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Cytotoxicity and Hypoglycemic Effect of the Japanese Jelly Mushroom *Auricularia auricula-judae*

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Abstract The aim of this study was to explore the biological activity of the polar and non- polar extracts of *Auricularia auricula-judae* and to evaluate the most active compounds and classes by GC-MS and LC-HRMS analysis. The jelly mushroom was collected from Hakozaki Higashi-ku Fukuoka-shi parks in Japan and successively extracted by elevating the polarity of the solvents. Non polar (n-hexane) extract which contained Oleic acid as a major compound (62.31%) was the most cytotoxic against HCT116 cell line, the recorded IC_{50} was (43.5) µg/ml. Among all extracts, ethyl acetate and methanol extracts were the most DPPH radical antioxidant with IC_{50} values (333.1) µg/ml and (371.7) µg/ml, respectively. By screening of the four extracts as antidiabetics, ethyl acetate was the most active and the obtained IC_{50} was (14.05±3.2) ppm. LC-HRMS analysis of the methanol extract evaluated that the most active compounds belonged to polyketides, and terpenoid classes and pyran derivatives.

Keywords Medicinal mushroom, *Auricularia auricular,* cytotoxicity, hypoglycemic and antioxidant

1. Introduction

Nowadays, Cancer is one of the major causes of mortality worldwide, and there is a steady increase in number of patients living with cancer; therefore, great efforts were oriented towards exploring and identifying novel potent compounds with an anticancer activity to be used in pharmacological research to improve design of anticancer drugs. Mushrooms induced anti-microbial, anti-oxidant, anti-inflammatory, and anti-diabetic [1-3]. Mushrooms are small pharmaceutical factories manufacturing various promising biologically active chemical compounds with potential anticancer and cytotoxic activities such as β-glucans, lentinan, krestin, hispolon, lectin, calcaelin, illudin S, psilocybin, schizophyllan, ganoderic acid , and laccase [4-8]. These compounds exist in the mushroom fruit bodies, cultured mycelium, and culture broth.

Auricularia auricula-judae or as commonly known Judas's ear or the jelly ear fungus, is an edible mushroom characterized by its brownish, ear-like jelly shape. The fruit bodies of *A. auricula* grow on wood and have been commonly used as a food and as antidiabetic, antihypertensive, antiinflammatory, immunomodulatory, anticancer, and antimicrobial medications in many Asian countries. Its fruit body is characterized by its high contents of carbohydrates, protein, and minerals such as calcium, phosphorous, potassium, and iron [9]. *A. auricula-judae* represents a promising source for novel chemical compounds of different biological functions. Many *in vitro* and *in vivo* studies have proven that acidic heteroglycans isolated from *A. auricula-judae* extracts possess anticancer activity against Sarcoma 180 tumors in mice [10-11], against (Acinar cell carcinoma (ACC) proliferation [12], Ehrlich Ascites carcinoma (EAC) model in mice [13], and bronchoalveolar cancer and gastric cancer cells [14].On the other hand, *A. auricula-judae* extracts were reported to have a hypocholesterolemic activity [15], hypoglycemic activity [16], anticoagulant activity [17], anti-inflammatory effect [18], and an antioxidant activity [19]. In this study, the cytotoxicity of *Auricularia auricula-judae* collected from Fukuoka, Japan was evaluated against HCT116 cell line. The non-polar extract was the most potent in cytotoxicity and the polar extracts showed antioxidant and antidiabetic activities.

2. Materials and Methods

2.1. Collection and Identification of Jelly Mushroom

The Mushroom sample was collected and cut from the bark surface of a dead tree. The tree was cultured in Hakozaki Higashi-ku Fukuoka-shi parks in Japan. It was identified as *Auricularia auricula-judae* according to the classification criterea described in the comprehensive guide to mushroom identification book [20]. The specimen was exported from Japan and deposited in the laboratories of Natural and Microbial products department.

2.2. Extraction of the Jelly Mushroom

The Mushroom (250 g) was extracted successively by different solvents. The weight was soaked in 500 ml of n-hexane for 48 h. The solvent was concentrated till dryness by the use of a rotatory evaporator. This step was repeated by increasing the polarity of the solvents; chloroform, ethyl acetate and 80% methanol were used successively. The dried solvent extracts were weighed and subjected for biological studies and the compounds were chemically identified by means of GC-MS and LC-HRESIMS analysis.

2.3. Cell Culture

HCT116 colon carcinoma human tumor cell lines were cultured in 95% humidity, 5% CO_2 and 37°C. The cell line was maintained in McCoy’s 5A medium supplemented with 10% fetal bovine serum.

2.4. Cytotoxicity Assay

The acid phosphatase assay was used to assess cytotoxicity according to the method described by Yang et al 1996 [21]. Human colon cancer cell line (HCT116) was used by seeding 10000 cell per well in 96 well plates, left to attach overnight, and then treated with different solvents extracts for three days. For one plate, a substrate solution was prepared where 20 mg tablet of pNPP (Sigma; cat. no. N2765) was dissolved in 10 ml buffer solution (0.1 M sodium acetate, 0.1% triton X-100, pH 5). Cell monolayers were washed with 250 µl PBS. 100 µl of pNPP substrate solution were added per well, then plates were incubated for 4 hours at 37°C. 10 µl of 1N sodium hydroxide stop solution were added per well. Absorbance was measured directly at wavelength 405 nm.

All samples were tested in triplicates, and 0.5% DMSO was used as negative control and 50 µM cisplatin was used as positive control. Extracts were tested at serial dilutions with final concentration of 400, 200, 100, 50, 25, 12.5, 6.25 µg/ml.

$$\text{Percent cytotoxicity} = [1-(D/S)] \times 100,$$

where D and S denote the optical density of drug and solvent treated wells, respectively.

2.5. DPPH radical scavenging activity assay

The free radical scavenging activity of mushroom fractions was evaluated by using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay described by [22]. Mushroom fractions were tested at final concentrations of 400, 200, 100, 50, 25, 12.5, 6.25 µg/ml using 0.1mM DPPH dissolved in methanol. After incubation for 30 min in dark at room temperature, the absorbance was measured at 517 nm. Ascorbic acid (vitamin C) was used as positive control at final concentrations of 20, 40 ug/ml. The DPPH solutions treated with 0.5% DMSO used as a negative control. The DPPH scavenging activity of mushroom fractions was calculated according to the following equation:

$$\text{Percentage reduction} = (1-(X/\ av(NC)) \times 100$$

where x indicates the absorbance of fraction and av(NC) indicates the average absorbance of the negative control. EC50 values were calculated using probit analysis utilizing the SPSS computer program (SPSS for windows, statistical analysis software package / version 9 / 1989 SPSS Inc., Chicago, USA).

2.6. α-Amylase Inhibitory Activity [23]

α-amylase of concentration 4 U/ml is prepared in phosphate buffer saline (pH 6.8).15 µl of sample at varying concentrations (0.6 to 220 ppm in the final volume) are mixed with 60 µl of 4 U/ml α- amylase and incubated for 15 min at 37 °C in a 96 well plate. 60 µl of 0.2% soluble starch solution (dissolved by heating in a microwave and then filtered) are added and incubated at 37 °C for 10 min. The reaction is terminated by the addition of 30 µl of 1M HCl. 150 µl KI/I_2 aqueous solution are added. α-amylase activity is determined spectrophotometrically at 595 nm by measuring the quantity of the released blue colour. The negative control has 15 µl of buffer solution in place of the test entity while acarbose is used as a positive control. The calculation and analysis of data is done using graphpad prism programme.

2.7. GC-MS Analysis

40 gm of the jelly Mushroom were macerated in 100 ml of n-hexane for 48 h then filtrated and concentrated. The analysis of the n-hexane extract was performed using a gas chromatography-mass spectrometry instrument stands at the Department of Medicinal and Aromatic Plants Research, National Research Center with the following specifications. Instrument: a TRACE GC Ultra Gas Chromatographs (THERMO Scientific Corp., USA), coupled with a THERMO mass spectrometer detector (ISQ Single Quadrupole Mass Spectrometer). The GC-MS system was equipped with a TG-WAX MS column (30 m x 0.25 mm i.d., 0.25 μm film thickness). Analyses were carried out using helium as carrier gas at a flow rate of 1.0 ml/min and a split ratio of 1:10 using the following temperature program: 60 ^{o}C for 1 min; rising at 3.0 ^{o}C /min to 240 ^{o}C and held for 1 min. The injector and detector were held at 240 ^{o}C. Diluted samples (1:10 hexane, v/v) of 0.2 μL of the mixtures were always injected automatically in splitless mode. Mass spectra were obtained by electron ionization (EI) at 70 eV, using a spectral range of m/z 40-450. Most of the compounds were identified using the analytical method: mass spectra (authentic chemicals, Wiley spectral library collection and NSIT library).The quantification of the components was based on the metabolites as detected by the mass spectrometer. Identification of the constituents was carried out by comparison of their retention times and fragmentation pattern of mass with those of published data [23] and or with those of the Wiley 9 and NIST08 mass spectra libraries.

2.8. LC-HRMS analysis

High resolution mass spectrometric data were obtained using a Thermo Instruments MS system (LTQ XL/LTQ Orbitrap Discovery) coupled to a Thermo Instruments HPLC system (Accela PDA detector, Accela PDA auto sampler, and Accela pump). The following conditions were applied: capillary voltage 45 V, capillary temperature 260°C, auxiliary gas flow rate 10-20 arbitrary units, sheath gas flow rate 40-50 arbitrary units, spray voltage 4.5 kV, mass range 100-2000 amu (maximum resolution 30000). For LC/MS, a Sunfire C18 analytical HPLC column (5 µm, 4.6 mm × 150 mm) was used with a mobile phase of 0 to 100% MeOH over 30 minutes at a flow rate of 1 $ml.min^{-1}$ [25].

3. Results and discussion

3.1. Cytotoxicity assay

In vitro cytotoxicity against HCT116 cell line was evaluated for *Auricularia auricula-judae* organic solvent extracts. Concentrations from (400 to 6.25) µg/ml were used and IC_{50} (µg/ml) was determined. The results were summarized in Fig. 1, cisplatin (50µM) was used as a positive control as presented in Fig.2. It was clearly shown that all fractions were 100% cytotoxic to the cells at the highest level (400 µg/ml) and retained the activity at 200 µg/ml except the methanol extract which lost 12.4% of its potency at this level. Moreover, a drop in the cytotoxicity (34.7%) was noticed by the polar solvent extract (80% Methanol) at 100 µg/ml while the metabolites of the other fractions (hexane, chloroform and ethyl acetate) exhibited potent cytotoxicity (97, 70.1 and 82.6) % respectively, at the same concentration. Hexane extract showed optimum IC_{50} which was obtained at (43.5) µg/ml as summarized in table 2. We attributed this result to the effect of Oleic Acid which represented the major compound (62.31%) of the n-hexane extract (Table1). Our findings correlate the results reported by Cury-Boaventura, *et al.*, 2005[26] who confirmed that Oleic acid had a toxic effect to Raji cells. The suggested mechanism of activity was to promote apoptosis, necrosis and mitochondrial depolarization of these cells. It was also found that Oleic Acid is a Key Cytotoxic Component of HAMLET-like Complexes that kills tumor cells and *Streptococcus pneumoniae* [27].

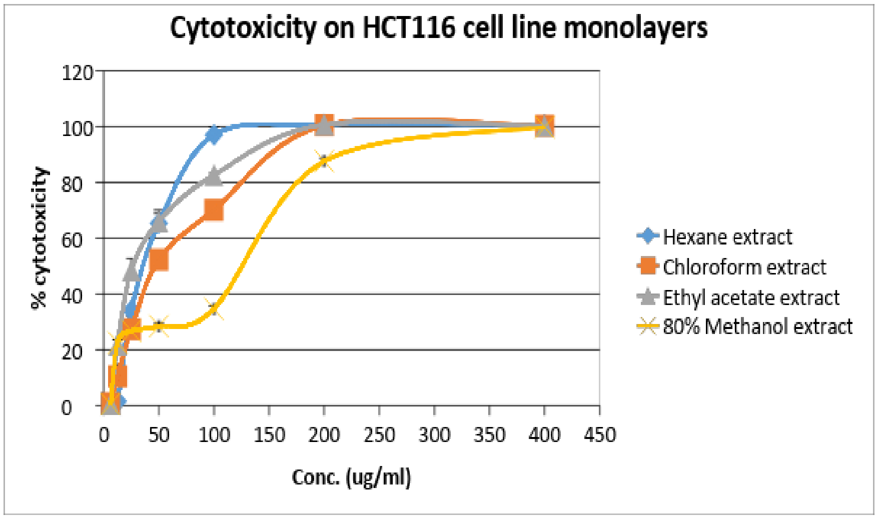


Figure 1: In vitro cytotoxic effect of Auricularia auricula-judae total metabolites to HCT11 cell line

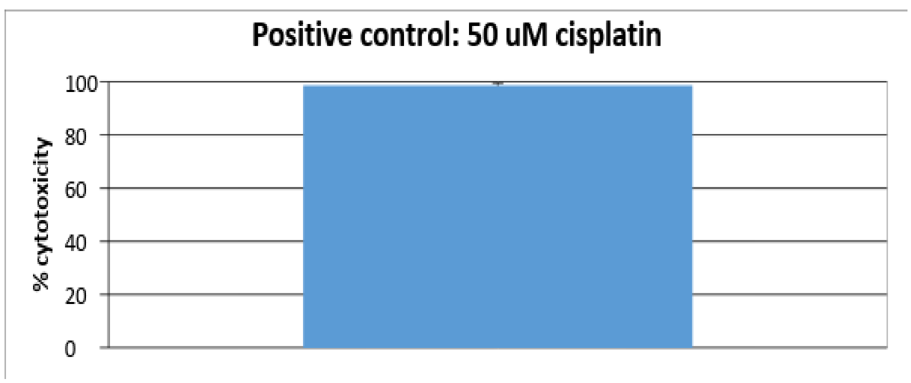


Figure 2: Cytotoxicity of cisplatin (50 µM) as a positive control

3.2. GC-MS analysis

By the use of GC-Ms, it was found that the total identified compounds represented 98.33 % of total peak area (oxygenated compounds 93.28 % and non oxygenated compounds 5.05%) (Fig. 3). In n-hexane extract, seventeen compounds were identified as presented in Table 1: Tetradecane (1.1%), Pentadecane (1.5%), Hexadecane (0.8%) as hydrocarbon compounds. Hexadecanoic acid, methyl ester (2.25%), cis- 9-hexadecenoic acid (0.8%), hexadecanoic acid (5.7%), 1-(5'-acetoxy-2',4'-dimethoxy-6'-hydroxyphenyl)-2-methylanthraquinone (1.35%), 9,12-octadecadienoic acid (z,z)-, methyl ester (2.63%), 9-octadecenoic acid (Z)-, methyl ester (7.05%), 11-octadecenoic acid, methyl ester (1.47%), octadecanoic acid, methyl ester (1.22%) . It was found that oleic acid (62.31%) was represented as the major compound, 6,9,12-octadecatrienoic acid, methyl ester (1.05%), meso-aminoacactaethylporph (1.65%), 2-(2,6-dimethoxy-4-(2,2-bis (ethoxycarbonyl) ethyl]phenyl)-9-(2,6-dimethoxyphenyl)-1,10-phenanthroline (0.89%), 1,2-benzenedicarboxylic acid, bis (2-ethylhexyl) ester (5.20%) and anthraergosta-5,7,9,22-tetren-3-ol (1.36%).

Table 1: GC-MS analysis of n-Hexane extract of jelly ear culinary-medicinal mushroom

Peak No.	Retention time (min.)	Relative %	Molecular Formula	Molecular weight	Compound name
1	18.50	1.1	$C_{14}H_{30}$	198	Tetradecane
2	21.58	1.5	$C_{15}H_{32}$	212	Pentadecane
3	24.53	0.8	C_{16}H34	226	Hexadecane
4	33.34	2.25	$C_{17}H_{34}O_2$	270	Hexadecanoic acid, methyl ester
5	34.01	0.8	$C_{16}H_{30}O_2$	254	Cis- 9-Hexadecenoic acid
6	34.47	5.7	$C_{16}H_{32}O_2$	256	Hexadecanoic acid
7	36.16	1.35	$C_{25}H_{20}O_6$	416	1-(5'-Acetoxy-2',4'-dimethoxy-6'-hydroxyphenyl)-2-methylanthraquinone
8	37.30	2.63	$C_{19}H_{34}O_2$	294	9,12-Octadecadienoic acid (Z,Z)-, methyl ester
9	37.45	7.05	$C_{19}H_{36}O_2$	296	9-Octadecenoic acid (Z)-, methyl ester
10	37.58	1.47	$C_{19}H_{36}O_2$	296	11-Octadecenoic acid, methyl ester
11	38.06	1.22	$C_{19}H_{38}O_2$	298	Octadecanoic acid, methyl ester
12	38.62	62.31	$C_{18}H_{34}O_2$	282	Oleic Acid
13	40.78	1.05	$C_{19}H_{32}O_2$	292	6,9,12-Octadecatrienoic acid, methyl ester
14	41.32	1.65	$C_{36}H_{47}N_5$	549	MESO-AMINOACTAETHYLPORPH
15	44.14	0.89	$C_{36}H_{36}N_2O_8$	624	2-(2,6-Dimethoxy-4-(2,2-bis(ethoxycarbonyl) ethyl]phenyl}-9-(2,6-dimethoxyphenyl) -1,10-phenanthroline
16	46.46	5.20	$C_{24}H_{38}O_4$	390	1,2-Benzenedicarboxylic acid, bis(2-ethylhexyl) este
17	53.85	1.36	$C_{35}H_{52}O_2$	504	Anthraergosta-5,7,9,22-tetren-3-ol

R_t = Retention time M+ = Molecular weight, MF= Molecular formula

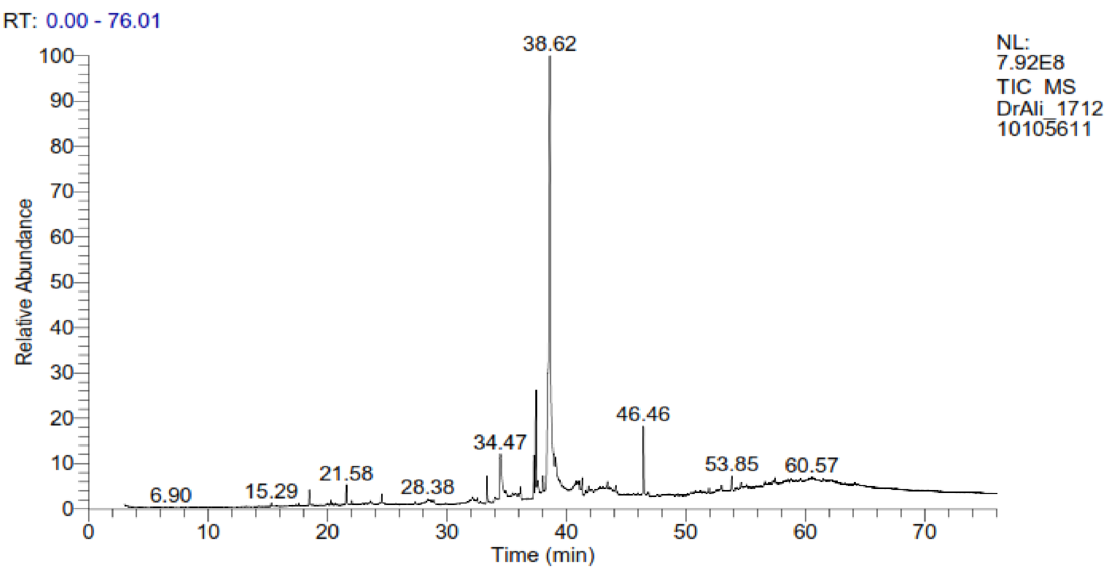


Figure 3: GC-MS analysis of n-Hexane extract of jelly ear culinary-medicinal mushroom

3.3. DPPH Radical Scavenging Activity

Different extracts were examined for the antioxidant of DPPH radical, Ascorbic acid was used as a positive control (Fig.5). The results presented in table 2 showed that ethyl acetate extract was the most potent with IC_{50} (333.1) µg/ml followed by the Methanol extract with IC_{50} (371.7) µg/ml. Antioxidant activity of the various extracts were compared to the positive control. The standard antioxidant was shown to have stronger activity than the examined extracts. Our results were similar to the results of Young, et al 2018 [27] who reported the potent antioxidant of *Auricularia auricula-judae* extract which promoted the biosynthesis of collagen precursor (procollagen) in HaCaT cells. Moreover, it was discovered that the total phenols and flavonoids that were extracted by the hot water of the mushroom had free radicle scavenging activity [29].

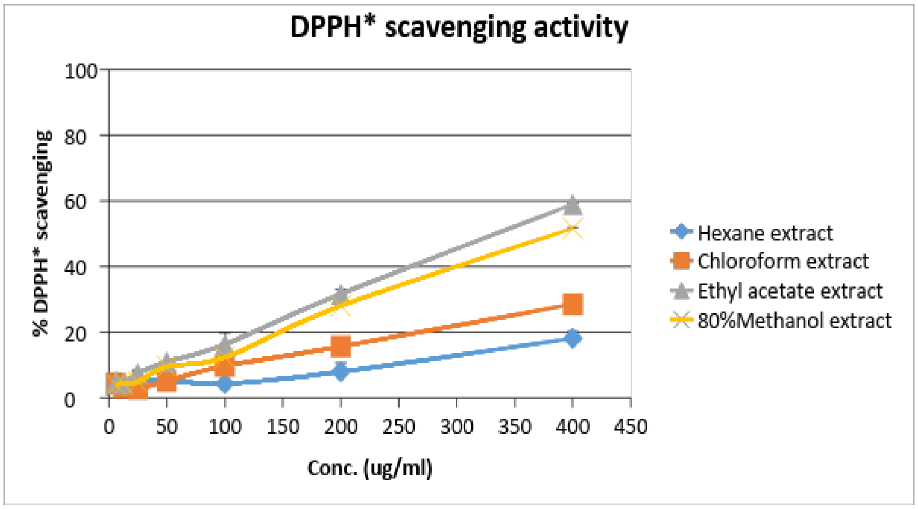


Figure 4: DPPH scavenging activity of Auricularia auricula-judae extracts

Table 2: IC_{50} of the antioxidant and cytotoxicity of *Auricularia auricula* extracts

Organic solvent extract	DPPH radical scavenging IC_{50} (µg/ml)	Cytotoxicity IC_{50} (µg/ml)
n-hexane	> 400	43.5
Chloroform	> 400	64.4
Ethyl acetate	333.1	45.3
80%Methanol	371.7	109.2

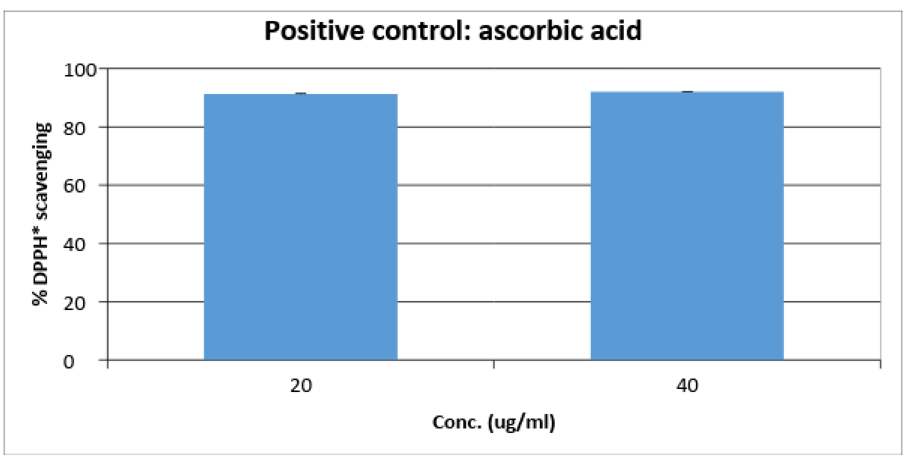


Figure 5: A positive control of DPPH scavenging activity

3.4. *In-vitro* α-amylase activity test

The four extracts were screened and their inhibition activity on α -amylase enzyme was evaluated. The results in Figs (6 and 7) and table (3) showed that the ethyl acetate extract was the most active with IC_{50} (14.05±3.2) ppm followed by the chloroform extract IC_{50} (14.3±4.49) ppm. It was noticed that the previous extracts (ethyl acetate and chloroform) were more potent than the positive control Acarbose which had a less IC_{50} value (21.08±3.3) ppm. A moderate activity (half the activity of the ethyl acetate extract) was obtained by n-hexane extract with IC_{50} (26.8±8) ppm. The LC-MS analysis illustrated that the bioactive ingredients might be related to polyketides, terpens and pyran derivatives, Figs(8 and 9). Our results were in accordance with the results of Sivajothi and Dakappa 2014 [30] who reported the antidiabetic activity of pyran ester derivative. They concluded that the isolated pyran derivative was more potent in blood glucose level (207.40±2.43) mg/dL (P<0.001) than the result obtained by a diabetic control (244.20±12.64) mg/dL. It was also reported that an isolated aromatic polyketide (NFAT-133) stimulated the uptake of glucose in skeletal muscle cells and lowered plasma glucose [31].

Table 3: The *in-vitro* inhibition activity of *Auricularia auricula-judae* extracts on α -amylase enzyme

Solvent extract	55ppm	27ppm	13.5ppm	6.75ppm	IC_{50}	Remarks
n-hexane	58%	56%	39%	31.7%	26.8±8ppm	Active
Chloroform	60%	60.9%	52.3%	37.9%	14.3±4.49ppm	Active
Ethyl acetate	80%	72%	53.8%	5.1%	14.05±3.2ppm	Most active
80%Methanol	5%	-	-	-	-	Inactive
Acarbose	85%	70%	30%	20%	21.08±3.3ppm	Positive control

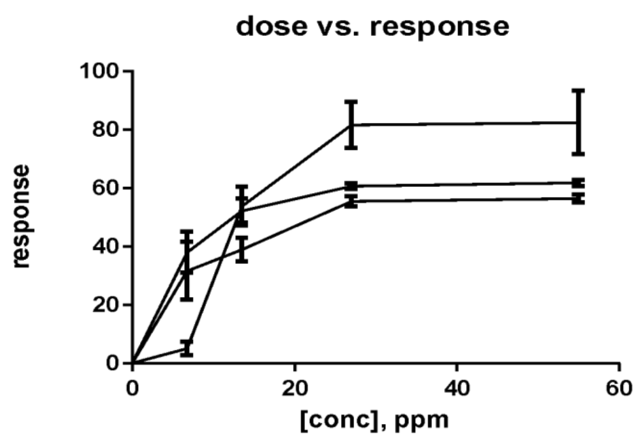


Figure 6: The inhibition activity of different extracts of Auricularia auricula-judae on α-amylase enzyme

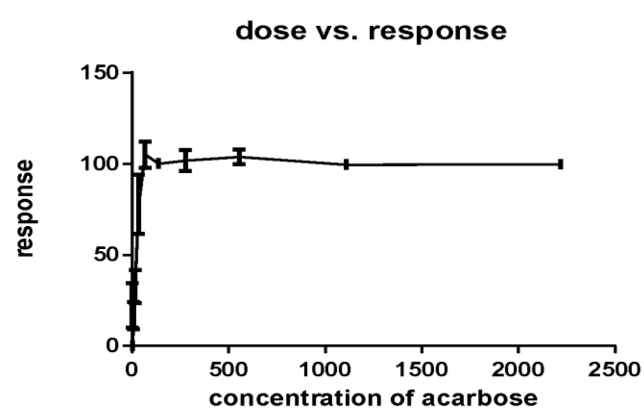
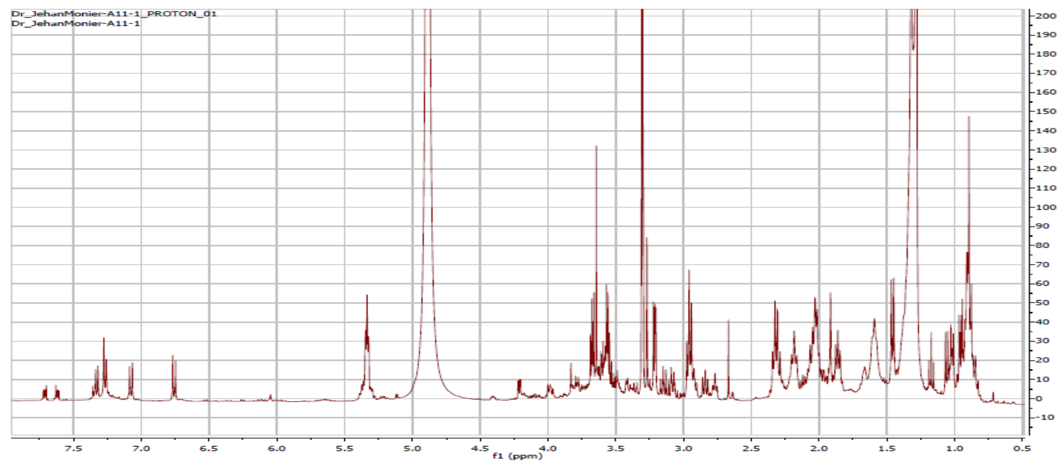
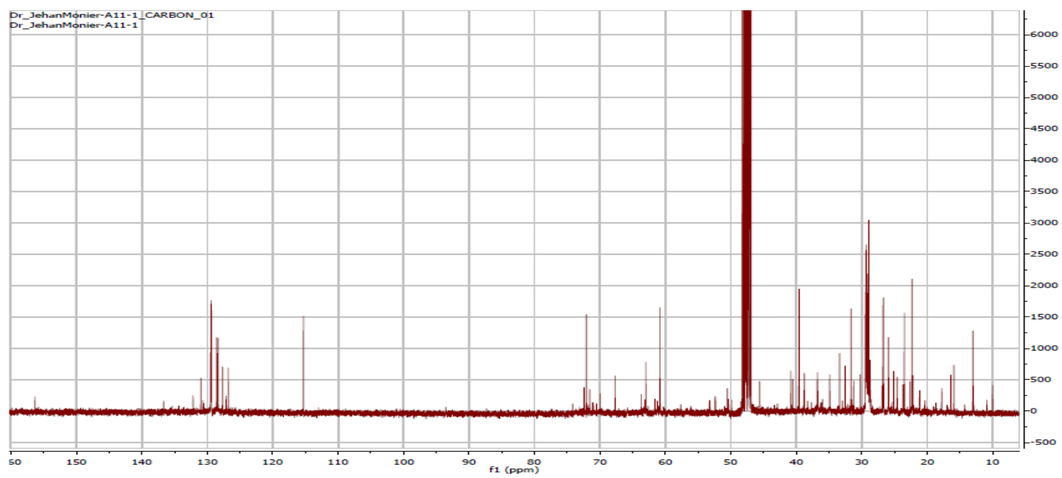


Figure 7: The inhibition activity of acarbose as a positive control on α-amylase enzyme

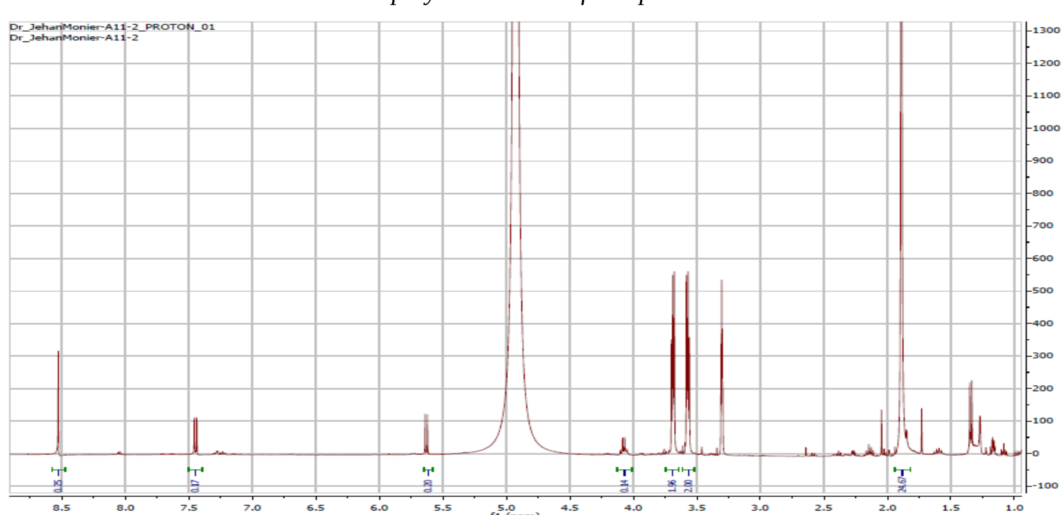


(A)

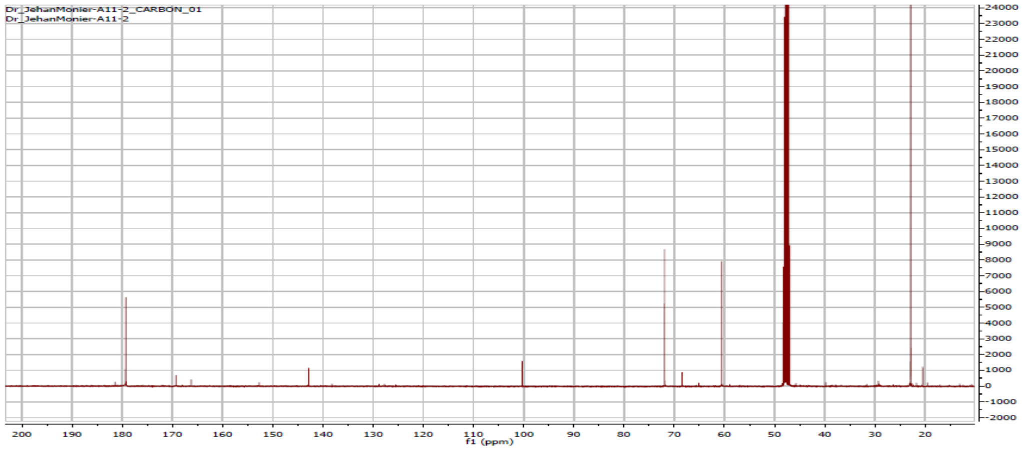


(B)

Figure 8: A: ^{1}H NMR , B: ^{13}C NMR spectra represent a fraction that contains few compounds belonging to terpene and polyketide class of compounds



(A)



(B)

Figure 9: A: ^{1}H NMR , B: ^{13}C NMR spectra represent a fraction that contains a pyran derivative

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