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Chaga Mushroom Extract as a Dual-Action Agent against Microbial and Cancerous Cells: An *In Vitro* Study

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ABSTRACT

The present study investigates the bioactive properties of the Ethyl Acetate Extract of *Inonotus obliquus* (EAEIO), sourced from the Chaga mushroom. Traditionally used in medicine, this mushroom is increasingly recognized for its potent antimicrobial and anticancer benefits. Our analysis sought to explore the impact of EAEIO on four diverse bacterial strains: *Escherichia coli* ATCC25922, *Bacillus cereus* EMCC1080, *Pseudomonas aeruginosa* ATCC10145, and *Listeria monocytogenes* NCTC7973, employing the disc diffusion method. We also investigated the potential cytotoxic effect of EAEIO on MCF7 breast cancer cells, HCT16 colon cells, and normal BHK cells using the MTT assay.

Our results underscore the effective antimicrobial properties of EAEIO, evidenced by inhibition zones between 16 mm to 28 mm and minimum inhibitory concentrations (MICs) ranging from 6.25 µg/mL to 1.563 µg/mL. In addition, the EAEIO demonstrated remarkable anticancer activity against MCF7 and HCT16 cell lines, with IC50 values of 7.56 µg/mL and 11.2 µg/mL, respectively.

To conclude, EAEIO - the Ethyl Acetate Extract of the Chaga mushroom, exhibited significant antimicrobial and anticancer properties while showing no toxic effect on BHK cells. These observations suggest EAEIO's potential as a valuable natural resource for antimicrobial and anticancer treatments. However, further research is essential to verify the safety and efficacy of EAEIO in cancer and infectious disease management.

1. INTRODUCTION

The Chaga mushroom (*Inonotus obliquus*), a staple in traditional medicine for many centuries, is cherished for its many health benefits. It thrives on birch trees in colder climates like Northern Europe, Russia, Korea, and Japan. Historically, it has been harnessed to mitigate a variety of ailments, spanning gastrointestinal conditions, pain, inflammation, and even cancer.

Investigations into the chaga mushroom's chemical composition have revealed an assortment of bioactive compounds, among which are polysaccharides, as documented Lu *et al.*, (2021). Jin *et al.*, (2022) have reported on the presence of triterpenes, and Drenkhan *et al.*, (2022) have pointed out the existence of betulin and betulinic acid.

These elements have shown antimicrobial properties, as highlighted by Basal *et al.,* (2021), Eid *et al.,* (2021), and Garádi *et al.,* (2021), antioxidant properties, as mentioned by Wang et al., (2018), and anticancer properties, as reported by Lee *et al.,* (2021). Shen *et al.,* (2022) demonstrated that the mushroom's polysaccharides have immunomodulatory and anti-inflammatory capabilities, as further supported by Sun *et al.,* (2022) and Zhang *et al.,* (2023). Géry *et al.,* (2018) and J. Kim *et al.,* (2020) linked triterpenes and betulinic acid to antitumor and anti-metastatic activities.

Despite significant advancements in cancer treatments like chemotherapy and radiation therapy, cancer remains a prevalent global health issue. These conventional treatment modalities often present various side effects, leading to the need for alternative, less harmful, and more efficient treatment methods. As such, Gielecińska *et al.,* (2023) and Soto *et al.,* (2023) indicated a surge of interest in natural remedies, including medicinal mushrooms like the chaga mushroom, as also highlighted by Asma *et al.,* (2022).

Previous research, both *in vitro* and in vivo, has explored the anticancer potential of chaga mushroom extracts, as per Abugomaa *et al.,* (2023). Their findings indicated these extracts' ability to inhibit a variety of cancer cell lines Lee et al., (2021), induce apoptosis or programmed cell death in cancer cells Su et al., (2020), and suppress tumor expansion and metastasis in animal models Arata *et al.,* (2016).

In addition to their anticancer capabilities, Glamočlija *et al.,* (2015) reported that chaga mushroom extracts possess significant antimicrobial action against a diverse array of bacteria. This action is linked to the mushroom's abundant polysaccharides and triterpenes. İnci *et al.,* (2022) carried out *in vitro* experiments showing that medicinal mushroom extracts exhibit broad-spectrum antimicrobial activity against bacteria such as Staphylococcus aureus, *Escherichia coli*, and *Bacillus cereus*. Cör *et al.,* (2018) and Zhou *et al.,* (2019) attribute the antimicrobial potency of chaga mushroom extracts to the presence of polysaccharides and triterpenes.

Further research is required to fully understand the potential of chaga mushroom extracts as antimicrobial and anticancer agents, despite promising findings in previous studies. The extraction of bioactive compounds from natural sources holds interest due to their potential health benefits. As per Ma *et al.,* (2013) and Xu *et al.,* (2015), ethyl acetate, a commonly used solvent, can extract bioactive compounds from various sources, including chaga mushroom, which shows potent antioxidants.

This research examines the antimicrobial and anticancer capabilities of the ethyl acetate extract from chaga mushrooms. The study tests the extract's cytotoxic effects on breast (MCF7) and colon cancer (HCT16) cell lines, its antimicrobial strength against various bacteria, and identifies its bioactive compounds. It's the first study to explore chaga mushroom's ethyl acetate extract's properties against MCF7 and HCT16 cancer cell lines. The goal is to shed light on the potential use of this extract as an alternative cancer treatment and prevention method and as an antimicrobial agent.

MATERIALS AND METHODS

1. Reagents and Reference Compounds:

Ethyl alcohol pure p.a. and LC-MS-grade methanol and formic acid (purity ≥ 98%) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The phosphate-buffered saline (FBS) and trypsin were obtained from Corning, while fetal bovine serum (FBS) was from Capricorn Scientific (Ebsdorfergrund, Germany). Sodium dodecyl-sulfate (SDS) was acquired from PanReac Applichem (Darmstadt, Germany), dimethylformamide (DMF), and dimethyl sulfoxide (DMSO, p.a.) from Avantor Performance Materials (Gliwice, Poland), while 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from Sigma-Aldrich (St. Louis, MO, USA). The chaga mushroom powder was purchased from Chi Chaga Foods (Brownsburg, QC, Canada).

The ethyl acetate organic solvent was purchased from Sigma-Aldrich.

2. Extraction of the Active Metabolites from Chaga Mushroom:

First, ten grams of chaga mushroom powder was mixed with 200 ml of ethyl acetate, and the mixture was incubated overnight at 50^{o}c with agitation at 200 rpm. Then the extracted solution was separated from chaga powder by centrifugation. The insoluble residue was treated twice again with the same method to increase the yield of the extracted compounds according to Nguyen *et al.,* (2023). Finally, the collected supernatants were evaporated using a vacuum rotary evaporator to obtain the crude extract which was used in further experiments.

3. LC-MS/MS Analysis:

The sample analysis was performed using liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) with an ExionLC AC system for separation and a SCIEX Triple Quad 5500+ MS/MS system equipped with electrospray ionization (ESI) for detection. The separation was carried out using an Ascentis® Express 90 Å C18 Column (2.1×150 mm, 2.7 µm) in both positive and negative ionization modes. The mobile phases consisted of two eluents A (5 mM ammonium formate at pH 3 for positive ionization mode and pH 8 for negative ionization mode) and B (LC grade acetonitrile). The mobile phase gradient was programmed as follows: 5% B at 0-1 min, 5-100% B from 1-20 min, 100% B from 20-25 min, 5% at 25.01, and 5% from 25.01-30 min, with a flow rate of 0.3 ml/min and an injection volume of 5 µl. For MS/MS analysis, negative ionization mode was applied with a scan (EMS-IDA-EPI) from 100 to 1000 Da for MS1 with the following parameters: curtain gas at 25 psi; IonSpray voltage at 5500 for positive ionization mode and -4500 for negative ionization mode; source temperature at 500°C; ion source gas 1 & 2 at 45 psi; and from 50 to 1000 Da for MS2 with a declustering potential of 80 for positive ionization mode and -80 for negative ionization mode, collision energy at 35 for positive ionization mode and -35 for negative ionization mode, and collision energy spread at 15. Compounds' identification was performed using MS-DIAL

4. Antibacterial Activity of The Extract:

EAEIO was subjected to tests to evaluate their antibacterial activities. These were done by use of the disc-diffusion method. Reference strains of bacteria from the American Type Culture Collection (ATCC, LGC Standards, Teddington, UK) and Centers for Disease Control and Prevention (CDC, Atlanta, GA, USA) were used in the study. These strains included two Gram-positive bacteria (*Bacillus cereus* EMCC1080, *Listeria monocytogenes* NCTC7973) and two Gram-negative bacteria (*Escherichia coli* ATCC25922, *Pseudomonas aeruginosa* ATCC10145). The purity of the bacteria was tested by culturing on nutrient agar and being maintained on nutrient agar slants.

The disc diffusion method was used. One mg of the chaga crude extract was dissolved in 5 ml DMSO, then the paper discs were impregnated with 5 µl of the dissolved extract. After that the discs were placed upon Müller-Hinton (MH) plates inoculated with 0.5 Mcfarland standard of the tested bacterial pathogens. Plates were incubated at 37^{o}c for 24 hr, then the inhibition zone diameters (mm) were measured. Negative control was only treated with DMSO. Positive control was treated with Chloramphenicol. Minimum inhibitory concentration (MIC) was carried out in Elisa plate with starting concentration of 100 µg/ml and diluted in bifold dilution, Chloramphenicol was used as a positive control.

5. Cell Culture:

Cell lines used for *in vitro* experiments included MCF7 (ATCC, human breast cancer), HCT16 (ATCC, human colon cancer), and BHK (ATCC, baby hamster kidney). The cells were cultured using Roswell Park Memorial Institute-1640 (RPMI-1640) medium RPMI medium supplemented with 10% (v/v) FBS, antibiotics (streptomycin 10 l g/ml, penicillin 100 U/ml). Cell lines were obtained from National Oncology Institute, Egypt. The cells

were cultured using Roswell Park Memorial Institute-1640 (RPMI-1640) medium RPMI medium supplemented with 10% (v/v) FBS, antibiotics (streptomycin 10l g/ml, penicillin 100 U/ml). Cells were trypsinized, sub-cultured and allowed to grow till confluency. Cells were maintained at 37ºC in a 5% CO2 atmosphere in a humidified incubator.

6. Cell Viability Measured by MTT Assay:

Different concentrations of ethanol extract of chaga mushroom were tested on all cell lines to evaluate the toxicity by MTT [3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide]- based colorimetric assay. Hela cells (1 x 10^4) cells were seeded wells of a 96-well microtiter plate and incubated for 24 h. Then, the cells were exposed to different concentrations of extract (10–500 lg/ml) at (37ºC, 5% CO_2 for 24 h incubation). After incubation, cells were washed with PBS, a concentration of (0.5 mg/ml) of MTT dye in each well and allowed the incubate dark at (37ºC and 5% CO_2 for 4 h). Finally, 100 ml of dimethyl sulfoxide (DMSO) was added to dissolve the purple formazan crystal in the reaction. The optical density (OD) was determined at 570 nm in an ELISA plate reader r (SpectraMax M5-Molecular Devices, USA).

7. Statistical Analysis

The present statistical analyses were executed using Statistical Package for Social Science (SPSS) software version 22. According to Kolmogorov–Smirnov test, data were normally distributed. An Independent t-test was applied to illustrate the statistical differences in the studied parameters in each of the experimental groups, as compared to the controls and nicotine-treated groups. $P<0.05$ represents significant differences. Data were displayed as mean ± standard error of the mean.

RESULTS

1. LC-ESI-MS/MS Analysis of EAEIO:

EAEIO was meticulously analyzed using LC-ESI-MS/MS under both negative and positive ionization conditions to elucidate the diverse spectrum of bioactive mycochemicals.

a. Major Bioactive Mycochemicals Detected in EAEIO (Negative Ionization):

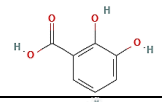
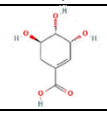
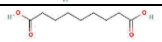
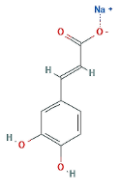
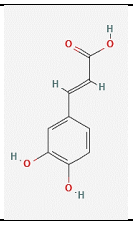
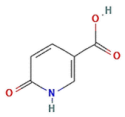
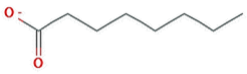
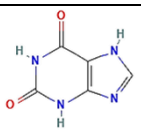
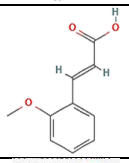
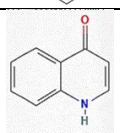
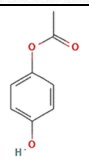
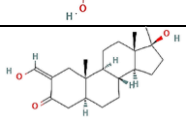
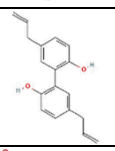
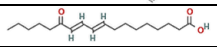
Figure 1 illustrates the LC-ESI-MS/MS profile for EAEIO, obtained under negative ionization. This analysis uncovered a variety of bioactive compounds, each represented by a unique peak in the profile. Table 1 details the major compounds identified, including their retention times. For instance, 2,3-Dihydroxybenzoic acid, Shikimic acid, and Azelaic acid were detected early in the analysis, with a retention time of 0.969883 minutes. Other compounds such as Octanoate and Xanthine were detected later, with retention times of 1.672433 and 3.122583 minutes respectively. More complex compounds, such as Oxymetholone and Magnolol, had retention times of 7.854533 and 13.35777 minutes, respectively. The longest retention time recorded in our analysis was 28.29398 minutes, identified for both Allopurinol and 3-Methylbenzoic acid.

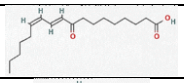
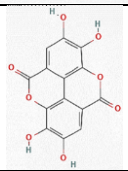
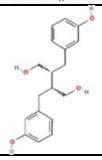
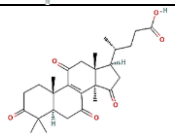
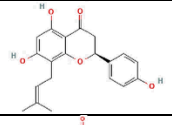
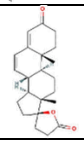
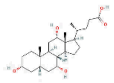
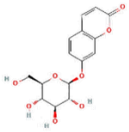
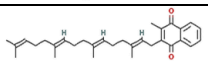
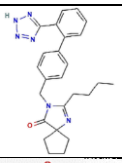
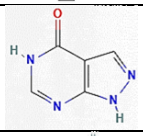
b. Major Bioactive Mycochemicals Detected in EAEIO (Positive Ionization)

When LC-ESI-MS/MS analysis of EAEIO was conducted under positive ionization conditions, as represented in Figure 2, a distinct set of bioactive mycochemicals was detected. The major compounds identified and their respective retention times are enumerated in Table 2. Lauryldiethanolamine was detected at 12.83568 minutes, and Berberine at the longest retention time of 22.58867 minutes.

The broad range of compounds revealed under both negative and positive ionization conditions illuminates the rich and complex mycochemical composition of *I. obliquus*, reinforcing its potential as a potent source of bioactive compounds with significant therapeutic implications.

Table 1: Major Bioactive Mycochemicals Detected in the EAEIO (Negative Ionization).

Sr. No	RT (min)	m/z	Type	Metabolite name	Chemical structure	Area%	Chemical formula
1	0.969883	153	[M-H]-	2,3-Dihydroxybenzoic acid		2.409344	$C_7H_6O_4$
2	0.969883	173.04	[M-H]-	Shikimic acid		0.191534	C7H10O5
3	0.969883	187.06	[M-H]-	Azelaic acid		0.527284	C9H16O4
4	1.044983	178.8	[M-H]-	Caffeate		0.295219	C9H7NaO4
5	1.109683	179.16	[M-H]-	Caffeic acid		0.942806	C9H8O4
6	1.6203	137.88	[M-2H]2-	6-Hydroxynicotinate		3.501874	C6H5NO3
7	1.672433	142.92	[M-H]-	Octanoate		0.429371	C8H15O2-
8	3.122583	150.96	[M-H]-	Xanthine		1.735176	C5H4N4O2
9	5.763967	177.04	[M-H]-	2-Methoxycinnamic acid		9.30528	C10H10O3
10	7.3277	144.12	[M-H]-	4-Hydroxyquinoline		0.443275	
11	7.7666	151.08	[M-H]-	4-ACETOXYPHENOL		1.054294	C8H8O3
12	7.854533	331.32	[M-H]-	Oxymetholone		0.365964	C21H32O3
13	13.35777	265.08	[M-H]-	Magnolol		0.135265	C18H18O2
14	13.43105	293.28	[M-H]-	13-KODE		2.636009	C18H30O3

15	13.6876	293.28	[M-H]-	9-KODE		1.228966	C18H30O3
16	18.70467	300.96	[M-H]-	Ellagic acid		0.339901	C14H6O8
17	18.70467	301.08	[M-H]-	Enterodiol		0.522419	C18H22O4
18	20.53217	455.04	[M-2H]2-	Lucidenic acid F		1.602289	C27H36O6
19	22.1532	339.12	[M-2H]2-	8-Prenylnaringenin		1.783949	C20H20O5
20	22.1532	339.24	[M-2H]2-	Canrenone		1.773427	C22H28O3
21	25.03673	407.16	[M-H]-	Cholic acid		0.141548	C24H40O5
22	27.43737	358.92	[M-H]-	skimmin		0.272809	C15H16O8
23	27.43737	443.28	[M-2H]2-	Menaquinone-4		4.636326	C31H40O2
24	27.50368	427.2	[M-H]-	Irbesartan		0.241125	C25H28N6O
25	28.29398	134.88	[M-H]-	Allopurinol		1.52181	C5H4N4O
26	28.29398	135	[M-H]-	3-Methylbenzoic acid		1.5796	C8H8O2

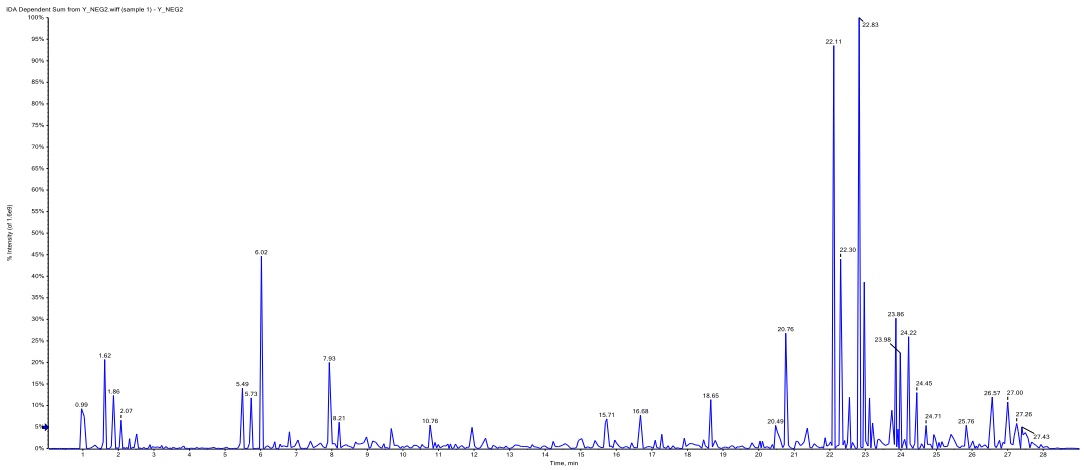
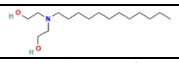
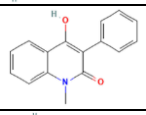
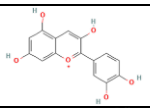
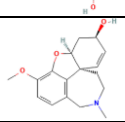
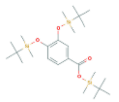
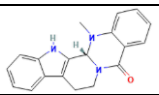
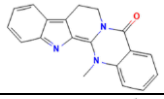
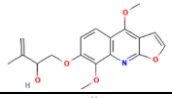
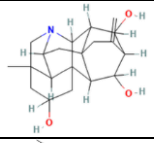
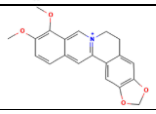


Fig. 1. LC-ESI-MS/MS profile of EAEIO: LC-ESI-MS/MS profile of mycochemicals obtained from EAEIO using negative ionization. Each peak in the profile corresponds to a specific compound, and only the bioactive compounds are listed in Table 1 for reference.

Table 2: Major Bioactive Mycochemicals Detected in EAEIO (Positive Ionization)

Sr. No	RT (min)	m/z	Type	Metabolite name	Ion	Area%	Chemical formula
1	12.83568	274.22	[M+H]+	Lauryldiethanolamine		9.088759	C16H35NO2
2	12.90345	274.08	[M+Na]+	n-methyl-2,4-dihydroxy-3-phenylquinoline		8.839259	C16H13NO2
3	12.98023	288.12	[M+H]+	Cyanidin		0.59528	C15H11O6+
4	12.98023	288.24	[M+H]+	Galanthamine		0.521426	C17H21NO3
5	13.38235	177	[M+H]+	Protocatechuic acid		2.307507	C25H48O4Si3
6	16.19653	304.2	[M+2H]2+	Evodiamine		0.282794	C19H17N3O
7	16.19653	304.32	[M+H]+	Dehydroevodiamine		0.267885	C19H15N3O
8	17.0684	330.12	[M+H]+	Evodine		0.547476	C18H19NO5
9	17.0684	330.36	[M+H]+	Hetisine		0.68868	C20H27NO3
10	22.58867	336	[M+H]+	Berberine		2.037699	C20H18NO4+

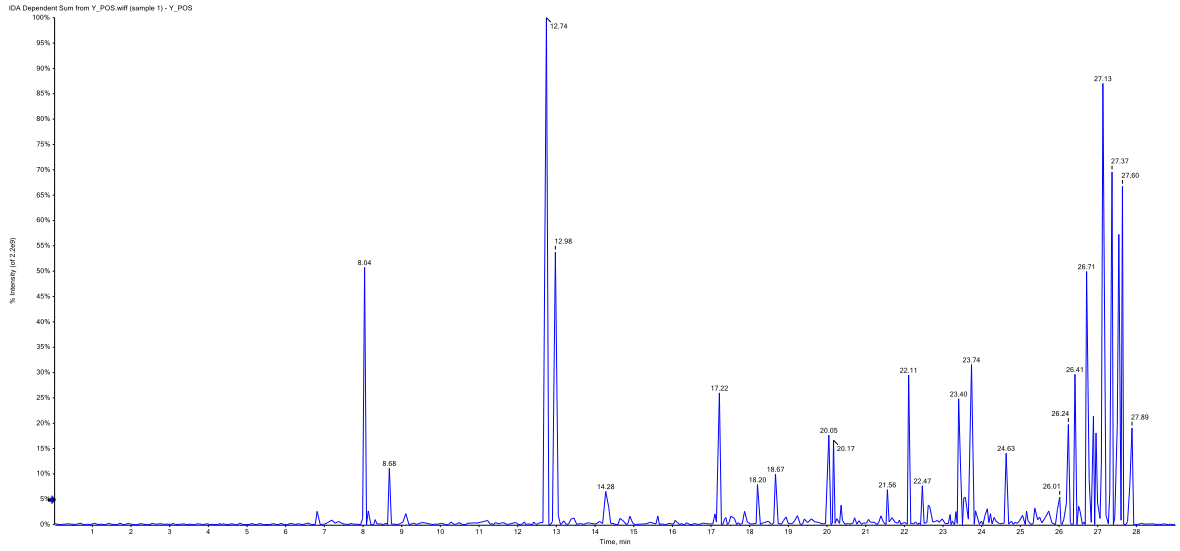


Fig. 2. LC-ESI-MS/MS profile of EAEIO: LC-ESI-MS/MS profile of mycochemicals obtained from EAEIO using positive ionization. Each peak in the profile corresponds to a specific compound, and only the bioactive compounds are listed in Table 2 for reference.

2. Antibacterial Activity of EAEIO against Select Bacterial Strains:

The antibacterial efficacy of EAEIO was scrutinized against four different bacterial strains, namely *E. coli*, *Bacillus*, *Listeria*, and *Pseudomonas*. Chloramphenicol, a broad-spectrum antibiotic, served as the positive control. The comparative analyses of both the inhibition zone diameters and minimum inhibitory concentrations (MICs) are presented below.

a. Inhibition Zone Analysis:

The inhibition zones of EAEIO and Chloramphenicol against the tested bacterial strains were measured and compared. For EAEIO, the average inhibition zones were found to be 18.00 ± 0.58 mm for *E. coli*, 25.00 ± 0.29 mm for Bacillus, 18.00 ± 0.46 mm for Listeria, and 16.00 ± 0.58 mm for Pseudomonas (Table 3).

In contrast, Chloramphenicol demonstrated smaller inhibition zones: 15.00 ± 1.15 mm for *E. coli*, 18.00 ± 0.58 mm for *Bacillus*, 15.00 ± 0.58 mm for *Listeria*, and 12.00 ± 0.58 mm for Pseudomonas (Fig.3). It's noteworthy that the inhibition zones for EAEIO were significantly larger for Bacillus, Listeria, and Pseudomonas with p-values of less than 0.05. However, against *E. coli*, the difference in inhibition zones was not statistically significant (p=0.08), despite EAEIO demonstrating a larger average zone.

Table 3: Inhibition Zone Diameters Indicating the Antibacterial Efficacy of EAEIO. The data presented are the mean values ± standard error of the mean (SEM) representing the diameter (in mm) of the zones of inhibition.

Inhibition zone (mm)	**Bacterial Pathogens**			
	E. coli	***Bacillus***	***Listeria***	***Pseudomonas***
EAEIO	18.00 ± 0.58	25.00 ± 0.29	18.00 ± 0.46	16.00 ± 0.58
Chloramphenicol	15.00 ± 1.15	18.00 ± 0.58*	15.00 ± 0.58*	12.00 ± 0.58*
P-value	0.08	0.000	0.015	0.008

*: represents a significant difference (p<0.000), as compared EAEIO alone.

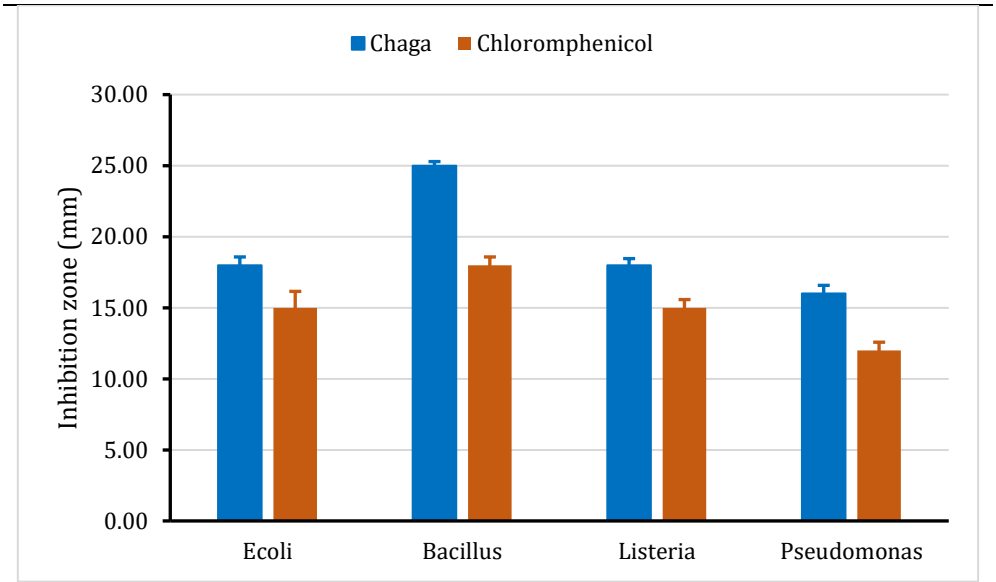


Fig.3: Bar Graph Illustrating the Antibacterial Activity of EAEIO Assessed via Inhibition Zones. Each bar indicates the mean diameter of the inhibition zone (in mm) ± standard error of the mean (SEM). An asterisk (*) signifies a statistically significant difference ($p<0.000$) when compared with Chloramphenicol.

b. Minimum Inhibitory Concentrations (MICs):

The MICs of EAEIO were also evaluated and compared to those of Chloramphenicol. EAEIO exhibited MIC values of 6.25 ± 0.43 µg/ml, 3.13 ± 0.25 µg/ml, 1.56 ± 0.27 µg/ml, and 6.25 ± 0.79 µg/ml against *E. coli*, Bacillus, Listeria, and Pseudomonas respectively (Table 4).

Chloramphenicol had considerably higher MIC values, with 7.80 ± 0.87 µg/ml against *E. coli*, and alarmingly high MICs of 250.00 ± 4.93 µg/ml, 125.00 ± 2.31 µg/ml, and 250.00 ± 9.81 µg/ml against Bacillus, Listeria, and Pseudomonas respectively (Fig.4). The differences in MIC values for Bacillus, Listeria, and Pseudomonas were statistically significant ($p<0.000$). Although the MIC for *E. coli* was marginally lower with EAEIO, the difference was not statistically significant ($p=0.185$).

These findings underscore the potential of EAEIO as a promising source of antibacterial agents, demonstrating significant inhibitory activity against the tested bacterial strains, superior to that of the conventional antibiotic Chloramphenicol.

Table 4: Minimum Inhibitory Concentrations (MICs) Reflecting the Antibacterial Potency of EAEIO. The data are portrayed as mean MIC values (in µg/ml) ± standard error of the mean (SEM).

MIC (µg/ml)	**Bacterial pathogens**			
	E. coli	***Bacillus***	***Listeria***	***Pseudomonas***
Chaga	6.25 ± 0.43	3.13 ± 0.25	1.56 ± 0.27	6.25 ± 0.79
Chloramphenicol	7.80 ± 0.87	250.00 ± 4.93*	125.00 ± 2.31*	250.00 ± 9.81*
P-value	0.185	0.000	0.000	0.000

*: represents a significant difference ($p<0.000$), as compared EAEIO alone.

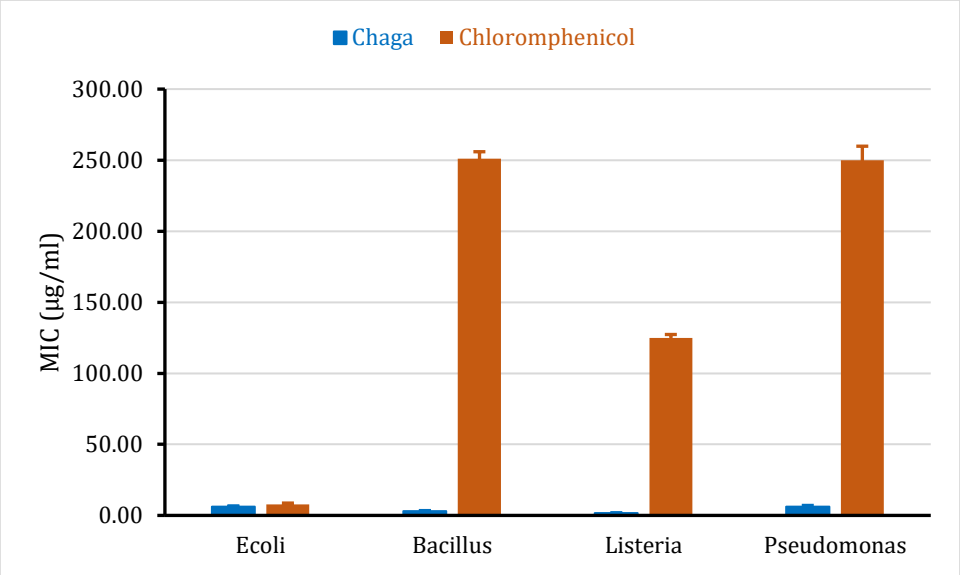


Fig. 4: Bar Graph Depicting the Minimum Inhibitory Concentrations (MICs) Indicative of the Antibacterial Potency of EAEIO. Each bar represents the mean MIC value (in µg/ml) ± standard error of the mean (SEM). An asterisk (*) denotes a statistically significant difference ($p<0.000$) when compared with Chloramphenicol.

3. Cytotoxic Impact of EAEIO Assessed Through IC50 Values Across Varied Cell Lines:

A significant facet of the present investigation revolved around understanding the cytotoxic potential of EAEIO. This was quantified through the determination of the IC_{50} values across different cell lines (Fig. 5). The IC_{50} value denotes the concentration of a substance required to inhibit 50% of cell proliferation.

In the context of the MCF-7 breast cancer cell line, EAEIO demonstrated a potent cytotoxic effect. The IC_{50} value was recorded at 7.56 µg/ml. This low IC50 value points to a strong suppressive influence of EAEIO on MCF-7 cell proliferation.

The anticancer efficacy of EAEIO was also examined against the Hct-16 colon cancer cell line. Here, the IC_{50} value was slightly higher, registering at 11.2 µg/ml. Despite the increase, this figure still denotes significant growth inhibition of the Hct-16 cells.

Finally, the EAEIO's impact was assessed against a non-cancerous cell line, the BHK (Baby Hamster Kidney) cells. The IC_{50} value, in this case, was measured at 22.5 µg/ml, suggesting that a higher concentration of EAEIO was required to inhibit the proliferation of these normal cells.

These findings, graphically represented in the subsequent figure, highlight the promising cytotoxic potential of EAEIO. This is particularly true in the case of breast and colon cancer cells, where the extract displayed marked growth inhibitory effects. While these are preliminary results, they lay the groundwork for further, more detailed investigations into the therapeutic potential of EAEIO as an anticancer agent.

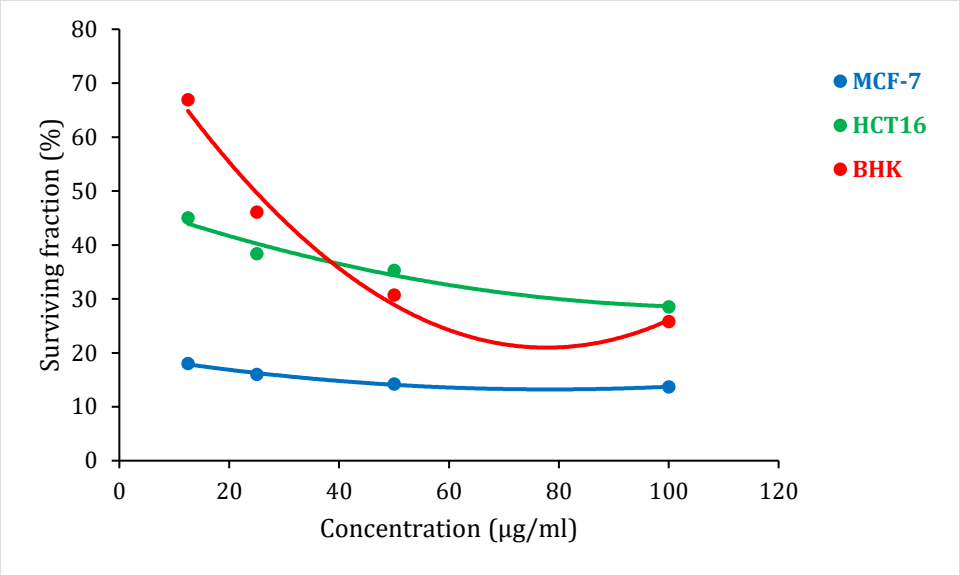


Fig 5. Cytotoxic potential of EAEIO across diverse cell lines. The graph illustrates the IC_{50} values of EAEIO against the breast cancer MCF-7 cell line, colon cancer Hct-16 cell line, and non-cancerous BHK cell line. IC_{50} values denote the concentration of EAEIO required to inhibit 50% of cell proliferation. Lower IC50 values correspond to greater anticancer efficacy. Error bars represent the standard error of the mean.

DISCUSSION

Fungi, especially the genus Inonotus, show tremendous promise in investigating natural products for medicinal applications Newman and Cragg, (2016), making this line of inquiry an increasingly popular one. The bioactive chemicals produced by this species are well-known Zheng *et al.*, (2010). Commonly known as Chaga, the medicinal mushroom I. obliquus has a high concentration of bioactive substances and has been studied extensively Glamočlija *et al.*, (2015). Because of these characteristics, it has attracted a lot of interest in the field of oncology. This research aimed to investigate the presence of bioactive mycochemicals and therapeutic qualities in an ethyl acetate extract of Inonotus obliquus (EAEIO). To completely characterize the EAEIO, this work used a three-pronged strategy that included LC-ESI-MS/MS analysis, antibacterial efficacy analysis, and cytotoxic evaluation.

There is growing evidence that bioactive compounds found in fungi could have medicinal applications Ratnaweera *et al.*, (2015). The bioactive components of fungal extracts have been the subject of extensive research, and the results have shown promising anticancer and antibacterial effects Keller, (2019; Liu *et al.*, (2020). Chaga extract contains bioactive components with potential anticancer action, including polysaccharides, betulinic acid, and polyphenols Drenkhan *et al.*, (2022). As a further advantage, chaga has been shown to have antibacterial activity against a wide range of harmful bacteria, providing new avenues in the fight against antibiotic resistance Garádi *et al.*, (2021).

LC-ESI-MS/MS analysis was performed under both negative and positive ionization conditions, revealing a broad range of bioactive compounds (Figures 1 and 2). It is consistent with prior studies highlighting the diverse mycochemical composition of Inonotus species Y. O. Kim *et al.*, (2005). Among the significant bioactive compounds identified under negative ionization (Table 1) were Shikimic acid and Azelaic acid. Shikimic acid is known for its anti-inflammatory properties and plays a crucial role in the biosynthesis of the antiviral medication Tamiflu Sheng *et al.*, (2023). On

the other hand, Azelaic acid has demonstrated significant antibacterial activity against acne-causing bacteria Spaggiari *et al.,* (2023). These findings underline the possible medical relevance of EAEIO. Other key compounds identified included Octanoate and Xanthine. Octanoate has been reported to modulate metabolic activities Zhao *et al.,* (2023) while Xanthine, a purine base, is involved in the biosynthesis of caffeine and has a role as a bronchodilator Baraldi *et al.,* (2007). Finally, the detection of complex compounds Magnolol suggests possible anti-inflammatory and antioxidant activities Peng *et al.,* (2023). These results imply the richness of EAEIO in potentially bioactive compounds. Under positive ionization (Table 2), a distinct set of mycochemicals was detected. Lauryldiethanolamine, identified at a retention time of 12.83568 minutes, is known for its potential role in lipid metabolism and signaling Hishikawa *et al.,* (2014). Similarly, Berberine, identified as having the longest retention time, has a wide spectrum of biological effects, including antimicrobial, anti-inflammatory, and antineoplastic activitiesGasmi *et al.,* (2023). The identification of such bioactive compounds implies the medicinal potential of EAEIO. The varying array of compounds identified under the different ionization conditions underscores the complexity of the mycochemical profile of EAEIO and the suitability of LC-ESI-MS/MS as a powerful tool for detecting and identifying metabolites.

The exploration of plants and fungi-based bioactive compounds as potential antimicrobial agents is a rapidly growing field of study Hashem *et al.,* (2023); Pastare *et al.,* (2023). As the current investigation showed, EAEIO demonstrated considerable antibacterial efficacy against different bacterial strains, including E. coli, Bacillus, Listeria, and Pseudomonas (Table 3, & Fig. 3). Interestingly, the inhibition zones for EAEIO were significantly larger for Bacillus, Listeria, and Pseudomonas when compared to Chloramphenicol, a broad-spectrum antibiotic commonly used in microbial susceptibility tests. This result indicates a potential edge of EAEIO over conventional antibiotics in combatting these bacterial strains. Although the difference against E. coli was not statistically significant, EAEIO demonstrated a larger average zone, suggesting comparable efficacy. The minimum inhibitory concentrations (MICs) provide an additional metric to gauge the antibacterial potency of EAEIO (Table 4, Figure 4). The significance of this thread. Lower MIC values for EAEIO, compared to Chloramphenicol, particularly against Bacillus and Pseudomonas, suggest a higher potency of the extract. This result further underscores the potential application of EAEIO in combating bacterial infections. However, further studies are needed to unravel the specific mechanisms through which EAEIO exerts its antibacterial effects.

In the MTT assay, our study further explored the anticancer potential of EAEIO, particularly against MCF-7 breast cancer and Hct-16 colon cancer cell lines. The observed cytotoxicity, as indicated by IC50 values, supports previous findings on the antitumor properties of *I. obliquus* extractsMa *et al.,* (2013; Youn *et al.,* (2008). Interestingly, the higher IC50 against the BHK non-cancerous cell line might hint towards a selective cytotoxic action of EAEIO, a desirable attribute in cancer therapies to minimize damage to healthy cells Fulda, (2010).

CONCLUSION

This study's findings reveal the diverse mycochemical composition of the ethyl acetate extract of Inonotus obliquus (EAEIO), its considerable antibacterial activity, and its cytotoxicity against two human cancer cell lines. The potential therapeutic advantages of EAEIO, as suggested by these results, underline the importance of continued research into its bioactive compounds and mechanisms of action. Such studies could further clarify the potential applications of EAEIO in both antibacterial therapies and cancer treatment.

In summary, our findings substantiate the potent bioactivity of EAEIO, highlighting its potential as a source of novel antimicrobial and anticancer agents. Future investigations

could help elucidate the mechanisms underlying these bioactivities and potentially contribute to the development of new therapeutic strategies based on *I. obliquus* bioactive compounds.

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