Global Scientific Journal of Biology 9 (1) 2024/ 119-127

Contents lists available at www.gsjpublications.com

Global Scientific Journal of Biology
Part A: Microbiology and Biotechnology
ISSN: 2524-227X
journal homepage: www.gsjpublications.com/gsjb

Isolation, Purification and Structural Characterization of Lentinan from a Newly Discovered Iraqi Strain of *Lentinula edodes* RSR

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ARTICLE INFO

Received: 10 Jan 2024,
Revised: 19 Jan 2024,
Accepted: 23 Jan 2024,
Online: 23 Feb 2024

Keywords:
Shiitake edible mushrooms, β-glucan, Lentinan, Lentinula edodes RSR

ABSTRACT

Background: One of the active ingredients purified from Shiitake edible mushrooms is lentinan, the backbone of β-(1, 3)-glucan with β-(1, 6) branches. It has been approved in Japan as a biological response modifier for the treatment of gastric cancer. **Objective**: This review discusses the purification and characterization of lentinan's potential mechanism of action, clinical application of lentinan, and future potential uses because a deeper comprehension of its biological activities should allow to use lentinan more effectively in the treatment of gastric cancer. **Method**: Lentinan is a high molecular weight β-glucan was purified from the fruiting body of Lentinula edodes RSR by ethanol precipitation fractionation and purified by gel chromotography column (2.5 cm X 7.8 cm length). **Results**: The absorbance of the separated 40 fractions were recorded at the wavelength at 280 nm, as well as the concentration of the lentinan was determent by ELISA .The results characterization of purified lentinan was proved that the glucan in Lentinan was composed of (1-3)-βD-linkages. **Conclusion**: The aim of the present study to purified and characterized of lentinan from new Iraqi strain for using it as an alternative compound to medicines and chemical compounds.

Introduction

Lentinan is a polysaccharide extracted from Shiitake mushrooms *Lentinula edodes* , which have been used for thousands of years in Asia to enhance general health (Sahib *etal*.,2023). In Japan, lentinan was approved as an adjuvant for stomach cancer therapy consists of β-(1–3)-glucose backbone with two (1–6)-β-glucose branches per five glucose units (Figure 3), with a molecular formula $(C_6H_{10}O_5)n$ with a high molecular mass of 5 x 10^5 Daltons . It consisted of glucose , mannose and galactose , free of nitrogen, phosphorus, sulphur, and any other element , except for carbon, oxygen, and hydrogen, Its alkali labile, water soluble, and heat stable (Chen and Seviour,2007).

Lentinan was isolated from *Lentinula edodes* mushroom for the first time and used in treatment of cancer in both China and Japan (Tomassen *et al.*,

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doi: 10.5281/gsjb.2024.10693647

2011; Ataollahi & Larypoor, 2022). Many studies confirmed about the active role of Lentinan beta glucan extracted from *Lentinula edodes* mushrooms as antioxidant and antidiabetes it could significantly reduce blood glucose levels by giving rats doses of the extracts and have immune modulatory activities against pandemic coronavirus disease 2019 (COVID-19) by using it in vitro models of lung injury and macrophage phagocytosis, also as antimicrobial against many pathogenic bacteria and anticancer medication chemotherapy increased the survival of patients with advanced gastric cancer when compared to chemotherapy alone (Zhang *et al.,*2018 ; Ren *et al.*,2018 ; Afiati ,2019; Murphy *et al.,*2020; Chen, 2021; Zhang et al., 2021)

Solvents such as hot water, alkaline solution, and polyethylene glycol have been used to isolate Lentinan from edible mushrooms *Lentinula edodes* (Jamil *et al.*, 2013). Although some fungal polysaccharides are polar and easily extracted with water, many β-glucans require strong treatments such as hot water or alkaline solutions, and others stay in the insoluble residue (Morales *et al.,* 2019). To isolated β-glucan and evaluate their unique bioactivities, many purification techniques were established. Solvent treatment, freeze-thawing separation, dialysis, ultrafiltration, and column fractionation were commonly used (Ruthes *et al.*, 2015). New advanced techniques, such as ultrasound-assisted extractions (UAE) and subcritical water extraction (SWE), make it possible to extract these and other polysaccharides using water as a non-polluting solvent (Rosello-Soto *et al.,* 2016**;** Marić *et al.,* 2018). Ultrasound-assisted extractions generate ultrasonic waves, which cause implosions of the cavitation bubbles produced, causing destruction of fungal cell walls. This procedure improved mass transfer and extraction yields (Kumar *et al.,* 2021).The aim of the present study to extract, purified and characterized of lentinan from new Iraqi strain for using as an alternative compound to medicines and chemical compounds.

2. Materials and Methods

Sample Collection

The current study was done on a wild cultivated mushroom *Lentinula edodes* RSR strain is Iraqi novel strain for first record in NCBI (National Center for Biotechnology Information) genbank and available for download at ID: MW375036.1.. The research was carried out at Department of Biology , College of Science , Mustansiriyah university.

Extraction of Lentinan from Edible Mushroom *Lentinula edodes* RSR Strain

Lentinan was extracted by using the method from Afiati *et al.*, 2019 with some modification. The dry mushroom powder 20 g was defatted three times with acetone the residue was added to 200 ml of hot water and boiled together for 1h at 80C˚, and left the sample for cooled. 95% ethanol (1:1) was added and it was left for overnight at 4 C˚ to precipitate the endopolysaccharides. The solution was centrifuged again under the same conditions. Phosphate Buffer Saline (PBS) solution was added to the pellet, which was then washed with distilled water. The mixture was concentrated and dialyzed to obtain a partially purified polysaccharide from fruiting body mushroom using dialysis bags with a cut-off separation rate 7000 Dalton was carried out with Phosphate Buffer Saline (pH 7.4) at 4°C for 24 hours with stirring and changing PBS for four times to increase the efficiency of dialysis process then immersed the dialysis bags in sucrose powder to concentrate the partial purified polysaccharide and kept in refrigerator until the use (Mahdi, 2015; Shafiq and Sahib, 2022).

Identification of Lentinan by ELISA Kit

Each fraction was measured by ELIZA (Enzyme-Linked Immunosorbent Assay) kit to determine which peak is containing a Lentinan compound. A plate with 96 wells (well-plate) is used in sandwich ELISA, Components of Lentinan antibody ELISA kit used in this and according to method (Mahdi,2015) was done ,pipet 100 µl of Standard solution was added to the first 6 wells of the first column and 100µl of sample was added to other wells. Then wells were covered and incubated for 60min at room temperature with gentle shaking. Then the solution was discarded and washed 4 times with wash solution. The washing was done by filling each well with washing buffer (300 µl) using a multi-channel pipette or auto washer. Complete removal of liquid at each step is essential to good performance. After the last washed, any remaining wash buffer was removed by aspirating or

decanting. Then the plate was inverted and blotted against a clean paper towels. After that 100 µl of ready to use conjugate Enzyme reagent (20X) was added to each well and Incubated for 30min at room temperature with gentle shaking then emptied the wells of the plate and added 300 µL of diluted washing solution. This procedure is repeated for three times. 100 µl of ready to use substrate solution was added to each well and the plate was incubated for 15 minutes at room temperature in the dark with gentle shaking. Then the solution was discarded and washing was repeated as in step 4. Lastly, 50 µl of stop solution was added to each well. The optical density of each well determined with a computerized micro-plate reader, and the results were read at 450 nm immediately.

Purification by Gel Chromatographic Column

A chromatography column was sealed with a piece of cotton wool on the bottom; Silica gel is a very fine powder and can easily get sucked into the rotary evaporator. Therefore plug the opening of the adapter or bump guard with glass wool or cotton to prevent "bumping" of the solid, that the silica will not drain out. .A small layer of sand was laid down below, and above the silica bed, Added across the diameter of the column (approximately 1-2cm). Silica gel was loaded onto the column. Vacuum was applied through the stopcock at the bottom of the column. The vacuum condensed the silica gel and packed the column tightly, then Phosphate buffer saline pH=7 was passed onto the column with a vacuum till all the silica adsorbent packed perfectly, and the all the solvent eluted. The column was filled with sufficient eluent to prevent the column drying. The column should not have any air bubbles before applying the crude extract on it. Repeat this procedure until the entire column is wetted.

The crude mixture was loaded on a top of the column using a pipette with 5ml volume, with flow rate 26 ml/hour at a volume of 2.5 ml for one fraction, and The absorbance of the separated 40 fractions were recorded at the wavelength at 280 nm, as well as the concentration of the Lentinan was determent by ELISA, Then The active fractions were pooled, and concentrated by dialysis bag , the concentrated fraction was characterized until used (Yeh *et al.*, 2021).

Structural Characterization of Lentinan

1- Fourier Transform Infrared Spectroscopy (FTIR)

FT-IR spectrum of the purified lentinan sample was determined by a Fourier transform infrared spectrophotometer IR Affinity -1 (SHIMADZU, Germany), the spectra were obtained from 1 mg of purified Lentinan sample in the middle infrared range from 4000 cm^{-1} to 400 cm^{-1} with a resolution of 2.0 cm^{-1} . Approximately 0.5 mg of the sample was used for the test at room temperature.

2-Nuclear Magnetic Resonance (NMR)

Scientists have used NMR technology to characterize polysaccharides, including anomeric configurations, patterns, and sequences of glycosidic linkages. NMR spectra were recorded on nuclear magnetic resonance Bruker spectrophotometer model Ultrasheild 400 MHz using tetramethylsilane internal standard and DMSO as solvent with a 5 mm inverse probe, and the analyses were performed at $70C^0$. The purified Lentinan sample (30 mg) were dissolved in DMSO and were centrifuged (10000 rpm, 22 ºC, 2 min) to remove insoluble material, therefore only the soluble fraction were analyzed.

3-UV-VIS analysis

0.02% w/v of purified Lentinan fraction was used for UV–Vis spectroscopy. UV spectra were recorded between 200 and 400 nm for visible region and between 700-1100 for IR-region at 25 °C using a Ultraviolet-visible (UV/Vis) spectroscopy was performed using an UltraViolet-1900i spectrometer (Shimadzu,Germany).

3. Results and Discussions

Extraction and Purification of Lentinan

Silica gel was chosen as stationary phase due to the high molecular weight of Lentinan. After extracting the crude polysaccharides from the fruiting body of the edible shiitake mushroom, it was confirmed that the crude extract contained the active compound of Lentinan by ELISA. A clear color change was obtained on the ELISA kit, evidence of the compound's presence regardless

of its concentration. ethanol precipitin extract were prepared in the second step to pass through the chromatography column , 5 ml of Curd polysaccharides extract was applied on gel column chromatography and eluted with phosphate buffer saline PH= 7.4 to obtain 40 fractions. Sugar molecules are very hydrophilic neutral molecules that are not affected by PH in regard of their degree of ionization, except at very high PH levels (Bawazeer *et al.*,2012). Fractions were collected and their absorbance measured by a spectrophotometer at a wavelength of 490nm, and determination of Lentinan concentration by ELISA (sandwich ELISA methods) as shown in table (2). While figure (2) showed Five peaks were drawn with chromatography column (X1, X2, X3, X4 and X5), and the highest peak was X2 with concentration 69.401 mg/dl as compared with other peaks (X1=2.313, X3= 1.011, X4= 2.505, X5= 3.369). Table (1) and Fig.(2) that appear high concentration in fraction number between 11 -18 ,the fractions were high specific and sensitive to antibody that coated in ELISA kit , therefore these fractions were selected to have pure Lentinan to preparation for used it in next steps and other biological applications. Table (2) and Figure (3) showed the concentrations of Lentinan during all purification steps which quantified and determined by ELISA technique. When a Lentinan molecule appears in peak No. 2 at a high concentration as shown in Table (1), that's indicates the molecular weight of Lentinan was too high to move down at the start of the separation process. This result was in agreement with Hou *et al.,* 2018, which demonstrated that molecules with the highest molecular weight are separated firstly on the silica gel column, followed by the smallest.

Table (1) Determination the Absorbance and Concentration of Lentinan fractions by ELISA

No. of fraction	Absorbance at 490 nm	Concentration lentinan by ELISA mg/dl
11	0	3.377
12	0.9	19.024
13	1.9	45.469
14	2.6	69.401
15	2.1	51.054
16	1.7	34.377
17	1.0	29.693
18	0	4.728

Table (2) Concentrations of Lentinan during purification steps

Purification steps	Lentinan Concentration (mg/dl)
Crude (ethanol precipitated)	11.836
After Centrifugation	13.575
Silica gel chromatography peaks (mg/ dl)	
X1	3.369
X2	69.401
X3	1.011
X4	2.313
X5	2.505
Final concentration of purified Lentinan	**1005**

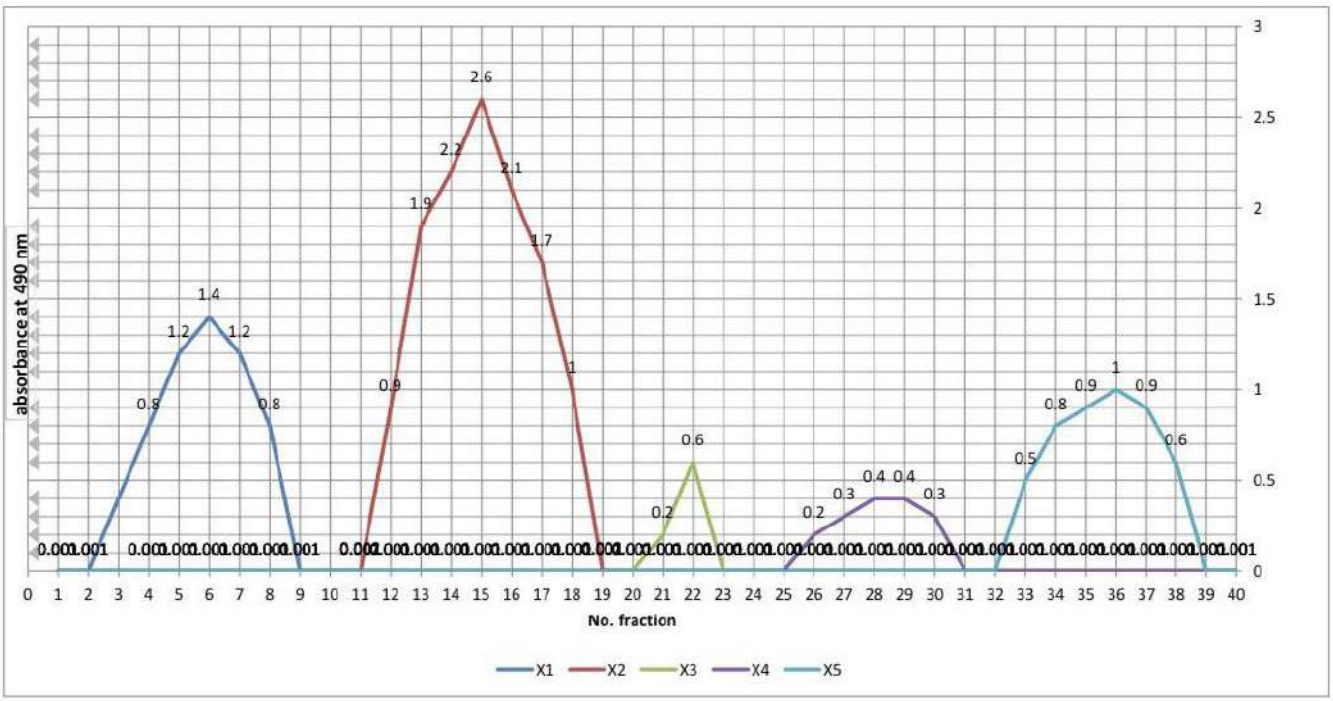


Figure (2) fractions were extracted from *Lentinula edodes* RSR strain

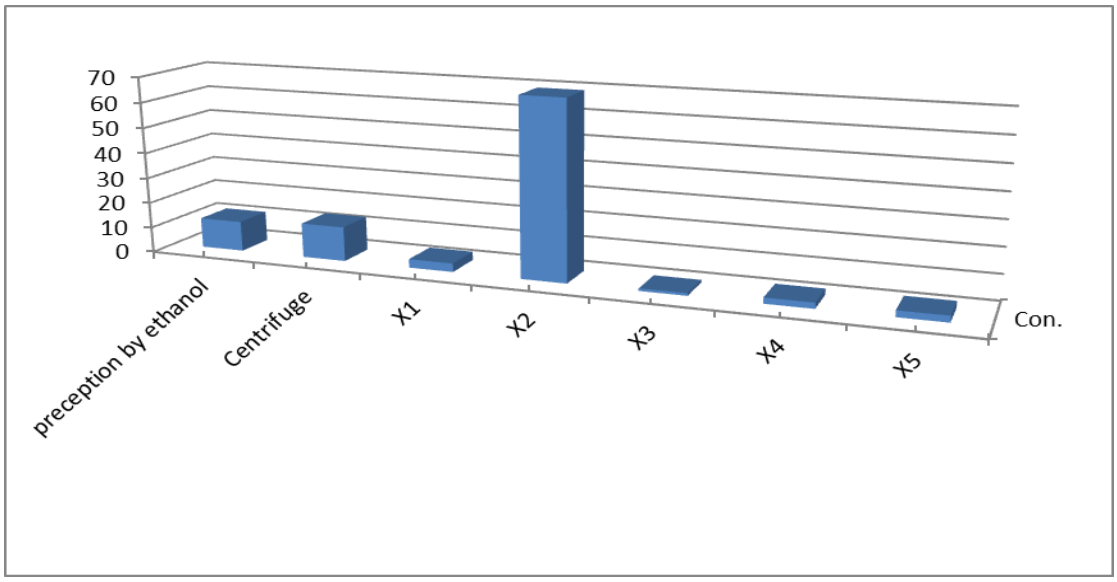


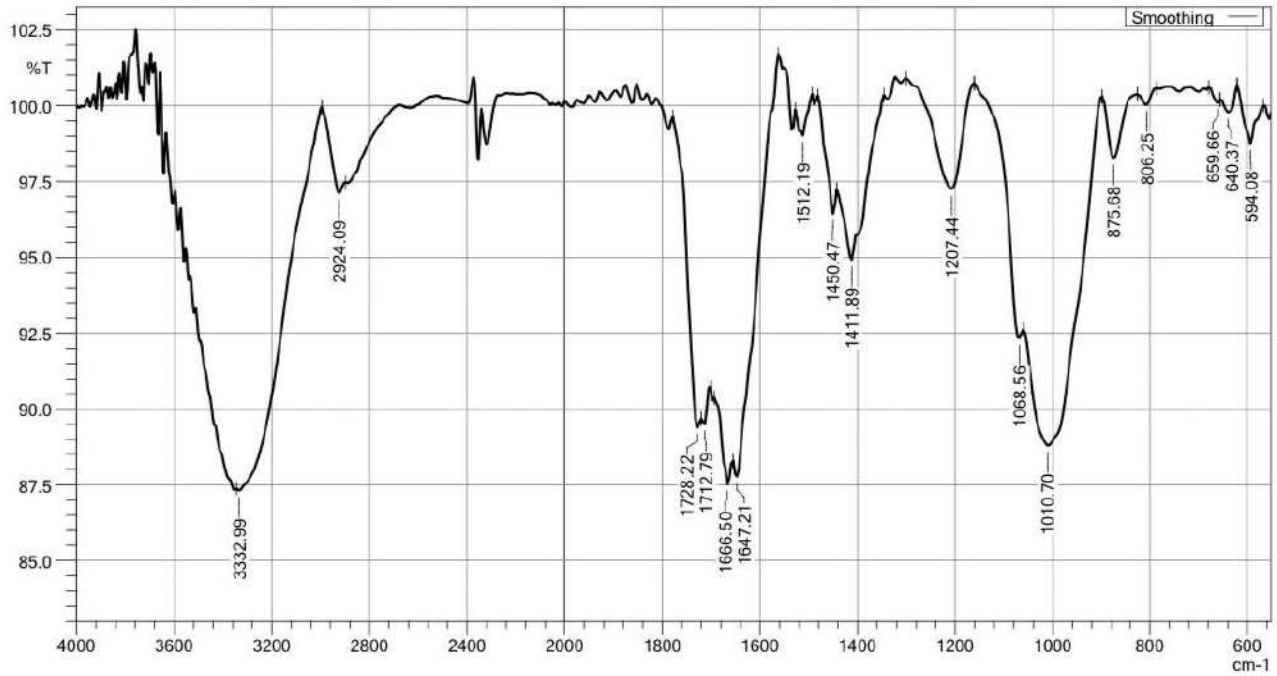
Figure (3) concentrations of Lentinan from *Lentinula edodes* RSR strain during purification steps

Structural characterization of Lentinan

FTIR analysis

In Figure 4, the location and anomeric configuration of the glycosidic linkage in the glucan of Lentinan extracts from L.edodes were determined using FTIR spectroscopy - The absorption region between 1250 cm-1 and 1650 cm-1 revealed that the substance was a polysaccharide. The broad peak at 3200 -3600 cm^{-1} indicated the existence of OH stretching; These bands come from the stretching of the hydroxyl

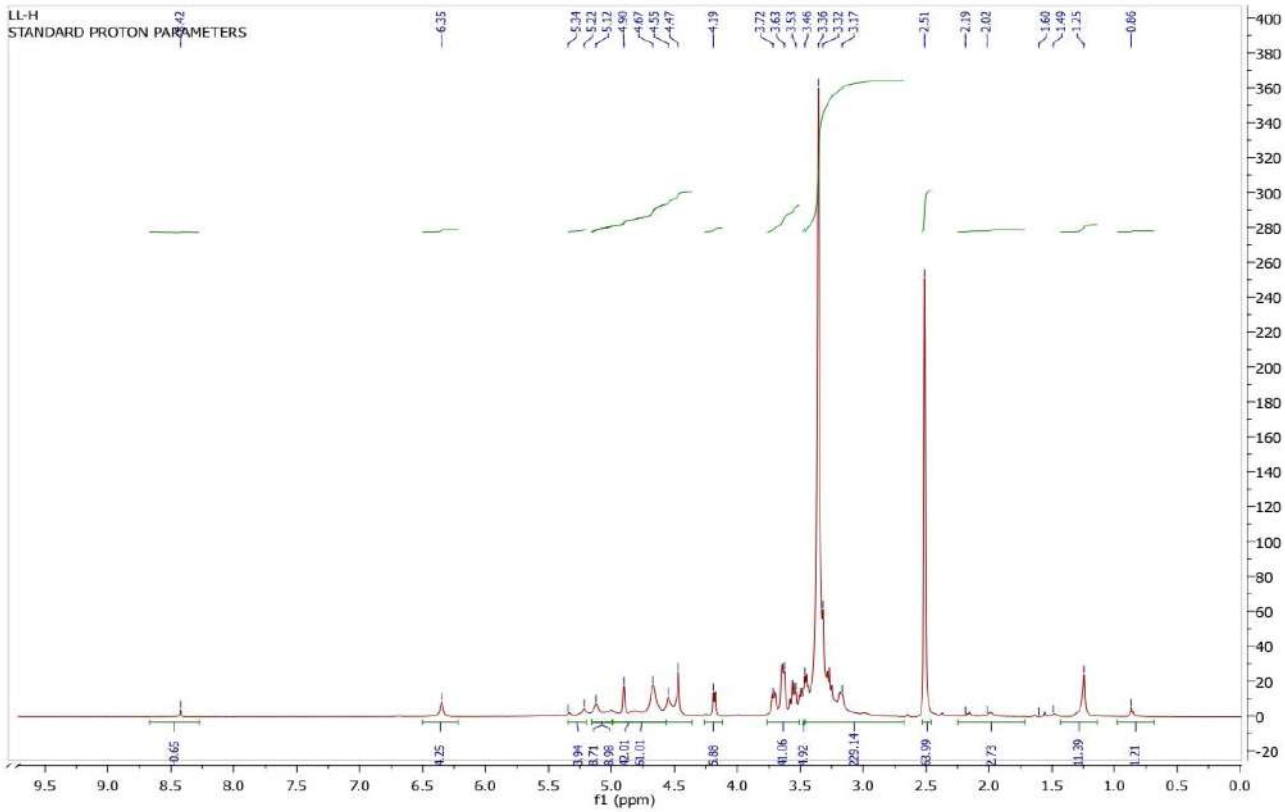
groups. The absorption peaks at 2924 cm^{-1} in the spectrum of Lentinan, were related with the stretching vibration of C–H in the sugar ring, indicating the presence of a methylene group CH2. N–H groups were absent and the bands' intensity for amide groups decreased because of the removal of proteins in first step of partial purification. The absorption peaks between1600-1800cm^{-1} demonstrated the water bending vibration in Lentinan. The absorbance peak at 1010 cm^{-1} also indicated the presence of C–O–C and C–O bonds stretching vibrations. So, the appearance of absorption peaks for C–O, C–O–C, and O–H stretching vibrations is identical to that of a polysaccharide structure. According to Usuldin *et al*., 2020; Wan-Mohtar *et al*., 2016 and Synytsya and Novak , 2014 the finger-print band from 750 cm^{-1} to 1000 cm^{-1} can determine the polysaccharide type and anomeric structure. The absorbance at 890 cm^{-1} indicated that the glycosidic linkage's anomeric structure was in configuration. Therefore, the structural characteristics of the Lentinan from the fruiting bodies of *Lentinula edodes* could be summarized based on the absorption peaks.



Figure(4) FT-IR spectrum of Purified Lentinan from *L.edodes* RSR strain

H-NMRanalysis

More information about the chemical structure of Lentinan was obtained by H-NMR spectrum as shown in (Figure 5) to verify the presence of β-glucan at 24C° with using D $_2$O as a solvent. The resonance signals with the chemical shift of 4.19-5.12 ppm were assigned for anomeric proton and carbon signals. Corresponding resonances at 4.4 - 4.6 ppm are characteristic of β-D glucans. This was agreed by Wan-Mohtar *et al*., 2016 and Abdullah *et al*., 2020 where they reported that the glucans had the spectrum chemical shifts between 3.9 ppm and 5.4 ppm. Therefore, based on the FTIR (Figure 4) and H-NMR results (Figure 5) proved that the glucan in Lentinan was composed of (1-3)-βD-linkages (a typical structure of β-glucan), as mentioned by Supramani *et al.*, 2019.



Figure(5) H-NMR spectrum of Lentinan Purified Lentinan from *Lentinula edodes* RSR strain

UV-VIS spectra analysis of Lentinan

The purity of the Lentinan polysaccharide from edible mushroom lentinula edodes was confirmed by UV-VIS spectrophotometry as shown in (Fig. 6). The figure represented the absorbance UV spectra of Lentinan between 190 and 1100 nm at 25 °C using a UV–Vis spectrophotometer (Shimadzu, Germany). And shows that maximum absorption about 1.46 at the wavelength194 nm and sharp decrease to the wavelength at 264 nm, absorption reach to 0.9 then absence of peaks between at 280 nm which indicated that the Lentinan was pure or free of nucleic acid and proteins. Absorbance between 190-264 nm was assigned to the present of glucose, and the other spectrum is still stable around 0.7 at the visible (400-700nm) and IR region (700-1100nm). The change in UV–Vis spectra characteristics at the mentioned wavelengths could be due to changes in carbonyl groups in the purified Lentinan fraction. Numerous studies (Ren *et al*., 2018; Samrot *et al*., 2020; Shaheen *et al*., 2022; Sombuor, 2022) confirmed the results mentioned above by FTIR and NMR.

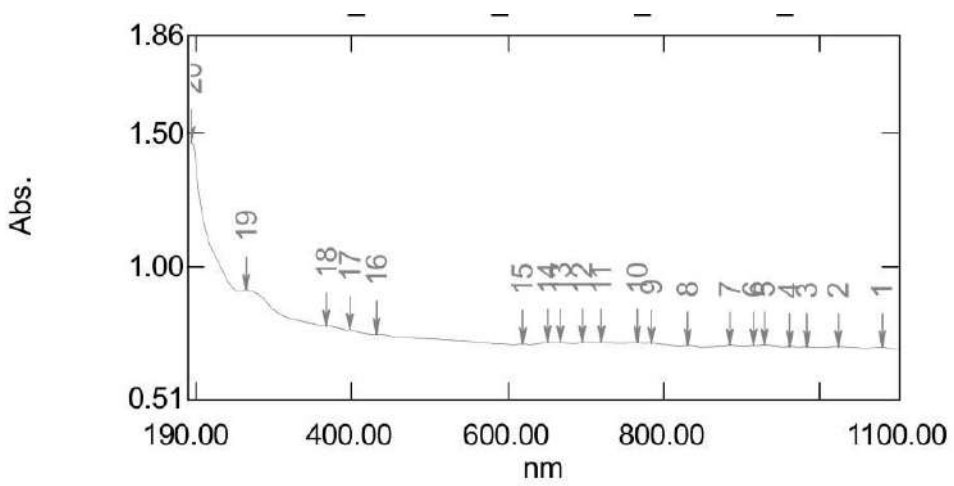


Figure (6) UV-VIS of Purified Lentinan from *Lentinula edodes* RSR strain

4. Conclusion

The polysaccharide Lentinan, which has a triple helix structure and a high molecular weight, is extracted out of the fruiting body of L. edodes. Particularly for its anti-tumor activity, the arrangement of glucose molecules in the helical structure is thought to be significant for both biological and pharmacological activities. Currently, Lentinan is used as a complement therapy in cancer clinics and certain products based on Lentinan.

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