Ultrasonic-assisted Extraction of Anthraquinones from an Edible Fungi Pleurotus ostreatus using Ethanol-Water mixtures and their application in treating Type II Diabetes

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Abstract

Context:

This study explored the utilisation of ultrasound-assisted extraction (UAE) to improve the extraction productivity of the old-style dissolvable extraction systems, for example, maceration and Soxhlet extraction to extricate against diabetic action mixes, anthraquinones, from the hyphae of P. ostreatus.

Objective:

The impacts of various extraction conditions were resolved, i.e., the temperature of (25, 45, and 60°), ultrasonic power, dissolvable sorts, and structures of ethanol in ethanol-water blends.

Materials and methods:

This method demonstrates that the yield increments with expanding extraction times and extraction temperatures. Besides, the utilisation of ethanol-water arrangement as extraction dissolvable expanded the yield of anthraquinones because of the relative extremity, the swelling impact of hyphae tissue framework by water, and expanded sound assimilation. To accomplish a similar recuperation as that accomplished by UAE, Soxhlet extraction and maceration required a longer time. Moreover, the metabolic fingerprinting of the compound derivatives was done utilising Gas chromatography Mass Spectrometry (GCMS), High-Performance Liquid Chromatography (HPLC), and Liquid Chromatography Mass Spectrometry (LCMS DXT).

Results:
This investigation prompted the detachment and distinguishing proof of five AQ derivatives. The secluded AQ was dissected for its anti-diabetic properties utilizing male Wistar rodents and the diabetic profile on intense lethality, blood glucose, lipid profile, kidney profile, and liver profile (urea, creatinine) lastly the liver tissue histopathology was completed.

Discussion and conclusion:
This examination uncovered that AQ extricate
amazingly turned around the streptozotocin actuated diabetes in the creature models in the brief term of 15 days.

Keywords: *Pleurotus ostreatus, UAE, LCMS, HMBC, Streptozotocin, Type II Diabetes*

Practical application:

Ultrasonic-assisted extraction technology is widely used in the enhancement of extracted components such as functional compounds, anthocyanins, polysaccharides, oils, aromatic compounds and polyphenolics when utilized in the unit process as a pre-treatment phase. Ultrasonic-assisted extraction of anthraquinone for enhancing components from PO edible fungi by GCMS, LCMS, HPLC, and FTIR extraction methods. The result showed the properties and medicinal uses of extracted Anthraquinone which is used in controlling type II diabetics.

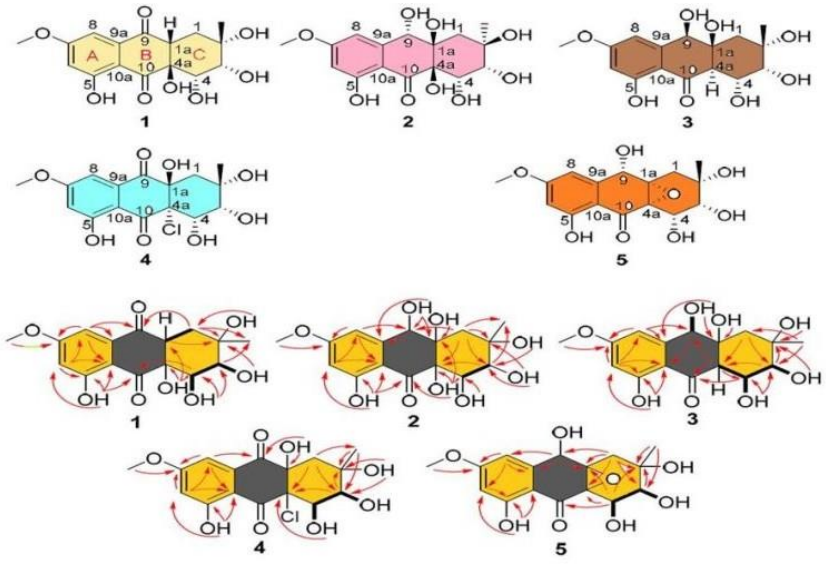

Figure 1: Graphical Abstract

1. Introduction:

Anthraquinones (AQs) are a significant collection of metabolites and establish the biggest gathering of normal quinones. AQ subsidiaries are chemotaxonomic markers in specific plant families: Rubiaceae, Rhamnaceae, Polygonaceae, Fabaceae, Liliaceae, and Verbenaceae[1]. *Pleurotus ostreatus* (PO) a palatable Oyster mushroom is known to have different medical advantages for diabetes, malignant growth, hypertension, joint inflammation etc. The glycemic record of this edible species is Zero, which makes it extraordinary dietary enhancement[2] and this organism shows an incredible cell reinforcement profile[3]. Subsidiaries of PO, for example, tetrahydro anthraquinone, estradiol, and pipercallisidone have significant cancer prevention agents, calming and antiproliferative exercises. Shellfish mushrooms like Pleurotus have a raised dimension of phenolics when contrasted with Agaricus species[4]. These bioactive segments exhibit properties like actuation of interleukin generation, nitric oxide synthase, free radical rummaging and iron chelating properties. It is excogitated that learning about these bioactive phytoconstituents gives insight into its organic exercises' past utilization of nourishment[5]. Anthraquinone removed from plants might be an appropriate substitute for manufactured colour thinking about its splendid yellow shading and solvency in water, in this way enabling them to fuse in sustenances for upgrading conceivable medical advantages in people. Indeed, even following quite a while of research, diabetes stays cryptic[6]. GC-MS examination of *Pleurotus ostreatus* uncovered a few phytoconstituents which appeared to have restorative properties[7]. As of late, Tetrahydro anthraquinone was accounted to be utilized for its restorative properties for treating diabetes and malignancy. Differentiated research on P. ostreatus analogs, for example, anthraquinone has been accounted for as have huge cancer prevention agent, mitigating and hostile to proliferative exercises against different malignant growth cell lines. Tetrahydro anthraquinone subsidiaries appeared to target incendiary pathways, along these lines averting neurodegenerative disease. One of the major worldwide wellbeing challenges is heftiness and research for the treatment of the equivalent is proceeding for quite a long time[8]. Weight is characterized by the WHO as a collection of extreme or unusual fat, which in this way leads to antagonistic well-being outcomes[9]. Different research on stoutness additionally demonstrates it is one of the major causes of Type II diabetes[10]. Because of the intriguing organic activities displayed by these AQs and their wide scope of uses in the pharmaceutical business, there is incredible enthusiasm to improve the procedures to extricate these mixes. Customary extraction techniques for AQs, include the utilization of a Soxhlet mechanical assembly with natural solvents of expanding extremity, beginning with hexane, trailed by benzene, ethyl acetic acid derivation, and ethanol[11]. Even though this various dissolvable extraction framework is quicker, less difficult, and devours less measure of dissolvable than other customary strategies (maceration, reflux, and so on.), it exhibits low selectivity for certain auxiliary metabolites, for example, AQs from *P. ostreatus*. Moreover, benzene and hexane are unsafe substances, because of their poisonous quality and combustibility. In this manner, it is critical to grow progressively particular and proficient methods for getting AQs, utilizing less hurtful solvents and requiring lower extraction times. In this sense, ultrasound-helped extraction (UAE) is an exceptionally fascinating option for the extraction and sanitization of these substances. It has been broadly used to segregate bioactive substances from plants[12].

All listed investigations of ultrasound extractions demonstrate higher yields just as shorter extraction times[13,14]. The bigger extraction productivity is chiefly because of cavitation impacts, which improve mass exchange and dissolvable entrance in the plant material by upsetting the cell dividers[15]. UAE to separate AQs from the underlying foundations of *Morinda citrifolia*. These creators considered the impacts of various extraction conditions: temperature, ultrasonic power, and dissolvable sorts. The outcomes demonstrate that the yield increments with expanding extraction times, extraction temperatures, and then utilization of ethanol-water arrangements as extraction dissolvable. In a past work, UAE was additionally connected for the extraction of AQs from the hyphae of *P. ostreatus*, in which this strategy was contrasted and MAE and Soxhlet, utilizing a succession of solvents with expanding extremity (hexane, benzene, and ethyl acetic acid derivation)[16]. The outcomes demonstrated that UAE expands the extraction yield of all-out AQs and lessens the time and measure of dissolvable utilized. In any case, the mix of UAE with benzene, in addition to MAE with ethyl acetic acid derivation at a consistent power, gave the best outcomes. In this work UAE of AQs from *P. ostreatus* utilizing ethanol-water blends as dissolvable is examined, to supplant hurtful dissolvable utilized in customary extractions. Ethanol is a non-harmful and economically dissolvable broadly utilized in the extraction of dynamic standards from plants[17]. Being a polar natural dissolvable, ethanol is on a basic level a satisfactory dissolvable for the somewhat polar AQs particles. To explore the impacts of various factors on extraction, including dissolvable fluid, dissolvable/example, temperature, and extraction time a scientific demonstration was connected[18]. While diverse numerical models have been connected to the UAE advance of common mixes[19,20] many studies have connected the Taguchi plan[21,22]. The extraction strategy for the segregation of anthraquinone relies upon whether the free aglycones or the glycosides are wanted. The anthraquinone can be detached by successive extraction with solvents of expanding extremity. Diverse concentrate arrangements can be additionally filtered by a fluid dividing step. As a first extraction stage, a non-polar dissolvable can be utilized, for example, ether, benzene, chloroform, dichloromethane or ethyl acetic acid derivation. The anthraquinone glycosides, nonetheless, ought to be extricated by utilizing water, ethanol, methanol or water-ethanol blends. Later the AQs are studied for their Type 2 Antidiabetic properties.

2. Materials and Methods

Fungi material *Pleurotus ostreatus* was purchased from Green Grow Cultivation Unit, Big Shop, Ooty, Tamilnadu (preserved in a hygienic, sterilized pliable sealed container) and the authentication was done by Dr Annamalai, Plant Biologist, Coimbatore and the voucher specimen (BIT/BT/POE5101/2018) was deposited in Department of Biotechnology, Bannari Amman Institute of Technology, Sathyamangalam, India. The fruiting hyphae were washed and fragmented and finally dried in an oven at 38 °C for 5 h.

2.1 Solvents

The solvents used in the extractions were: ethanol (Porta, 96%v/v) and distilled water.

2.2 Ultrasonic-assisted extraction

Hyphae of *P. ostreatus* (0.25 g) were air-dried. At that point, these hyphae were triturated precisely utilizing a blade plant (Retsch K.G. 5657Haan West-Germany) with a work n◦5 (sifter opening 4 mm). After this pre-treatment, these hyphae were submitted to Ultrasonic-helped extraction by utilizing ethanol-water solutions. The ultrasonic light analyses were completed in a TESTLAB SRL sonomatic cleaning shower (model-TB02TACF) working at 80 W power and 40 kHz recurrence. Measurements of the tank were 150 × 140 × 100 mm. The extraction of the organism material was assessed at various conditions: dissolvable creation (60, 80, and 96% v/v of ethanol), temperatures (35, 45, and 55◦C), extraction time (15, 30, 45 min) and dissolvable/example proportion (10:1, 20:1, and 30:1). These investigations were performed by triplicate. At last, the concentrates were separated and dried under a vacuum. The grouping of AQs in the concentrates was controlled by High-Performance Liquid Chromatography (HPLC).

2.3 Regular Soxhlet Extraction

Dried hyphae (29 g) were precisely triturated and treated with an ethanol-water arrangement (435 ml), in a fixation that was chosen from the outcomes acquired in the UAE tests, which relate to the proportion with more noteworthy extractive limit (60%v/v of ethanol). The measure of dissolvable and test utilized in these extractions was controlled by the elements of the Soxhlet contraption[23], while the timeframe utilized (9 h) guaranteed the depletion of the vegetable material. The concentrates acquired were dried under a vacuum. The grouping of AQs in the concentrates was dictated by HPLC.

2.4 Quantitative Analysis of Total Anthraquinones

Refined water, 50%, 70% and 95% ethanol were utilized for extraction of all out anthraquinones as indicated. All out anthraquinone content in each concentrate was controlled by UV-vis spectrophotometric technique. A dissolvable advanced concentrate with the most astounding yield of complete anthraquinones was chosen as the proper dissolvable.

2.5 High-performance liquid chromatography

HPLC is a versatile, robust, and widely used technique for the isolation of natural products, it is a chromatographic technique that can separate a mixture of compounds and is used in phytochemical and analytical chemistry to identify, quantify and purify the individual components of the mixture. HPLC analysis was performed on an Agilent 1260 Infinity system(Agilent Technologies, Waldbronn, Germany), which consisted of a 1260 quaternary pump, an autosampler and a multiple wavelength detector connected to a Hector-M C18 column (250 mm x 4.6 mm i.d., RStech, Daejeon, Korea). The mobile phase was prepared from HPLC grade acetonitrile and 85% Phosphoric acid 0.01 M in the ratio of 1:1 to Eliot the anthraquinone derivatives. The flow rate was kept at 2.0 ml /min and detection was carried out at 254 nm. The outer alignment strategy was connected to evaluate each AQ in each concentrate, by inserting the zone under each peak for each compound from the adjustment bends. Seven alignment bends (n = 3) were direct (connection coefficients >0.99).

2.6 Liquid chromatography/mass spectrometry (LCMS/DXT) analysis of anthraquinone

The triple quadrupole mass spectrometer allows for increased sensitivity and specificity. The distillate was further analysed for volatile compounds using a Hewlett-Packard 1100 chromatograph (Agilent Technologies, Santa Clara, CA) with a quaternary pump and a UV detector (UV). The column is coupled with an MSD Ion Trap XCT mass spectrometer (Agilent Technologies, Santa Clara, CA) equipped with an electrospray ionization interface (ESI). Fractions were injected into a C-18 column (4.6×25 cm, 5m; Phenomena UK, Macclesfield, UK). The solvents used were water (A) and a 90:10 ratio of Acetonitrile: isopropyl alcohol. The elution gradient was isocratic 10% B for 5 min, 10-100% B over 20 min, 100% B for 6 min, and re-equilibration of the column, using a flow rate of 800 L/ min. Spectra were recorded in positive ionization mode between m/z 50 and 1200.

2.7 Fourier Transform Infrared (FTIR) Spectroscopy of anthraquinone

The interferogram measured from zero path difference to a maximum length based on the solution required. The interferogram is converted to a spectrum by Fourier transformation. This requires it to be stored in digital form as a series of values at equal intervals of the path difference between the two beams. To measure the path, difference a laser beam is sent through the interferometer, generating a sinusoidal signal where the separation between successive maxima is equal to the wavelength. This can trigger an analog-to-digital converter to measure the IR signal each time the laser signal passes through zero. Alternatively, the laser and IR signals can be measured synchronously at smaller intervals with the IR signal at points corresponding to the laser signal zero crossing being determined by interpolation. This approach allows the use of analog-to-digital converters that are more accurate and precise than converters that can be triggered, resulting in lower noise and specific structural prediction. For a maximum path difference d adjacent wavelength, λ and λ will have n and (n+1) cycles respectively in the interferogram. The corresponding frequencies are $\nu 1$ and $\nu 2$:

$d = n\lambda 1$ and $d = (n+1)\lambda 2$ (1)

$\lambda 1 = d/n$ and $\lambda 2 = d/(n+1)$ (2)

$\nu 1 = 1/\lambda 1$ and $\nu 2 = 1/\lambda 2$ (3)

$\nu 1 = n/d$ and $\nu 2 = (n+1)/d$ (4)

$\nu 2 - \nu 1 = 1/d$ (5)

The spectrometer was continuously purged with dry air to remove excess water vapour. The samples were placed in a liquid cell between two windows (CaF2) separated with Mylar spacers (0.6 μm). The AQ concentration for FTIR spectroscopy was 0.25 mm.

2.8 Experimental Animal

Inbred Wistar rats (10-12 weeks), weighing 220-240 gm, were resided under a standard condition of temperature, 12 hours light/dark and fed with a standard pellet diet and water ad libitum. Animals were acclimatized to laboratory conditions at least 12 days before conducting the experiments. After acclimatization for 12 days, streptozotocin was administered by bolus intravenous injection to 45 male rats fasted overnight for 12 hours. To identify the diabetic a stabilization period of 14 days was followed by a glucose tolerance test (GTT).

All animal experiments were conducted according to the rules and regulations of the Animal Ethics Committee, Government of India.

2.9 Acute Toxicity Studies

The acute toxicity study was carried out according to the OECD (Organisation for Economic Co-operation and Development) guideline (423). Tetra hydro anthraquinone extract at a dose range of 50 mg–500 mg/kg was administered intravenously to different groups of animals(six rats in each group). The animals were observed continuously for 2 hours, for any symptoms of toxicity (behaviour pattern, tremors, sleep, coma) and or death. They were under observation for a period of further 2 weeks[24].

2.10 Experimental Design

Group I: Normal control (sterile deionized water), n=9
Group II: STZ + sterile deionized water (in equal volumes) treated control (200mg/kg.iv),n=9
Group III: STZ + 250 mg/kg anthraquinone extract, n=9
Group IV: STZ + 500 mg/Kg anthraquinone extracting=9
Group V: STZ+150 mg/Kg metformin, n=9

2.11 Induction of Diabetes in Experimental Animals

Freshly prepared solutions of streptozotocin (STZ) monohydrate (Sigma Aldrich Chemicals, Pvt., Ltd., Bangalore) dissolved in sterile normal deionized water at a dose of 50 mg/kg body weight were injected into the overnight fasted rats. After 72 hours of STZ injection, the rats with serum glucose levels of >250 mg/dl were included in the study. Treatment with tetra hydro anthraquinone extract was started 72 hours after STZ injection.

2.12 Collection of Blood Samples:

The o-toluidine method of blood glucose estimation was carried out. On day 15, blood was collected from the heart under mild ether anaesthesia from overnight fasted rats and serum was isolated. Serum was analyzed for total cholesterol, triglyceride, HDL, LDL, creatinine, urea and SGOT and SGPT were estimated.

2.13 Statistical Analysis

The statistical profiling was carried out using Graph Pad Prism, Version 4.01. (Graph Pad Software, San Diego, California, USA). *$p < 0.05$, **$p < 0.01$ was considered statistically significant. Data were also analyzed statistically by one-way ANOVA following SPSS statistical software.

3. Results and discussion

Anthraquinones and their subordinates are a gathering of pigmented polyketides broadly created by growths. Aside from their brilliant shading credited to the common conjugate framework in their structure, they have additionally pulled in the consideration of researchers because of their decent variety of structures and wide scope of pharmacological activity, for example, their enemy of infective, calming, and glucosidase inhibitory exercises and cytotoxicity against malignant growth cells. Impact of dissolvable structure on the extraction of anthraquinones by ultrasound-assisted extraction.

Subsequent, to deciding the ideal conditions for the extraction of AQs, the impact of dissolvable organization on the AQ yield was noted down. Tests of 0.5 g hyphae of *P.ostreatus* were extricated in the ultrasonic shower at the ideal temperature (55 ◦C), dissolvable/example proportion (20:1) and time (15 min) controlled by Taguchi is strategy, and utilizing distinctive ethanol-water compositions (50, 60, 70, 80, and 96% v/v of ethanol). The various proportions of ethanol-water arrangements were considered dissolvable for examining the dissolvable piece impact. Ethanol is a non-poisonous and economically dissolvable generally utilized in the extraction of dynamic standards from plants[25]. Being a polar natural dissolvable, ethanol is on a fundamental level a sufficient dissolvable for the somewhat polar AQs particles. There is an expansion in the AQs yield with the water content in the dissolvable up to 40% v/v (60% v/v of ethanol) and after that a lessening for a half v/v blend. These outcomes are in concurrence with the ideal dissolvable structure controlled by Taguchi's strategy. Cavitation, which is the fundamental marvel engaged with ultra-sound assisted extraction, is influenced by certain physical properties of the dissolvable, for example, surface strain, thickness, and vapour weight. The estimation of these properties for ethanol + water blends plays a remarkable[26,27]. Surface strain diminishes with the expansion in ethanol concentration, while consistency demonstrates a most extreme at about half v/v of ethanol[28] and vapour weight increments consistently with ethanol focus till the azeotropic synthesis (97% v/v at 50 ◦C). On a fundamental level, cavitation would benefit from higher surface strain, lower thickness, and lower vapour weight of the cavitating medium. Be that as it may, for a given sonic recurrence, there is an ideal vapour weight where the motivation weight and temperature acquired by the breakdown of a hole are most extreme[29]. As indicated by the trial results, the ideal vapour beyond any doubt for the conditions considered compares to a dissolvable with a 60% v/v ethanol. Even though the thickness is about at its most extreme incentive for this focus, and the surface strain of the water + liquor arrangement is generously lower than that of unadulterated water, a medium estimation of the vapour weight worth is by all accounts the predominant factor in the cavitation impacts. Similarly, the impact of time was examined by playing out a progression of UAE tests at various occasions (0, 15, 45, 60, 75, and 90 min), and keeping up the other extraction components as indicated by the ideal conditions (dissolvable piece 60% v/v of ethanol, dissolvable/example proportion 20:1 and temperature 55 ◦C). All tests were done in triplicate and the outcomes were. As it very well may be watched, the yield increments with expanding contact time between the plant material and dissolvable, up to 30 min. After this time, the yield of AQs stays consistent (6.56 mg AQs/100 g plant material), demonstrating that the plant material is depleted.

3.1 Correlation of UAE with ordinary Soxhlet extractions

In this examination, UAE was contrasted and the customary Soxhlet extraction strategy. The UAE analyses were done under ideal conditions. To think about the two methods, the Soxhlet extractions were performed utilizing a similar dissolvable structure. It demonstrates that the absolute AQs yield acquired by UAE is practically twofold that gotten by Soxhlet extraction. Moreover, it tends to be seen that, in the two strategies, Soranjidiol is the AQ with the significant fixation in the concentrate. Regarding extraction time, UAE is likewise the quickest extraction technique, requiring only 30 min to deplete the hyphae material. In this way,

proficiency (yield of total AQs/time of extraction) is more prominent in the UAE than in Soxhlet extraction. Also, note that the AQs yield extraction obtained is like yield acquired by customary extraction methods for AQs, which include the utilization of a Soxhlet apparatus with natural solvents of expanding extremity, beginning with hexane, trailed by benzene, ethyl acetic acid derivation, and ethanol. Be that as it may, the UAE utilizing ethanol shows the upsides of lower extraction time (30 min for UAE and 16h for conventional strategy) and the utilization of less hurtful dissolvable.

3.2 Gas chromatography Profile of crude *Pleurotus ostreatus*

The PO was found to accommodate numerous middle-chain aliphatic alcohols, aldehydes, and ketones which are supposedly believed in the fatty acid degradation. Major peaks were found to contain numerous aldehydes, ketones and aliphatic compounds (Figure 2). The mass spectra of all the phytoconstituents were predicted using the NIST library as listed in Table 1. Evaluation of tetra hydro anthraquinone reversed the endogenous level of dopamine toxicity, acting against selective loss of dopaminergic neurons in Parkinson's Disease[30]. Being fungal, the mushrooms contributed mainly to polysaccharides, leading to their dry weight. It is also predicted that carbohydrates hydrolyse the cell wall components thereby increasing cell wall permeability and yielding higher extraction yield.

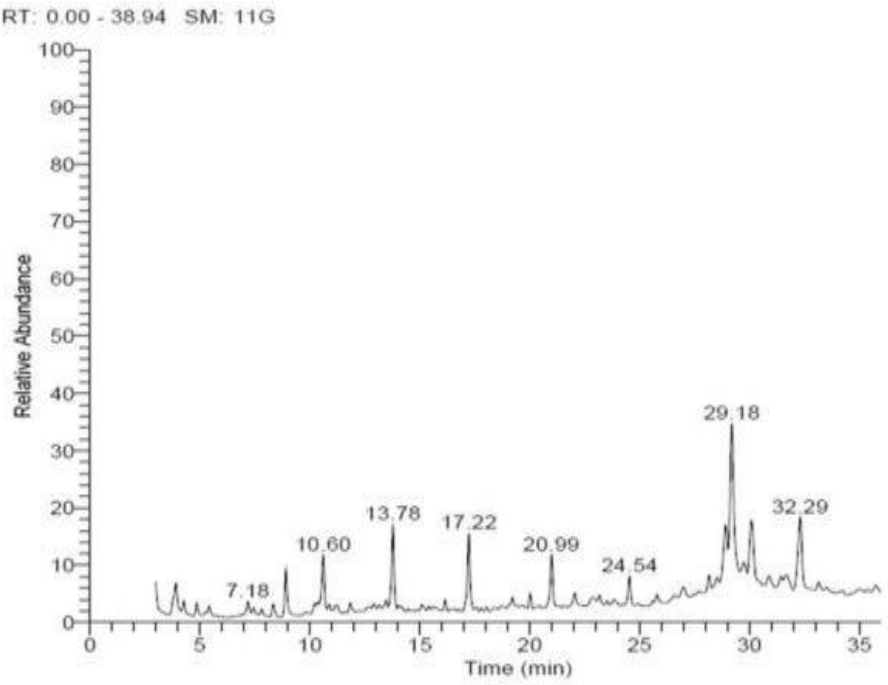

Figure 2 GC-MS Chromatogram of ethanolic extract of Pleurotus ostreatus

Table 1 Bioactive components identified in ethanolic extract of Pleurotus ostreatus by GC-MS analysis

Sl No	Retention time (RT)	Name of the compound	Molecular formula	Molecular weight	Kovats Indices
1	3.90	2,2,5-TRIMETHYL-5-HEXEN-3-ONE	$C_9H_{16}O$	140	765
2	5.43	1-HEXANOL, 2-ETHYL	$C_8H_{18}O$	130	992
3	7.18	3-DODECENE, (Z)-	C12H24	168	1187
4	8.34	CYCLOHEXANE, HEXYL-	C12H24	168	1224
5	8.91	ACRYLIC ACID OCTYL ESTER	C11H20O2	184	1273
6	10.60	1-TETRADECENE (CAS)	C14H28	196	1392
7	13.78	CYCLOHEXADECANE (CAS)	C16H32	224	1876
8	17.22	(CIS)-2-NONADECENE	C19H38	266	1921
9	19.21	5,6-DIHYDRO-5,6-DIMETHYLBENZO[C]CINNOLINE	C14H14N2	210	1986
10	20.02	10-NONADECANONE	C H O	282	2045
11	20.99	ENDO-12-HYDROXY[4](1,1')[3](2,3')FERROCENOPHANE	C17H20FeO	296	2134
12	25.80	RICINOLEIC ACID	C18H34O3	298	2351
13	29.18	7-HYDROXY-1-METHOXY-6-METHYL TETRA HYDRO ANTHRAQUINONE	C16H12O4	268	2392
14	30.10	PIPERCALLOSIDINE		303	2476

3.3 HPLC analysis of Pleurotus ostreatus

The validation of the analytical method is a key requirement before the estimation of actual samples for an analyst. Tetra hydro anthraquinone was found in the hyphae of the plant Pleurotus ostreatus (PO) in a retention time of 5.9 in the chromatogram of fractions collected from conventional chloroform extraction for tetra hydro anthraquinone (Figure 3). HPLC methods have also been developed to analyse phytosterols, but studies with mycosterols are rather scarce. The analysis of sterols might be performed with polar (e.g. silica, DIOL, amino, CN) or reversed phase (e.g. C18 = ODS, C8, or phenyl) columns (Moreau et al. 2002). A. bisporus showed the highest ergosterol content when expressed in mg/g of fat (158±2) or mg/100 g of dry weight (352±1). Nevertheless, the maximum value in mg/100 g of fresh weight was found in L. edodes (44.0±0.3). Otherwise, M. esculenta presented the lowest content independently of the units: 9.9±0.3 mg/g of fat; 43±2 mg/100 g of dry weight; 4.0±0.3 mg/100 g of fresh weight.

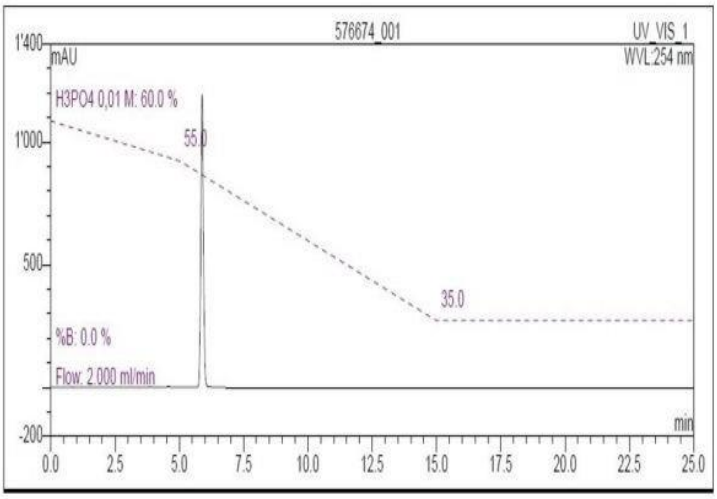

Figure 3 HPLC Chromatogram of anthraquinone

3.4 LCMS:

Under positive ionization, the molecular weight obtained was 209 m/z was detected. Adding up of ions increasing the H+ makes the actual 208.20 m/z of tetra hydro anthraquinone to 209.20 m/z. The amount of tetra hydro anthraquinone was determined by plotting the calibration curve from 100 ppm to 10 ppm. The concentration of AQ was approximately 29.9 ppm. Characterization of physiochemical properties (logP) and molecular mass of mushroom isolated AQ was carried out using Agilent SQ system, LCMS DXT (Triple quadrupole). Before LCMS analysis AQ extract was reconstituted at a concentration (500:500) of 50% of 20 Mm ionizing water and 20% (200:800) of acetonitrile: isopropyl alcohol. The LCMS output included retention time, peak area as relative quantification and molecular mass of AQ extract (Figure 4). The molecular mass range of 100-500 m/z was chosen by the range of the PCAP model which is used in calculating Tmax from logP[31]. Compound characterization was carried out according to the molecular mass obtained by positive ionization[32].

Pearson's correlation analysis between total phenolic and LCMS relative quantification of STD concerning AQ extract was performed[33]. The functional calibration curve was prepared using AQ std. The calibration was found to be linear with an R2 value of 0.995. Diabetes mellitus is probably the single most important metabolic disease and is universally a potent factor which ends up in death and disability if not treated. It affects every cell in the body and the body's essential biochemical processes, and it is a major public health problem in developing countries[34].

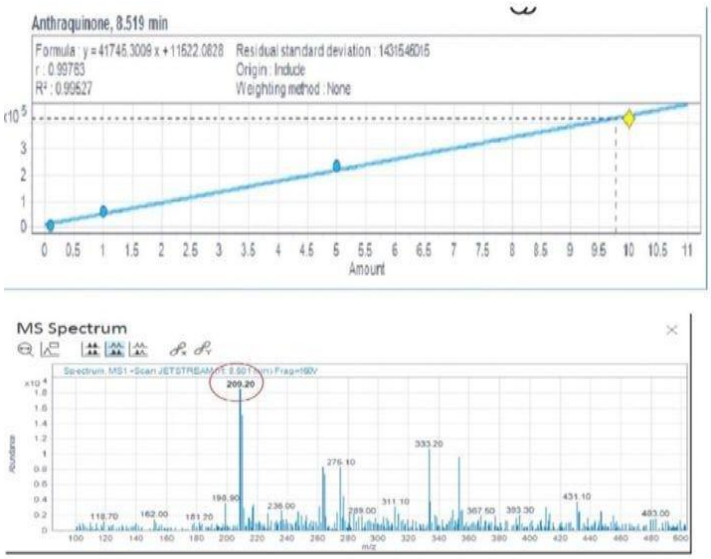

Figure 4 Extracted ion chromatogram (EIC) at m/z in positive ion mode

3.5 FTIR:

The functional group analysis by IR spectrum was formed in a fixed cell with an appropriate solvent. Since the extracted mixture was methanolic extract, the FTIR was performed accordingly. The fixed cell size was about 0.1mm thickness and a spacer of 0.025 was used in the detection. The functional groups were analysed using FTIR(Shimadzu) in the region of 4000-500 cm (-1) (Figure 5). FTIR characterization of AQ exhibited aromatic rings/functional groups indicating the aroma flavour of tetra hydro Anthraquinone. Similarly, Wasser (2002) reported mushrooms are found mostly as glucans with different types of glyosidic linkage like(1---3),(1---6)Beta glucans and (1---3) alpha glucans along with heteroglucans. Four specific regions of FTIR spectra (4000-1800cm-1,1800-1500cm-1,950- 750cm-1,850-610 cm-1) are observed. In the region between 4000-1800cm-1, the prominent band centred around 3319.49 assigned to O-H stretching vibration of glycosidic structures. These O-H stretching vibrations could be overlapped by inter and intra-hydrogen bonds that are available in polysaccharides. The bands around 1800-1500 cm-1 in turn assigned to C=O, C=Stretching of fatty acids from the cell wall[35] are more predominant in the mushroom spectrum. In the region between 950-750 cm, two major bands C-X and C-H (aromatic)assigned to chloride and aromatic proteins[36]. The final region of 850-610 cm-1 indicated C-H and C-H (Bromide/Iodide) in the spectrum. This may be due to the solvent extraction leading to vibrational stretch in the spectrum.

It also constitutes the aromatic configuration of polysaccharides in particular around 808.17cm-1 to alpha glycosides. The presence of aromatic groups, beta-glucans and glucan protein complexes is evident from the FTIR spectrum.

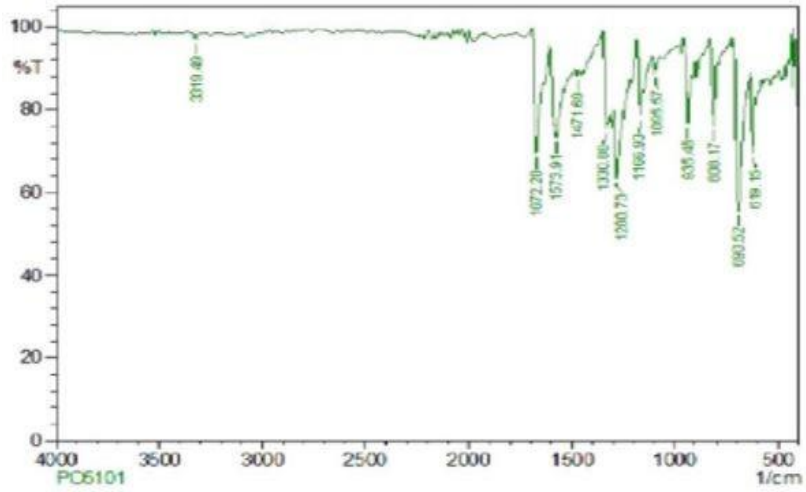

Figure 5 FTIR spectra of anthraquinone

3.6 Effect of Anthraquinone Extract on Body Weight and Organ Weight of STZ-Induced

Diabetic Mice STZ mediated body weight reduction was remarkably reversed by the delivery of AQ extract. Intravenous administration of the extract resulted in increased body weight, which was comparable to the increase in the body weight of normal control (Table 2). The liver and kidney indices were significantly different in the diabetic control compared to the normal, healthy control animals, and the liver index was significantly higher in the treated group compared to the diabetic group. Even though, there are different types of oral hypoglycaemic agents with insulin for the treatment of diabetes, demand by patients to use natural products with antidiabetic activity is always at a peak rate[37]. In today's life, the current drug that we use for diabetes is becoming life-threatening due to their drawbacks in long-term use, thus there is always a demand to find safer and more effective antidiabetic drugs[38]. Weight loss is also one of the parameters measured during diabetes due to the inactivation of metabolic pathways or due to the excessive breakdown of tissue proteins. Similarly, STZ-caused body weight loss was regained to its normal control values by *Barleria lupulina* extract treatment[39,40]. Wistar rats treated with the extract showed a progressive increase in their percentage mean body weights. This could be explained by protein-sparing action i.e. gluconeogenesis from the muscle protein would result in a decrease in total protein [41]. Tetra hydro anthraquinone extract-treated rats showed increased liver weight gain. Increased breakdown of glycogen, pronounced gluconeogenesis, and enhanced catabolic processes such as glycogenolysis, lipolysis and proteolysis triggered by a lack of insulin or cellular glucose in diabetes might be responsible for the reduction in liver weight of diabetic animals. Glomerular enlargement leads to microvascular renal obstacles involving a series of metabolic changes that are part of the pathogenesis of diabetic neuropathy.[42]

3.7 Effect of anthraquinone extract on Blood Glucose Level, lipid profile and liver profile of STZ Induced Diabetic Mice

Hyperglycaemia is the main attribute of diabetes mellitus. Diabetic control mice showed 309±5.66 mg /dL of blood glucose level when compared to AQ-treated mice showed 167.56±4.3 mg/dl on the 15th day (Table 2). After supplementation with AQ extract, the alteration in lipid metabolism was partially attenuated as evidenced by decreased serum total cholesterol level. The level of HDL cholesterol was significantly reduced in untreated diabetic rats and these lowered levels of HDL cholesterol were enhanced significantly in AQ extract treated animals. Hypolipoproteinaemia is usually complicated by diabetes mellitus[43]. The present study showed that the level of serum total cholesterol was significantly elevated in the diabetic control group as compared to the normal healthy control. After supplementation with tetra hydro anthraquinone extract, the alteration in lipid metabolism was partially attenuated as evidenced by decreased serum total cholesterol level. Similarly, it has been shown that intraperitoneal administration of *Boerhaavia diffusa* Linn extract to diabetic rats significantly ($p<0.05$)normalized the levels of erythrocyte membrane cholesterol and phospholipids and restored the status of total cholesterol level. The effect of AQ extract on SGPT and SGOT of STZ-induced mice is presented in Table2. Normal blood glucose reduction metabolism within the body is maintained during normal health metabolism, but in diabetic patients, it is vice versa. The increased levels of serum urea in diabetic mice were significantly ($p<0.01$) restored to near-normal levels in the AQ extract-treated mice. An elevated level of serum urea in diabetic mice was found to be slightly declined in the treated mice (Table 2). It has been experimentally proven that leaves of Anacardium occidentale exhibited hepato-protective activity by lowering the levels of SGPT and SGOT and this may be due to antioxidant properties exerted by flavonoids present in the fruit extract[44]. The increased levels of serum urea in diabetic mice were significantly ($p<0.01$) restored to near-normal levels in the AQ extract-treated mice. In the diabetic mice, the increased serum creatinine was noticed and it was found to be reduced significantly in the treated mice. Aqueous extract of Clitoria ternatea (CTL) and flowers (CTF) increased the total protein and lowered the serum urea and creatinine levels by enhancing the renal function that is generally impaired in diabetic rats[45]. Dyslipidaemia, identified with low HDL-cholesterol is one of the identity parameters in diabetes. LDL plays an important role in atherosclerosis and hypercholesterolemia which is associated with a defect relating to the lack of LDL receptors [46]. Compared to the normal control group, the levels of LDL cholesterol and VLDL cholesterol levels were significantly ($p<0.01$) elevated in the diabetic control group clearly showing that the response was better after the administrations of the tetra hydro anthraquinone extract in the treated group i.e. both LDL and VLDL cholesterol levels were decreased to a normal level. This causes an increase in LDL particles and results in an increase in LDL-cholesterol value in diabetic mellitus. The increased VLDL-cholesterol and triglyceride levels decrease the level of HDL-cholesterol and increase the concentration of small dense LDL-cholesterol particles by activation of lipoprotein lipase and lecithin acyl-cholesterol transferase. Triglyceride accumulation in the liver of diabetic rats is due to enhanced synthesis or decreased output from the liver as VLDL or a combination of both [47].

In diabetic mice, the level of SGPT was elevated significantly and the enzyme level reached to normal level after the intravenous administration of AQ extract (Table 2). Glycaemic control is the major indicator of triglyceride serum level. Hyper-triglyceridemia is a common finding in diabetic patients and is responsible for vascular complications [48]. Deficiency of lipoprotein lipase activity may contribute significantly to the elevation of triglycerides in diabetes [49]. Insulin is a potent inhibitor of lipolysis since it inhibits the activity of the hormone-sensitive lipases in adipose tissue and suppresses the release of triglycerides [50]. Furthermore, the treatment of diabetes with insulin served to lower plasma triglyceride levels by returning lipoprotein lipase levels to normal [51]. The diabetes-induced hyperlipidaemia might be due to excess mobilization of fat from the adipose tissue because of the underutilization of glucose. Histopathological Examination of Liver. Liver parenchyma with hepatocytes of normal healthy liver tissue of rats was carried out, which appeared healthy. Diabetic rats treated with AQ extract revealed a remarkable improvement of hepatic tissues, diminished necrosis and even the cellular arrangement of hepatocytes appeared undamaged which was seen in diabetic rats (Figure 6). The main histopathological changes found in diabetic mice were perioral infiltration. Treatment with AQ extract brought back the cellular arrangement to near normal. Neither erythrocyte haemorrhage nor inflammatory cell infiltration were encountered. Similarly, the effect of T.arjuna stem bark extract on the histopathology of the liver in STZ-induced diabetic rats where reported that the normal liver tissue section shows sinusoidal cards of hepatocytes with central vein and portal tracts and the diabetic rat liver tissue section shows distortion in the arrangement of cells around the central vein, perioral fatty infiltration with focal necrosis of hepatocytes.

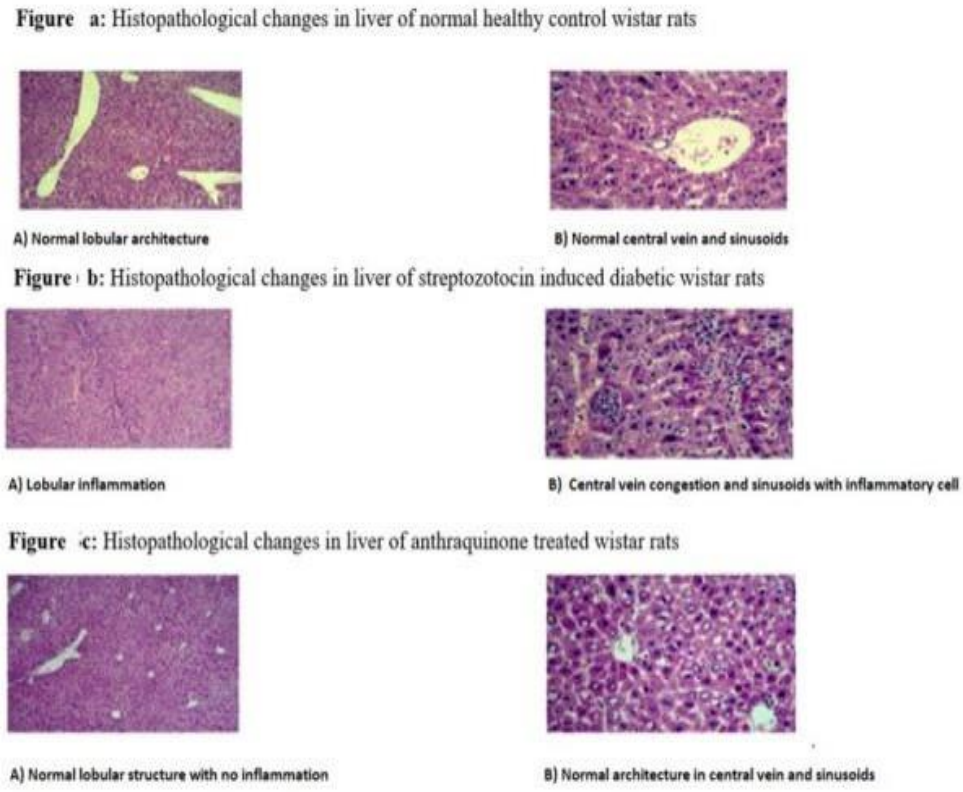

Figure 6 Histopathology analysis

Table 2 Effect of anthraquinone extract on biochemical parameters

Treatment groups		**Normal control**	**STZ + sterile deionized water**	**STZ + 250 mg/kg tetra hydro anthraquinone**	**STZ + 500 mg/Kg tetra hydro anthraquinone**	**STZ+250 mg/Kg metformin**
Mean initial body weight (g)		190.24±4.11	193.43±6.24	189.21±7.36	185.61±5.33	197.48±7.34
Mean final body weight (g)		196.55±5.36	172.84±4.24	195.37±3.14	193.83±3.38	213.32±8.39
Mean weight Gain (G↑)/loss (L↓) (g		6.31↑	20.59↓**	6.16↑[a]	8.22↑[a]	15.84↑[a]
Fasting blood glucose (mg/Dl)	**Initial**	66.78±1.92	247.56±2.74	231.14±1.24	213.12±5.28	230.66±6.16
	Final (after 14 days)	73.45±1.54	265.16±4.36	145.36±4.66[b]	122.33±4.57[b]	105.53±4.25[b]
Organ weight(g)	**Liver (g)**	3.213± 0.17	1.43±0.13[b]	2.54 ±0.06**	2.32± 0.16**	1.88± 0.14[b]
	Kidney(g)	1.048± 0.01	0.82±0.04[b]	0.93±0.07**	0.98±0.00**	0.88± 0.17b
Insulin (mIU/mL)		20.421±1.030	9.290±0.948**	14.290±1.670[a]	18.508±1.250[c]	19.710±1.090[c]
Glucose (mg/dL)		72.81±3.54	197.39±2.61**	133.66±1.56[a]	112.51±1.84[c]	107.38±1.84[c]
Urea (mg/dL)		12.06±1.04	29.73±1.85*	21.58±1.26	14.55±1.38	13.15±1.56[a]
Creatinine (mg/dL)		0.73±0.12	2.45±0.35*	1.86±0.48	1.29±0.15	1.05±0.11[a]
HbA1c (%)		4.51±0.52	9.79±0.25**	7.16±0.76[ns]	5.15±0.81[a]	5.20±0.62[a]
Protein (g/dL)		7.83±0.16	6.12±0.19*	6.56±0.15[a]	8.16±0.65[a]	7.86±0.38[ns]
Albumin (g/dL)		4.53±0.14	3.74±0.21*	4.02±0.11	4.76±0.53	4.03±0.45
Globulin (g/dL)		3.33±0.11	2.48±0.24	2.74±0.14	3.30±0.52	3.83±0.24
SGPT (µ/L)		18.53±1.52	97.18±3.44*	38.56±1.16	27.59±1.74	21.94±1.02
SGOT (µ/L)		20.26±1.2	89.15±1.81*	49.56±1.66	30.41±1.84	25.78±1.55

	3				
ALP (µ/L)	184.93±3.55	226.16±4.74*	204.08±4.46[a]	191.56±2.79[a]	173.94±2.06[a]
TC (mg/dl)	98.6 ±5.34	177.60±2.33	132±18.18**	112±0.4**	104.56±4.4[a]
HDL (mg/dl)	34.33 ±3.12	28.34±5.2	46.2±1.13**	35.4±8.8**	40.21±2.04[a]
LDL (mg/dl)	47.28 ±2.6	118.19±0.84	28.73±11.6**	55.92±2.4**	45.6±2.14[c]
VLDL (mg/dl)	18.92 ±0.18	34.46±0.19	28.9±0.16**	23.39±0.27**	18.4±1.24[a]
TG (mg/dl)	89.56±0.01	172.05±1.12	119±2.6**	106±1.03**	92.12±1.84[a]

4. Conclusion

In summary, the AQ extraction from edible fungi was extracted and studied for its Type II antidiabetic properties. This, AQ merits further exploration both chemically and biologically to exploit its relevant therapeutic effect to substantiate its ethno-medicinal usage.

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Disclosure statement

The authors have declared no conflict of interest.

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