	11.1±0.364^{ab}	14.9±0.690b	2.05±0.476	149±2.30^{b}	*B.pubescens* dry extract

All values are given per g of freeze dried extract. All values are means ± standard deviation (n = 3); mean values within a column marked with different letters (a,b) are significantly different at $p < 0.05$. Differences between means were assessed with ANOVA and Post Hoc Tukey test. [1] Aox GA eq. mg/g antioxidant activity expressed as Gallic acid equivalent mg/g

Although the ratio of dried raw material and solvent were the same for both samples the yield of freeze dried dry extract from samples differed slightly. 50 g of *B. pendula* conks resulted in 1.26 g of dry extract and the same amount of *B. pubescens* gave 1.45 g of dry extract.

There were no significant differences in the content of sitosterol and betulinic acid. Also antioxidant activity evaluated using DPPH method did not reveal any differences between these extracts (Table 1). Significant differences were revealed between the content of lanosterol, inotodiol and betulin in the extracts from conks from *B. pubescens* and *B. pendula*. The content of inotodiol in these samples was negatively correlated with the content of betulin and lanosterol, while betulin and lanosterol content had a strong positive correlation.

 doi:10.20944/preprints202405.0419.v1

2.2. Cytotoxic Activity of Chaga

The cytotoxic activity of chaga (growing on *B. pendula* and *B. pubescens*) extract against thirty-one human cancer cell lines (Table 2) was evaluated by a sulforhodamine B assay [21,22]. The results of the cytotoxic assays are summarized in Table 3. These data showed that the strongest inhibitions were observed with chaga (growing on *B. pendula*) extract on the HepG2 and CAL-62 cell line and with chaga (growing on *B. pubescens*) extract on HepG2 cell line, with IC_{50} values of 37.71, 43.30, and 49.99 μg/mL, respectively. Chaga (growing on *B. pendula*) extract had no effect on NTERA-2, SK-LU-1, CL141, Huh-7R, NCI-N87, ACHN, AGS cancer cells (with IC_{50} values of > 100 μg/mL), and exhibited moderate inhibitory activity on other cell lines with IC_{50} values in the range of 55.29–97.73 μg/mL. Similarly, no activities were detected against MDA-MB-231, LNCaP, SK-LU-1, Hep3B, Huh-7, Huh-7R, MKN7, NCI-N87, AGS, SW626, SW480, HT29, ACHN, OCI/AML3 tested cell lines, and moderate cytotoxicities on the other cancer cell lines were observed with chaga (growing on *B. pubescens*) extract.

Table 2. Abbreviations of 31 human cancerous cell lines.

Basic characteristics	Name
Human carcinoma in the mouth	KB
Human breast adenocarcinoma	MDA-MB-231
Human breast carcinoma	MCF7
Human prostate carcinoma	LNCaP
Human lung carcinoma	SK-LU-1
Human lung adenocarcinoma	CL141
Human lung carcinoma	A549
Lewis lung carcinoma - high metastasis	LLC
Human hepatocellular carcinoma	HepG2
Human hepatocellular carcinoma	Hep3B
Human hepatocyte-derived carcinoma	Huh7
Drug resistant human hepatocyte-derived carcinoma	Huh 7R
Human acute leukemia	HL-60
Human differentiated human gastric adenocarcinoma	MKN7
Human gastric carcinoma	NCI-N87
Human gastric adenocarcinoma	AGS
Human stomach carcinoma	SNU-1
Human ovarian adenocarcinoma	SW626
Human cervix carcinoma	Hela
Human colon adenocarcinoma	SW480
Human colorectal adenocarcinoma	HT-29
Human rhabdomyosarcoma	RD
Human malignant melanoma	SK-Mel-2
Human kidney adenocarcinoma	ACHN
Human acute myeloid leukemia	OCI/AML3
Human chronic myelogenous leukemia	K562
Human acute T cell leukemia	Jurkat

 doi:10.20944/preprints202405.0419.v1

Human undifferentiated thyroid carcinoma	8505c
Human thyroid anaplastic carcinoma	CAL-62
Human urine bladder carcinoma	T24
Pluripotent human embryonal carcinoma	NTERA2

Table 3. Cytotoxic activity of chaga extracts on various cancer cell lines.

Cancer cells's line	Concentration (μg/mL)	Growth inhibition, %					
		Betula pendula		*Betula pubescens*		Ellipticine	
		Average	SD	Average	SD	Average	SD
KB	100	69.38	1.12	90.50	2.86	90.50	2.86
	20	20.49	1.65	70.14	1.96	70.14	1.96
	4	6.88	0.42	65.56	1.60	65.56	1.60
	0.8	-1.11	0.12	16.65	1.02	16.65	1.02
	IC_{50}	**63.35±2.89**		**74.90±1.38**		**0.49±0.03**	
MCF-7	100	56.34	2.08	53.92	2.04	90.55	3.21
	20	26.76	1.50	15.24	1.16	83.67	1.44
	4	12.11	1.19	5.27	1.15	53.23	1.08
	0.8	7.55	0.36	1.36	0.23	19.33	0.97
	IC_{50}	**77.92±4.49**		**90.87±3.82**		**0.61±0.05**	
MDA-MB-231	100	51.21	2.15	45.24	1.01	90.55	3.21
	20	12.79	1.01	14.74	0.63	83.67	1.44
	4	10.43	0.96	1.59	0.14	53.23	1.08
	0.8	4.33	0.14	-3.78	0.22	19.33	0.97
	IC_{50}	**97.73±3.78**		**>100**		**0.68±0.07**	
LNCaP	100	60.19	1.36	46.39	2.31	97.83	4.44
	20	26.13	0.78	12.97	1.13	80.77	1.51
	4	12.42	1.19	9.13	0.24	58.59	1.92
	0.8	6.63	0.51	1.54	0.12	31.77	1.42
	IC_{50}	**70.46±2.17**		**>100**		**0.44±0.04**	
SK-LU-1	100	43.08	1.59	28.57	1.82	97.72	2.28
	20	12.17	1.04	10.90	1.03	92.29	2.51
	4	5.35	0.13	-0.15	0.02	65.40	1.57
	0.8	0.74	0.04	-2.26	0.11	13.09	0.66
	IC_{50}	**>100**		**>100**		**0.45±0.05**	
CL141	100	46.10	1.37	53.20	2.52	98.79	1.56
	20	21.59	1.73	12.33	1.05	85.23	1.34
	4	4.39	0.37	-1.53	0.17	61.63	1.08
	0.8	1.72	0.12	-2.77	0.21	14.49	1.06
	IC_{50}	**>100**		**92.81±4.55**		**0.55±0.03**	
A549	100	54.11	2.07	57.18	1.31	89.70	2.90

 doi:10.20944/preprints202405.0419.v1

Cancer cells's line	Concentration (μg/mL)	Growth inhibition, %					
		Betula pendula		*Betula pubescens*		Ellipticine	
		Average	SD	Average	SD	Average	SD
	20	24.85	1.18	22.49	0.75	69.97	1.19
	4	12.96	0.83	5.52	0.56	53.98	1.10
	0.8	7.56	0.56	-0.94	0.05	25.15	1.04
	IC_{50}	**85.44±5.27**		**78.32±3.31**		**0.62±0.04**	
LLC	**100**	80.74	3.19	76.82	1.63	100.26	3.92
	20	23.07	1.78	22.49	1.06	90.22	2.18
	4	8.68	0.55	13.33	0.39	73.21	1.96
	0.8	-0.95	0.09	2.03	0.57	24.77	1.32
	IC_{50}	**52.29±3.57**		**55.89±2.37**		**0.35±0.03**	
HepG2	**100**	90.94	3.13	85.96	3.13	91.65	3.42
	20	31.91	1.26	24.65	1.63	83.80	2.45
	4	17.16	1.06	5.60	0.56	69.66	1.39
	0.8	2.74	0.23	4.65	0.53	26.16	1.06
	IC_{50}	**37.71±2.08**		**49.99±1.94**		**0.37±0.02**	
Hep3B	**100**	60.78	1.46	45.95	1.73	99.31	1.76
	20	17.38	1.40	17.92	0.80	82.09	2.26
	4	5.93	0.13	4.66	0.53	53.19	1.03
	0.8	-1.11	0.12	-3.92	0.28	18.23	1.92
	IC_{50}	**76.70±3.42**		**>100**		**0.73±0.04**	
Huh-7	**100**	52.24	1.34	43.02	1.75	98.82	2.42
	20	10.47	1.92	10.48	0.57	84.41	2.98
	4	2.55	1.96	-2.34	0.23	57.02	1.77
	0.8	-3.78	0.36	-4.14	0.31	16.32	1.03
	IC_{50}	**95.34±3.36**		**>100**		**0.56±0.05**	
Huh-7R	**100**	42.81	0.85	39.22	2.27	96.45	2.40
	20	13.62	1.01	6.67	0.40	82.09	1.21
	4	0.35	0.03	4.16	1.80	51.64	1.28
	0.8	-2.77	0.19	-0.56	0.03	18.18	1.52
	IC_{50}	**>100**		**>100**		**0.66±0.06**	
HL-60	**100**	64.65	1.87	71.73	1.74	82.15	3.43
	20	10.24	1.09	12.01	1.06	80.94	1.25
	4	0.60	0.04	0.67	0.08	54.42	1.83
	0.8	-1.59	0.12	-1.66	0.11	20.25	1.19
	IC_{50}	**79.02±2.82**		**71.21±2.41**		**0.64±0.05**	
MKN7	**100**	69.44	2.80	47.07	2.80	95.10	2.15
	20	15.72	1.54	11.74	0.69	91.13	1.57
	4	5.45	0.21	5.63	0.26	64.56	1.00

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Cancer cells's line	Concentration (μg/mL)	Growth inhibition, %					
		Betula pendula		*Betula pubescens*		Ellipticine	
		Average	SD	Average	SD	Average	SD
	0.8	-1.47	0.10	0.36	0.02	32.41	1.59
	IC_{50}	**68.85±4.06**		**>100**		**0.36±0.02**	
NCI-N87	**100**	47.57	1.10	35.08	2.73	85.64	2.98
	20	12.68	1.29	15.63	1.15	79.12	1.42
	4	7.82	0.36	0.98	0.03	51.92	1.76
	0.8	2.18	0.17	-1.11	0.12	16.90	1.33
	IC_{50}	**>100**		**>100**		**0.75±0.06**	
AGS	**100**	46.35	2.10	34.89	2.10	92.21	3.67
	20	13.34	0.63	13.69	1.47	81.27	1.32
	4	2.09	0.17	2.95	0.26	52.92	1.42
	0.8	-3.48	0.26	-2.66	0.12	21.04	0.54
	IC_{50}	**>100**		**>100**		**0.68±0.03**	
SNU1	**100**	52.32	1.70	51.02	1.59	94.73	3.70
	20	8.62	0.58	16.76	1.32	92.96	2.35
	4	0.79	0.08	4.42	0.48	67.30	1.81
	0.8	-1.56	0.13	0.97	0.05	20.36	1.40
	IC_{50}	**95.82±2.97**		**97.02±4.61**		**0.44±0.02**	
SW626	**100**	55.52	2.13	46.25	2.79	100.09	2.13
	20	21.43	1.58	10.00	1.32	92.09	2.51
	4	2.90	0.25	6.11	1.78	61.96	1.40
	0.8	-1.69	0.13	1.23	0.12	22.17	0.86
	IC_{50}	**82.88±5.84**		**>100**		**0.51±0.03**	
Hela	**100**	62.80	1.86	51.56	1.66	98.00	2.00
	20	23.77	0.95	19.53	0.88	97.00	1.09
	4	6.12	0.12	2.12	1.14	61.55	2.61
	0.8	1.89	0.16	-2.69	0.23	18.85	1.53
	IC_{50}	**68.50±3.45**		**94.28±4.69**		**0.50±0.04**	
SW480	**100**	53.84	1.62	45.25	2.71	96.33	2.91
	20	22.61	1.17	18.19	1.94	88.79	1.14
	4	10.23	1.02	4.07	0.36	55.73	1.50
	0.8	3.66	0.23	2.22	0.19	25.32	0.87
	IC_{50}	**87.10±5.19**		**>100**		**0.53±0.02**	
HT29	**100**	56.32	1.74	45.71	2.24	95.82	2.67
	20	15.85	1.38	26.46	0.73	93.62	1.72
	4	5.88	0.46	13.07	1.10	66.36	1.87
	0.8	1.66	0.12	5.15	0.28	30.68	1.59
	IC_{50}	**86.08±6.03**		**>100**		**0.37±0.03**	

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Cancer cells's line	Concentration (μg/mL)	Growth inhibition, %					
		Betula pendula		*Betula pubescens*		Ellipticine	
		Average	SD	Average	SD	Average	SD
RD	100	56.70	2.83	62.14	2.69	92.16	1.76
	20	17.47	1.43	10.09	1.25	79.32	1.45
	4	5.02	0.56	2.59	0.21	45.26	1.20
	0.8	1.33	0.11	-0.91	0.05	22.54	0.82
	IC_{50}	**84.12±5.95**		**82.08±3.96**		**0.74±0.02**	
SK-Mel-2	100	66.82	1.98	63.12	2.67	97.00	4.08
	20	24.41	1.06	20.91	1.34	95.90	2.33
	4	6.55	0.65	4.15	0.13	61.23	1.36
	0.8	1.87	0.12	1.22	0.10	14.20	0.56
	IC_{50}	**62.82±3.20**		**71.18±4.61**		**0.57±0.02**	
ACHN	100	46.83	2.35	39.18	1.33	84.62	2.85
	20	15.76	1.00	15.13	1.00	71.59	2.94
	4	5.19	0.34	7.18	0.67	52.21	1.06
	0.8	1.63	0.18	-1.69	0.12	18.95	1.12
	IC_{50}	**>100**		**>100**		**0.77±0.02**	
OCI/AML3	100	59.86	1.15	34.58	1.86	85.35	1.15
	20	35.60	1.73	27.65	1.29	84.74	1.44
	4	20.31	1.29	14.80	1.17	54.36	1.03
	0.8	8.64	0.65	4.69	0.24	24.32	1.25
	IC_{50}	**55.57±4.33**		**>100**		**0.57±0.03**	
K562	100	77.43	2.43	65.77	1.96	91.72	2.15
	20	19.91	1.38	16.59	1.01	81.50	1.23
	4	10.24	1.02	10.34	0.91	65.13	2.14
	0.8	5.36	0.25	5.64	0.34	20.50	0.81
	IC_{50}	**61.41±3.28**		**75.00±3.17**		**0.46±0.04**	
Jurkat	100	69.35	2.14	66.17	1.99	91.26	1.07
	20	22.01	1.11	10.84	1.07	81.23	0.46
	4	7.45	0.54	1.33	0.77	52.63	1.84
	0.8	2.94	0.13	-3.56	0.22	21.02	1.11
	IC_{50}	**63.48±3.15**		**76.16±2.88**		**0.68±0.05**	
8505c	100	54.91	1.11	41.47	3.19	101.87	1.24
	20	11.08	0.90	11.15	1.06	73.65	0.52
	4	5.09	0.51	2.54	0.39	53.80	2.47
	0.8	1.94	0.15	-1.74	0.16	19.73	1.84
	IC_{50}	**91.50±1.80**		**69.42±3.10**		**0.59±0.02**	
CAL-62	100	73.84	1.82	66.49	1.61	80.96	1.63
	20	35.08	1.22	17.63	1.43	71.77	1.84

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Cancer cells's line	Concentration (μg/mL)	Growth inhibition, %					
		Betula pendula		*Betula pubescens*		Ellipticine	
		Average	SD	Average	SD	Average	SD
	4	15.03	1.02	2.86	0.18	52.56	1.03
	0.8	6.87	0.35	-2.22	0.21	26.94	0.97
	IC_{50}	**43.30±2.52**		**69.42±3.10**		**0.59±0.02**	
T24	**100**	61.17	2.41	52.46	3.90	92.74	1.77
	20	18.11	1.58	13.94	1.00	87.92	1.07
	4	5.08	0.53	0.22	0.02	57.97	1.16
	0.8	1.93	0.15	-2.95	0.26	18.12	1.24
	IC_{50}	**76.76±4.71**		**93.81±5.66**		**0.54±0.02**	
NTERA-2	**100**	40.10	2.37	65.20	2.52	96.98	1.82
	20	21.59	1.73	18.33	1.35	95.23	0.34
	4	4.39	0.37	-1.53	0.17	61.63	1.38
	0.8	-4.71	0.24	-3.75	0.41	13.49	1.36
	IC_{50}	**>100**		**70.11±3.93**		**0.57±0.03**	

3. Discussion

The most abundant of the identified triterpenes was inotodiol as has been reported in the earlier studies [23]. The content of lanosterol, inotodiol and betulin varied between the extracts from *B. pubescens* and *B. pendula* significantly. Inotodiol content was higher in *B. pendula* extract and lanosterol and betulinic acid content in *B. pubescens* extracts. Correlation analysis revealed positive correlation between inotodiol content in the extract and the effect on growth inhibition on most of the tested cell lines. Negative correlation was revealed between inotodiol and growth inhibition only on cell lines NTERA-2 and 8505c.

Also, in previous study [4] the concentration of inotodiol was much more higher than other triterpenes lanosterol, betulin and betulinic acid. Upska et al. studied the extractability of chaga active ingredients using different methods and both polar and non-polar solvents. The best extractability of inotodiol was obtained by Soxhlet extraction using cyclohexane [24]. Inotodiol and lanosterol are both important compounds in chaga's anti-cancer effects [25,26]. Inotodiol and other lanostanes were effective against A549 cancer cells [27,28]. Inotodiol and lanosterol isolated from the conks of *Betula* spp. have immunological effect [13,29]. Inotodiol and lanosterol may also have cosmetic importance as they activate tyrosinase and increase pigment synthesis in skin cells [30]. Tian et al. showed that triterpenes of chaga may be an effective natural aid for treating and protecting various kidney diseases [31].

The antioxidant activity measurements performed using a DPPH assay showed same results for chaga from both *Betula* spp. The level of antioxidant activity may depend on the solvent used to extract chaga and the origin of the raw material. The difference in the DPPH scavenging activity may be up to 40 times different in chagas from various countries [10]. The scavenging activity of the water and ethanol extracts of chaga conks showed a similar level, but the methanol extract had the maximum IC_{50} values 18.96, 16.25, and 24.90 mg/mL, respectively [32].

In an earlier study [11], the methanol extract of chaga collected from the herb farm Kubja Ürditalu, Estonia, N59.054344, E25.963234, the commercial sample was purchased from a retail pharmacy in Tartu, Estonia, revealed potent cytotoxic effects against promyelocytic leukaemia and lung adenocarcinoma cells, with IC_{50} values of 32.2 and 38.0 μg/mL, respectively. Moreover, the extract showed moderate cytotoxicity (41.3–57.7 μg/mL) against cells from colon adenocarcinoma,

 doi:10.20944/preprints202405.0419.v1

liver hepatocellular carcinoma, oral epidermoid carcinoma, and prostate cancer [11]. This study assessed the anti-cancer properties of chaga (growing on *B. pendula* and *B. pubescens*) extracts across a diverse range of cancer cell lines. The results demonstrated a broad spectrum of cytotoxic effects, against 24 cancer cell lines for chaga (growing on *B. pendula*) extract including KB, MCF-7, LNCaP, A549, LLC, HepG2, Hep3B, HL-60, MKN7, SW626, Hela, SW480, RD, SK-Mel-2, OCI/AML3, K562, Jurkat, CAL-62, T24, Huh-7, HT29, 8505c, SNU1, MDA-MB-231, and against 17 cancer cell lines for chaga (growing on *B. pubescens*) extract including KB, MCF-7, CL141, A549, LLC, HepG2, HL-60, SNU1, Hela, RD, SK-Mel-2, K562, Jurkat, 8505c, CAL-62, T24, NTERA-2. Both chaga growing on these two host species possessed the most potential cytotoxic activities against HepG2 cell line, with IC_{50} values of 37.71 and 49.99 μg/mL, respectively. Furthermore, the extract of chaga (growing on *B. pendula*) exhibited remarkable cytotoxicity against the CAL-62 cell line, with an IC_{50} value of 43.30 μg/mL. Notably, liver cancer in men and thyroid cancer in women rank among the five most common types of cancer [20].

Here are some comparative data obtained using the same method in other natural products, which help to orient the strength of chaga's cytotoxic activity. The strongest anticancer effect of *Matricaria chamomilla* methanol extract against SK-MEL-2 cells was IC_{50} 40.7 μg/mL, but the extract of *Calendula officinalis* showed weak cytotoxic activity against SK-MEL-2 and KB cells (IC_{50} 62.6 and 79.2 μg/mL, respectively) [33]. The essential oils of *Anthemis sylvestris* roots and aerial parts had the strongest anticancer activity on KB cells (IC_{50} 19.7 μg/mL and 19.8 μg/mL, respectively), while the methanolic extract had no effect [22]. The *Pinus sylvestris* needle methanol extract suppresses the viability of MDA-MB-231 cells on the level IC_{50} 35 μg/mL [34]. In this context of anticancer activity, the effect of chaga's extract, having the strongest values between 37.7-43.3 μg/mL, showed remarkable but not strong effects.

Chaga has received considerable attention from many researchers due to its numerous biological attributes, particularly its potential anticancer effects. According to Ma et al., the petroleum ether and ethyl acetate extracts of chaga displayed significant cytotoxic activities against the human prostatic carcinoma cell PC_3 and breast carcinoma cell MDA-MB-231 [15]. The inhibitory effects were mainly attributed to ergosterol peroxide and trametenolic acid [15]. The aqueous extract of chaga in France containing a high content of betulin and betulinic acid and inotodiol showed an effect on human lung adenocarcinoma cells [18]. Noticeably, chaga extract was reported to markedly reduce HepG2 cell viability due to G0/G1-phase arrest and apoptotic cell death, thus leading to down-regulation of p53, pRb, p27, cyclins D1, D2, E, cyclin-dependent kinase (Cdk) 2, Cdk4, and Cdk6 expression [35]. Through matrigel-coated filters, the methanolic extract and its EtOAc-soluble fraction from Chaga collected in Japan showed the significant invasion inhibition of human fibrosarcoma HT 1080 cells. Importantly, compound 3*β*-hydroxylanosta-8,24-dien-21-al exhibited a strong inhibitory effect on HT 1080 cells. Furthermore, the methanolic extract of this sample significantly suppressed the formation of lung tomors in mice at 500 mg/kg/d [17]. The methanolic extract of Russian chaga purchased in Korea showed inhibitory effects toward four human lung adenocarcinoma cell lines (including A549, H1264, H1299, and Calu-6) through induction of apoptosis accompanied by caspase-3 cleavage. Significantly, compounds 3*β*-hydroxylanosta-8,24-dien-21-al, trametenolic acid, and 3*β*-hydroxy-5*α*lanosta-8,25-dien-21-oic acid isolated from this extract showed cytotoxicities against these cell lines, with IC_{50} values ranging from 75.1 to 227.4 μM in the same pattern [36]. The aqueous extract of chaga (in Korea) displayed anti-proliferative activity on HCT116 and DLDl cell lines, and reduced in intestinal polyps in $APC^{Min/+}$ and colon tomors in AOM/DSS-treated mice through downregulation of Wnt/β-catenin and NF- κB pathways [37]. The extract of chaga collected from birch trees in Japan successfully reduced tomors in both tomor-bearing mice and metastatic mice by promoting energy metabolism [16]. Additionally, the administration of chaga obtained in Japan could induce necrotic lesions, resulting in a decrease in the growth of the tomor and the weight of dog bladder cancer organoid-derived xenografts [19].

Current work and some others [4,11] indicate that *Inonotus obliquus* origin and the fungus host affects the source of useful bioactive compounds. *Inonotus obliquus* grows naturally on *Betula* spp. and on *Alnus* spp. as well as some other broadleaved trees in northern Baltic conditions [2,3]. In this area,

 doi:10.20944/preprints202405.0419.v1

the fungus grows on *B. pendula* and *B. pubescens* [2,3,38], and *B. pendula* is widely known host to the chaga [4], but not *B. pubescens*. However, nothing is known about the bioactive compounds of the chaga growing on *B. pubescens*. Current work shows evidently that the. bioactive compounds of chaga differ significantly according to the origin of quite close host species as *B. pendula* and *B. pubescens*. It indicates that the fungus origin and background are important for further analyses including effects on different human cancer cell lines.

These findings highlight the potential of chaga as a source of bioactive compounds with selective anti-cancer properties, encouraging further exploration of its therapeutic mechanisms and potential applications in cancer treatment.

4. Materials and Methods

4.1. Sample Sites, Fungal Isolation, Detection and Preparation

The conks of *I. obliquus were* collected from *B. pendula* and *B. pubescens* originate from Estonia between 21.12.2019 and 03.02.2022 (Table 4). The isolates were optained from fresh conks to grow on 2% malt-extract agar (Biolife, Milano, Italy) plates.

The DNA of *I. obliquus* isolates were extracted using a GeneJET Genomic DNA Purification Kit (Thermo Scientific, Vilnius, Lithuania) and the fungus was detected as described by Drenkhan et al. [39]. ITS-PCR products from the isolates were sequenced at the Estonian Biocentre in Tartu. The ITS sequences were edited using the BioEdit program, Version 7.2.5 [40] and deposited in a Genbank (see Table 4). BLAST searches for the fungal taxa confirmation were performed in the GenBank database (NCBI). All the pure cultures were deposited to the Fungal Culture Collection (TFC) and GenBank (NCBI).

Table 4. Origin and hosts of *Inonotus obliquus*, isolated from the conks and used in this study's fungal strains.

Accsession no in GenBank**	Fungal Collection code*	Sampling date	Geographical coordinates	Host	Strain no
OP019325	TFC101258	21.12.2019	N58.90373, E26.44672	*Betula pendula*	PAT29045
OP942253	TFC101271	30.12.2019	N58.06711, E26.42738	*Betula pendula*	PAT29055
PP346417	TFC101304	11.02.2020	N58.2567, E26.6659	*Betula pendula*	PAT29051
OP942256	TFC101274	09.11.2021	N58.52718, E22.91413	*Betula pendula*	PATKA880
OP942259	TFC101277	11.11.2021	N58.91147, E22.35235	*Betula pendula*	PATKA896
OP942263	TFC101281	27.01.2022	N57.82154, E27.48639	*Betula pendula*	PATKA1567
OP942264	TFC101282	27.01.2022	N57.82154, E27.48639	*Betula pendula*	PATKA1568
OP942268	TFC101286	25.01.2022	N59.01350, E27.60455	*Betula pendula*	PATKA1575
OP942272	TFC101290	03.02.2022	N59.27381, E25.36868	*Betula pendula*	PATKA1679
OP942273	TFC101291	03.02.2022	N59.27381, E25.36868	*Betula pendula*	PATKA1680
OP942269	TFC101287	25.01.2022	N59.01654, E27.44207	*Betula pubescens*	PATKA1576
OP942270	TFC101288	25.01.2022	N59.01367, E27.53098	*Betula pubescens*	PATKA1577
OP942260	TFC101278	11.11.2021	N58.98419, E22.72165	*Betula pubescens*	PATKA900
PP346418	TFC101305	17.10.2020	N58.9065, E26.0808	*Betula pubescens*	PATRD3354
PP346419	TFC101306	29.10.2020	N58.3203, E25.7080	*Betula pubescens*	PATRD3364
PP346420	TFC101307	05.03.2021	N58.3776, E25.9267	*Betula pubescens*	PATRD3402
-	TFC101308	15.03.2021	N58.9566, E25.4320	*Betula pubescens*	PATRD3355_1
PP346421	TFC101309	27.10.2021	N58.6825, E25.6854	*Betula pubescens*	PATRD3356
PP346422	TFC101310	17.12.2021	N59.4096, E26.6767	*Betula pubescens*	PATRD3386

PP346423	TFC101311	17.12.2021	N59.4932, E26.5887	*Betula pubescens*	PATRD3385

* Tartu Fungal Collection in Estonian University of Life Sciences, Estonia (TFC). **ITS sequences in GenBank (NCBI).

Before extractions and biochemical analysis, the conks were kept in a freezer at -20 °C. Then, the collected conks were dried in a laboratory oven (MMM Medcenter Einrichtungen GmbH, Planegg, Germany) at 50 °C, and all the material was ground to a coarse powder using a cutting mill Retch SM 300 with a 1 mm sieve (Retsch, Haan, Germany). Extracts were prepared from the mix of 10 individual conks samples per host species in equal proportions (see Table 4). For extract preparation 500ml of 80% ethanol was added to 50g of dried and milled sample and agitated at room temperature for 12 hours using Biosan ES20 orbital shaker. After vacuum filtration, the extracts were concentrated on rotary evaporator RV 10 control Flex (IKA-Werke GmbH & Co. KG. Germany) to remove most of the ethanol and further dried in the freeze-drier VirTis AdVantage 2.0 EL (SP Scietific, USA). Bioactive compounds detection from *I. obliquus* was done according to [4].

Antioxidant activity measurements were performed in triplicate using a 2.2-diphenyl-picrylhydrazyl (DPPH) assay [41]. The absorbance values of the samples were measured at 515 nm using a spectrophotometer (UV-1800, Shimadzu, Japan). The results were expressed in mg of gallic acid equivalent per g of dry weight (mg GA eq./g).

Qualitative and quantitative analyses were performed on a Shimadzu Nexera X2 UHPLC with mass spectrometer LCMS 8040 (Shimadzu Scientific Instruments, Kyoto, Japan). The UHPLC system was equipped with a binary solvent delivery pump LC-30AD, an autosampler Sil-30AC, column oven CTO-20AC and diode array detector SPD-M20A. Five triterpenoids (betulinic acid, betulin, lanosterol, inotodiol and sitosterol) were identified by comparing the retention times and parent and daughter ion masses with those of the standard compounds. Chromatographic separation was performed using Ascentis Express column (C30 50 x 4,6mm, Merck, Germany) at 40 °C. The flow rate of the mobile phase was 1 mL/min, and the injected sample size was 1 µL. Mobile phases consisted of 1% formic acid in Milli-Q water (mobile phase A) and 1% formic acid in acetonitrile (mobile phase B). Separation was performed for 10 min at isocratic conditions with 92% of mobile phase B and 8% of mobile phase A. All samples were kept at 4 °C during the analysis. MS data acquisitions were performed on LCMS 8040 with the APCI source. All samples were analysed in triplicate, and the results were expressed as mg per g of dry weight (mg/g).

The standards betulin, betulinic acid and lanosterol were purchased from Cayman Chemical Company (USA) and inotodiol from Aobious (USA). All other standards (sitosterol, gallic acid) and chemicals (formic acid, methanol) were used of analytical grade and purchased from Sigma (Germany).

The dry matter content in the samples was determined at 105 °C using moisture analyser Precisa EM 120 HR (Precisa Gravimetrics AG, Switzerland).

4.2. Cytotoxic Assay

A total of 31 human cancer cell lines (Tables 1 and 2) were cultivated at 37 °C in a humidified atmosphere containing 5% carbon dioxide. SRB cytotoxic assay for monolayer cells has been analyzed according to the method described in [42–44].

The 80% ethanolic dry extracts were firstly stocked in dimethyl sulfoxide (DMSO) 100% at the concentration as 20 mg/mL. The samples were then prepared in a range of diluted concentrations as 2000 µg/mL - 400 µg/mL - 80 µg/mL - 16 µg/mL using basic RPMI medium (w/o FBS) in 96 sample plate. Then, 10 µL from each well (with the diluted sample above) will be added into the tested cell pre-seeded (190 µL) well to evaluate cytotoxic activities. Thus, the final concentration of the sample will be 100 µg/mL - 20 µg/mL - 4 µg/mL - 0.8 µg/mL. Ellipticine was tested with the final concentration ranging as 20-4-0.8-0.16 µg/mL.

The effects of *Betula* chaga hydroethanolic extracts on the viability of malignant cells were determined by sulforhodamine B cytotoxic assay [43,44]. Briefly, cells were grown in 96-well microtiter plates, each containing 190 µL of medium. After 24 h, 10 µL of test samples dissolved in

 doi:10.20944/preprints202405.0419.v1

DMSO were added to each well. One plate with no samples served as a day 0 control. The cells were continuously cultured for an additional 48 h, fixed with trichloroacetic acid, and stained with sulforhodamine B, followed by determining optical densities at 515 nm using a Microplate Reader (BioRad, California, USA). The percentage of growth inhibition was calculated using the following equation:

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

where OD is the optical density or absorbance values. The potent anticancer agent ellipticine was used as a positive control.

4.4. Statistics

Biochemical analysis data were analysed using Jamovi software [45]. Metabolite concentrations were analysed by one-way analysis of variance (ANOVA). Differences between means were assessed with Post Hoc Tukey test. The IC_{50} values were analyzed using TableCurve 2Dv4 software.

5. Conclusions

The strongest inhibitions were observed with both hosts (*Betula pendula* and *B. pubescens*) based chaga extracts on the HepG2 cell line. In general, the cytotoxic activity of chaga extracts has been at a moderate or weak level.

The *B. pendula* origin chaga extracts exert somewhat stronger effects on most cancer cell lines studied than *B. pubescens* parasitizing chaga extracts. This effect can be attributed to higher content of inotodiol in *B. pendula* origin extracts in comparison to *B. pubescens.* This is also the first comparative investigation of the chemical composition of *I. obliquus* parasitizing on *B. pendula* and *B. pubescens*.

The results of the study highlight the potential of chaga as a source of bioactive compounds with selective anti-cancer properties, encouraging further exploration of its therapeutic mechanisms and potential applications in cancer treatment.

Author Contributions: Conceptualization, A.R., H.K., O.K., H.T.N., and H.K; methodology, A.R., R.D., H.K., K.V.N. and H.T.N.; software, H.K..; validation, H.K., U.B. and K.V.N.; formal analysis, H.K., A.R.,A.A., and K.V.N.; investigation, H.K., U.B., M.S., K.A., A.A., M.E., and K.K.; resources, M.S., K.A., K.K., M.V., A.A., and M.E.; data curation, K.J., H.K., A.R., O.K., H.T.N. and R.D.; writing—original draft preparation, A.R., H.K., K.V.N., H.T.N., O.K., M.S. and R.D.; writing—review and editing, A.R., O.K. and R.D.; visualization, H.K., U.B. and A.R.; supervision, R.D., H.T.N. and A.R..; project administration, R.D., H.K., and A.R.; funding acquisition, R.D. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Inopure OÜ, Estonian University of Life Sciences project PM220078MIME, the Estonian Research Council, Grant PRG1615 and the European Regional Development Fund's project "PlantValor—full-scale product development service in synergy with the traditional activities of Polli Horticultural Research Centre" 2014-2020.5.04.19-0373.

Acknowledgments: The research was conducted using the „Plant Biology Infrastructure – TAIM" funded by the Estonian Research Council (TT5).

Conflicts of Interest: The authors declare no conflict of interest.

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