Broad spectrum bioactivity of a β-glucan rich heteropolysaccharide- Pestalopine isolated from endophytic fungi Pestalotiopsis chamaeropsis CEL6

Hiran Kanti Santra
Vidyasagar University
Debdulal Banerjee (debu33@gmail.com)
Vidyasagar University

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Abstract

A biologically potent exopolysaccharide (EPS); Pestalopine was produced by *Pestalotiopsis chamaeropsis* CEL6, an endophytic fungal isolate of *Chloranthus elatior* Sw. Pestalopine is composed of glucose, arabinose, fucose, rhamnose, and galactose in a molar ratio of nearly 10:1:2:2:4 having an Mw ~ 3.29 x 10^5 Da. Pestalopine exhibited a radical scavenging effect and significantly increased antioxidant parameters (malondialdehyde, superoxide dismutase, glutathione peroxidase, reduced glutathione oxidized glutathione) in peritoneal macrophage cells in a concentration-dependent manner, with a maximum effect at 500 mg mL^{-1}. Pestalopine is hepatoprotective in nature and improves the liver function profiles; - total bilirubin, direct bilirubin, hepatic enzymes- alkaline phosphatase, gamma-glutamyl transferase, aspartate aminotransferase, alanine transaminase of liver fibrosis induced (through Thioacetamide) male Wistar rats in comparison to control. Pestalopine-fed rats are reported to have higher counts of beneficial *Lactobacillus* sp. Present findings suggest that Pestalopine may have promise as a non-toxic exogenous antioxidant with hepatoprotective and probiotic efficacies.

Contribution to the field

Secondary metabolites synthesized by endophytic fungi hold numerous pharmaceutical potentialities. Especially exopolysaccharides produced in culture broth are majorly antioxidative in action. In the current situation, there is a huge scarcity of potent antioxidants that can replace contemporary synthetic ones which hold numerous detrimental side effects. Natural products are always the first choice to replace the existing ones and other than plant sources, novel metabolites from potent endophytic fungi are acting as prominent sources nowadays. Here an effort has been made to explore secondary metabolites from fungal endophytes of medicinal plants for the production of potent antioxidants. In the present investigation, β-glucan rich heteropolysaccharide (named Pestalopine) produced by an endophytic fungi *Pestalotiopsis chamaeropsis* CEL6 (isolated from *Chloranthus elatior* Sw.) exhibited in-vitro free radical scavenging activity, antioxidative activity, and in-vivo hepatoprotective activity. Also, Pestalopine modulated gut microbial flora of treated Male Wistar rats. Investigational outcomes shades light on the immense health benefits of endophytic fungal secondary metabolites, mainly macromolecules like exopolysaccharides. The present outcome holds future potential for the commercialization of Pestalopine as a major in-vitro and in-vivo antioxidant.

Introduction

The inauguration of glycoscience was almost two hundred years ago milestoning from the discovery of chitin from edible mushrooms (Muzzarelli et al., 2012). The domain of carbohydrate-based polymer research has a footprint in multiple arenas of modern-day therapeutics as antitumors, immunomodulators, probiotics, antiallergics, anti-radiants, and antimicrobials. Their structural diversity, unique linkages, rheological properties, biodegradability, and biocompatibility have also promoted their use as inert polymers for nano-drug delivery, therapeutic diagnosis, bioactive compound encapsulation, and also in wound healing (Santra and Banerjee, 2021). The thickening and gelling properties of

polysaccharides have made them suitable candidates to be used in cosmetics and pharmaceuticals as an alternative to synthetic polymers. Polysaccharides either remain stitched to the cell surface (CPS, capsular polysaccharide) or are secreted in the extracellular (EPS, extracellular polysaccharide) medium (Sajna et al., 2021). The diverse array of both intra and extracellular polymers with remarkable biological activity has been reported from a variety of sources. Exopolysaccharides are always prioritized due to their simple isolation, purification, and smooth administration to the *in-vivo* system (Mahapatra and Banerjee, 2016).

Polysaccharides from plants and macrofungi have been extensively studied (Maity et al., 2020, Singha et al., 2021, Khatua and Acharya, 2022). In the last two decades, there has been a surge in the exploration of bioactive exopolysaccharides from uncanny natural sources and endophytic fungi score at the top of that novelty list (Saxena and Strobel, 2021). Endophytes are hidden microorganisms that reside within the host plants' internal tissues and provide broad-spectrum protection of the host from both biotic and abiotic stresses (Wen et al., 2022). They are considered the plants' in-house doctors and are thought to be co-evolved with their green partners. Interestingly they are specialised hubs for the synthesis of miraculous substances with both pharmaceutical and agricultural utility (Tiwari and Bae, 2022). Still, exploitation of fungal endophytes for bioactive exopolysaccharide production is not plenty and hence requires special attention.

The proper use of microbial secondary metabolites from an industrial perspective depends on both structural characterisation and process optimisation. Exopolysaccharide synthesis from endophytic fungi may be impacted by a variety of fermentation circumstances, including medium composition, initial medium pH, incubation period, and incubation temperature. Diverse bioactive EPS, such as fructans, polygalactans, gellan, alginate, kefiran, and α-D-glucans and β-D-glucans, are clustered with diverse combinations of monosaccharides, such as amino sugars (D- glucosamine, D- galactosamine), hexoses (D- allose, D-fructose, L- fucose, D- glucose, D- galactose, D- mannose, L- rhamnose,), pentoses (D- arabinose, D-ribose, D- xylose), and different organic (hyaluronic acid, glucuronic acid) and inorganic substituents (acetate, ether, pyruvate, phosphate, lactate, and sulfate, etc.) (Joulak et al., 2019).

In the present investigation endophytic fungi *Pestalotiopsis chamaeropsis* CEL6 isolated from *Chloranthus elatior* Sw. has been explored for exopolysaccharide production. β-glucan rich heteropolysaccharide was designated as Pestalopine here and two different optimization attributes, one variable at a time (OVAT) method and response surface methodology (RSM) were adopted to optimize the Pestalopine production. In-vitro and in-vivo antioxidative activity, hepatoprotective action, and efficacy to alter intestinal bacterial flora of Pestalopine were also evaluated.

Materials and Methods

Isolation and identification of exopolysaccharide (EPS) producing endophytic fungi

Pestalotiopsis chamaeropsis CEL6 was isolated as an endophyte from leaf tissues of *Chloranthus elatior* Sw., collected from Passighat forest areas of Arunachal Pradesh, in water agar medium following the standard procedures of sterilisation and inoculation (Banerjee et al., 2010). Isolate was screened as the most potent EPS producer. The macroscopic, microscopic morphology of the isolate was studied and the phylogenetic position of CEL6 was examined by obtaining ribosomal DNA (ITS region) sequences. PCR run was performed with universal primers ITS-1 and ITS-4 with necessary reagents obtained from Invitrogen, Waltham, USA (White et al., 1990). Amplified gene products were sequenced (Bioserve Biotechnologies Pvt. Ltd., a Repro Cell Company, Hyderabad, India) and analysed by nucleotide BLAST. A phylogenetic tree was constructed retrieving close-matching sequences from BLAST results (Tamura et al., 2021). The nucleotide sequence bears a GenBank Accession number of OP316869.

Production and optimization of EPS

P. chamaeropsis CEL6 was grown in 250 mL Erlenmeyer flasks containing 50 mL of the potato dextrose broth (PDB) medium, and incubated at 25°C with 120 rpm shaking speed for 14 days for EPS production. The one variable at a time (OVAT) method was adopted to optimise the necessary fermentation parameters. To investigate the required parameters CEL6 was grown in different fermentation temperatures, incubation periods, initial pH of the medium, additional carbon (2 g L^{-1} of glucose, rhamnose, galactose, raffinose, starch, fructose, and maltose), nitrogen (0.5 g L^{-1} of yeast extract, peptone, ammonium chloride, ammonium nitrate, urea, and glycine) and salt requirements (metal ions- 0.05 g L^{-1} $CaCl_2$, $MnCl_2$, $MgCl_2$, $FeCl_3$, KCl, NaCl, $HgCl_2$ and phosphates- 0.05 g L^{-1} K_2HPO_4, KH_2PO_4 and NaH_2PO_4) were investigated. During the optimization of fermentation conditions; medium compositions were used as optimized in its previous experimental step (Mahapatra and Banerjee, 2013a). Oxygen requirement was evaluated by growing CEL6 in 250 mL Erlenmeyer flasks with different medium volumes (25, 50, 75, and 100 mL). The necessity of O_2 for growth and EPS production were indirectly predicted by calculating surface area, headspace volume, medium volume, total volume, and medium depth in the Erlenmeyer flask following the procedures of Wonglumsom et al. (2000). After OVAT optimization, the response surface methodology (RSM) was adopted through Box–Behnken design (BBD). From OVAT outcomes the four most influential factors at three different levels (-1, 0, and + 1) were selected. A second-order polynomial equation-$Y = \beta_0 + \Sigma\beta_1 x_1 + \Sigma\beta_{ij} x_i x_j + \Sigma\beta_{ii} x_i^2$ with multiple regression techniques predicted the EPS production through an empirical model. Here Y is the predicted response or dependent variable, x_i, and x_j are independent factors, β_0 is the intercept of the regression equation, β_i is the linear coefficient, β_{ii} is the quadratic coefficient and β_{ij} is the interaction coefficient.

Estimation of EPS

The fungal culture broth was filtered (by Whatman filter paper no. 4) and centrifuged (Reimi, India) at 10,000 × g to separate fungal biomass. The mycelial pellets were collected, dried, and weighed. The supernatant was concentrated in a rotary evaporator (Bacca Buchi, Switzerland) under low pressure at

40°C. Chilled absolute ethanol was mixed with the concentrated supernatant (5:1 ratio) and refrigerated at 4 ∘C for 24 h. The precipitated crude EPS was procured by centrifugation at 10,000 × g for 10 min, and dialyzed in a cellulose membrane (molecular weight cut off 10,000) against distilled water for 24 h. EPS concentration was estimated by the phenol sulfuric acid method (Dubois et al., 1956) with glucose (10–500 µg mL^{-1}) as the standard.

Characterization of the extracted EPS

Extracted EPS was termed as Pestalopine and total glucan, β-glucan, and α-glucan were estimated following the protocols of the Yeast β-Glucan Assay Kit (Megazyme Institute Wicklow, Ireland). EPS was pressed with KBr (SRL, India) into pellets for FT-IR analysis in the frequency range of 400–4000 cm^{-1}. The molecular weight of the EPS was determined by gel permeation chromatography (GPC) on a Sepharose-6B column (65 cm × 2 cm) using distilled water as eluent (flow rate-04 mL min^{-1}) and dextrans (40, 70, and 200 kDa) as standard (Mahapatra and Banerjee, 2012). The monosaccharide composition of Pestalopine was detected using GC-MS following the standard procedures (Santra and Banerjee 2022a).

In brief, using a water bath at 70°C for 15 min, 100 mg of dried exopolysaccharide was combined with 1 mL MeOH, 20 µL ribitol (which serves as an internal standard), and 20 µL nor-leucine. The entire mixture was then centrifuged for 5 min at 10,000 rpm, and the supernatant was immediately dried and dissolved in 20 µL of methoxy-amine HCL for 120 min at 37°C. The final 1 µL of the derivatized EPS sample was loaded onto the GC-MS for analysis of monosaccharide composition after 40 µL of TMS (Tri methyl siloxane) had been added. The instrument was set up with a 30 m x 0.25 mm DB-5 Ultra Inert column. With a split ratio of 25:1, the inlet temperature was 230°C, and the MS transfer line temperature was 250°C. A constant flow mode was used for the column's flow, which was 1.3 mL min^{-1} with an average linear starting velocity of 39 cm sec^{-1}. Helium was used as the carrier gas, and ZB-1701 served as the guard column. The program was isothermal, holding at 70°C for 5 min, increasing by 10°C per minute to 180°C, holding for 2 min, increasing by 10°C per minute again to 220°C, holding for 1 min, and finally ramping up by 2.5°C per minute. Up to 265°C with a 1-minute hold, then a ramp up to 285°C with a 1-minute hold, and finally a climb of 1°C per min up to 290°C with a 0.6-minute hold. The mass spectrum was obtained in scan mode from 40 to 650 amu with a detection threshold of 100 ion counts while the detector was in positive ion mode.

Evaluation of antioxidative potentialities

In-vitro antioxidative ability of the EPS was evaluated against common free radical generator DPPH, ABTS, H_2O_2, and FRAP with ascorbic acid and EDTA as the standard antioxidants following the spectrophotometric procedures (Santra and Banerjee 2022b). In brief for DPPH radical scavenging assay fungal ethyl acetate extracts were measured by using DPPH (diphenyl picryl hydrazyl) (Blois 1958). 200

µL of 0.1 mM of DPPH solution (prepared by dissolving DPPH in methanol) was added to different concentrations of EA extracts (0, 10, 25, 50, 100 µg mL^{-1}) of fungal isolates. The two liquids were mixed thoroughly and incubated for thirty minutes in dark conditions at normal room temperature. Post-incubation tasks include the measurement of absorbance at 517 nm of wavelength using a UV spectrophotometer. EA (ethyl acetate) mixed with methanolic DPPH was used as blank. The radical scavenging potential was calculated in the form of a percentage using the formula = [1- (Abs S_1/ Abs S_2)] * 100. Abs S_1 = absorbance of the sample at 517 nm; Abs S_2 = absorbance of the control at 517 nm. Ascorbic acid was used as a positive control.

For ABTS radical scavenging assay first ABTS was prepared by mixing aqueous ABTS (2mM) with potassium persulfate (2.45mM) and stored at room temperature for 24 hours in dark. Prior to the detection of antioxidative activity of the different concentrations (12.5µg/mL-100 µg/mL) of Pestalopine or standard the ABTS solution was set at an absorbance of 0.750 ± 0.025 at 734 nm diluting with sodium phosphate buffer (0.1 M, pH 7.4). Mixing of diluted ABTS with culture extract was followed by 30 minutes of incubation and the absorbance was measured at 734 nm. The scavenging capability of ABTS radical was calculated by the following equation: (1- (A_1/ A_2)* 100) where, A_1 was the initial concentration of the ABTS and A_2 was the absorbance of the remaining concentration of ABTS in the presence of culture extract or standard (Gülcin, 2006b).

For the detection of H_2O_2 scavenging activity different concentrations of Pestalopine (20µg/mL-100 µg/mL) were tested for their H_2O_2 scavenging activity following the methods of Ruch et al. (1989). Various concentrations of the culture extract mixed in phosphate buffer (0.1 M, pH 7.4) were added to 43mM H_2O_2 and the absorbance was measured at 230 nm. Blank one was devoid of culture extract. H_2O_2 scavenging percentage was obtained by the following Eq. (1- A_1/ A_2)* 100 here, A_1 and A_2 were represented as the absorbance of the control and different concentrations of culture extract or standards (ascorbic acid) (Elmastas et al., 2005).

For Fe3+ (Ferric ions) reducing antioxidant power assay (FRAP) Pestalopine extracts (25µg/mL–400 µg/mL) were evaluated for their reducing power following the methods of Gu¨lcin et al. (2010). Ethylacetate culture extracts were mixed with sodium phosphate buffer (0.2 M, at pH 6.6), potassium ferricyanide [$K_3Fe(CN)_6$] (1%), and incubated at 50 C for 20 minutes. The post-incubation procedure includes the addition of 10% trichloroacetic acid, double distilled water, and 0.1% $FeCl_3$, and absorbance was measured at 700nm. Higher OD values indicate enhanced reducing power and ascorbic acid was used as a standard.

Also, total antioxidant capacity was estimated in terms of the gm equivalent of ascorbic acid (Khatua and Acharya, 2017). Peritoneal macrophages isolated from male Wistar rats were treated with different concentrations (1-1000 µg mL^{-1}) of Pestalopine and the non-cytotoxic nature was confirmed upon 99% survival of the macrophages even under 1000 µg mL^{-1} dosage (Mahapatra and Banerjee 2013b). The ability of Pestalopine to improve the enzymatic antioxidant parameters and lipid peroxidation was tested

on peritoneal macrophages. In brief, macrophages were distributed in eight groups (group-I-control group-without any Pestalopine treatment, group-II-VIII- treated with different concentrations of Pestalopine- 1, 25, 50, 100, 250, 500, and 1000 µg mL^{-1}), and each group with 4×10^6 cells in three Petri dishes. Superoxide dismutase (SOD) activity (CS1000), lipid peroxidation activity (MAK085), reduced glutathione (MAK364) and oxidised glutathione level (CS0260), and GPx (CGP1) activity were performed following the standard procedures mentioned in kits of Sigma Aldrich, USA.

Determination of hepatoprotective action

Male Wistar rats were grouped into six sets and each set with five rats. Group-I-control set without any Pestalopine and Thioacetamide (TAA) treatment, Group-II only receives liver fibrosis-causing agent TAA at 150 mg kg^{-1} body weight dosage for eight weeks (three times a week). Groups III and IV- receive TAA treatment along with Pestalopine treatment of 300, and 500 mg Kg^{-1} of body weight. Group V- TAA treatment along with Silymarin of 25 mg/kg of body weight. Histopathological and was done. Analysis of Aspartate aminotransferase (ASAT), Gamma-glutamyl transferase (GGT), Alanine transaminase (ALT), Alkaline phosphatase (ALP), total bilirubin (TB), total protein (TP), triglycerides (TGL), total cholesterol (TC) and albumin was done along with the estimation of liver and spleen indices following the protocols mentioned in the kits of Swemed BioMedicals Pvt. Ltd., Bangalore, India).

Detection of probiotic efficiency

Male Wistar rats were grouped into five sets and each set with five rats. Group-I- was controlled without any Pestalopine treatment, and group II-V receives different concentrations of Pestalopine treatment (100, 200, 300, and 500 mg EPS/kg of body weight). In brief, feces from each rat were collected immediately after dropping onto clean paper underlying the cage for the analysis of intestinal bacterial analysis following the methods of Mahapatra and Banerjee (2013a). Total aerobic and anaerobic bacteria, *Lactobacillus* sp., *Escherichia* coli, and *Clostridium perfringens* were isolated in their respective media and enumerated from 0 days to 30 days till the completion of the experiment.

Statistical analysis

All experiments were performed in triplicate, and the results are presented as means ± standard errors (SE). Data were analysed by Prism GraphPad software version 9.2.0 (332) (San Diego, CA, United States). Minitab (version 20.2) statistical software was used for response surface methodology experiments (Box– Behnken design).

Results

Isolate CEL6 was identified as Pestalotiopsis chamaeropsis

Endophytic fungus CEL6 was isolated from symptomless leaf tissue of *Chloranthus elatior* and was identified as *Pestalotiopsis chamaeropsis* based on morphological and molecular characteristics (Fig. 1a-c). The fungal colony was whitish in appearance and bears black-coloured pycnidium with a tuft of dark-brown-coloured septate (3–5 in number) conidia. The occurrence of two apical hyaline appendages/ setulae at the two terminal positions is the identifying character of *Pestalotiopsis* species (Fig. 1a, b). The ITS sequence of the fungus showed sequence similarity with different species of *Pestalotiopsis* but the maximum similarity was with *P. chamaeropsis* (Fig. 1c).

Molecular identification of the isolate was done based on two primers ITS1 and ITS4. The amplified gene products of CEL6 were sequenced. A total of 556 nucleotides were presented in the final dataset of CEL6. Gaps and missing data were eliminated from the dataset. Sequenced data was compared with the already available database of GenBank using the BLAST tool and evolutionary distances were calculated by the neighbour joining method. Bootstrap analysis was performed to construct the phylogenetic tree which depicts that the endophytic fungal isolates are phylogenetically related to the *Pestalotiopsis chamaeropsis* (Fig. 1c) supported by high bootstrap values of 97% (Saitou and Nei, 1987; Tamura et al., 2004). Cladograms were constructed using five hundred bootstrapping repetitions. The phylogram is prepared using nineteen isolates grouped into seven sections (Felsenstein, 1985) based on different species of *Pestalotiopsis* utilised to set up the tree. Various strains of eight different species of the isolate were represented in the phylogenetic tree: *Pestalotiopsis chamaeropsis*, *P. vismiae*, *P. trachyarpicola*, *P. papuana*, *P. microspora*, *P. heterocornis*, *P. biciliata*, and *P. trachycarpicola*. Another member of Mucoromycota- *Mucor circinelloides* was taken as the outgroup. The tree starts with MH425905 *Pestalotiopsis chamaeropsis* LB61-3. along with other three taxa OP315332, KM199326, and MH25903 along with endophytic isolate OP316869. These five species constitute Group 1. Group 2- Group 7 consists of different taxa of *P.* vismiae, *P. trachycarpicola*, *P. papuana*, *P. microspora*, *P. heterocornis*, *P. biciliata*, *P. diploclosiae* respectively. The total branch length of the phylogenetic tree was calculated as 0.1634924517. The EPS (Pestalopind)-producing endophytic fungal isolate CEL6 in this study completely matches (a 99% query cover) with *P. chamaeropsis* MH425905 and, hence, can be concluded as *C. chamaeropsis* CEL6.

Optimisation of Pestalopine production from endophytic P. chamaeropsis **CEL6**

OVAT optimisation results indicated that maximum Pestalopine production was detected at 12 g L^{-1} of glucose concentration, 1.75 g L^{-1} yeast extract concentration, 7 medium pH, and 7 days of fermentation time (Table 1). 50 mL of this optimised medium in a 320 mL Erlenmeyer flask with a headspace volume of 270 mL, and a medium depth of 2.1 cm yielded maximum Pestalopine production (Table 2). Here, After the OVAT optimisation, the RSM technique was applied using a three-level BBD, which involved the most important four factors-glucose concentration, yeast extract concentration, MpH, and fermentation time. Maximum Pestalopine production was noted at five replicated centre points (Table 3) and the statistical model suggests the following equation for optimum Pestalopine production $Y_{EPS} = 3.1893 - 1.0519x_1 - 1.6371x_2 - 1.1204x_3 - 1.1742x_4 - 1.0942x_1x_2 - 1.8391x_1x_3 + 1.1753x_1x_4 - 1.1936x_2x_3 + 1.9821x_2x_4 -$

$1.4031x_3x_4 – 1.1982x_1\ 2 – \ 1.1652x_2^2 – 1.7301x_3^2 – 1.3726x_4^2$; x_1, x_2, x_3, and x_4 are the coded factors of glucose concentration, yeast extract concentration, medium-pH, and fermentation time, respectively. The goodness of fit of RSM and experimental outputs were evaluated by regression analysis (Table 4).

The model's significance was demonstrated by the F-test results of 3961.38. The adjusted determinant coefficient (R^2 Adj) was found to be 0.997, indicating that there is a strong correlation between the experimental and predicted values and that the second-order polynomial prediction equation can accurately anticipate more than 99% of the response variance. According to the indicated Adeq precision value of 77.67, the model is suitable. The model's fitness was perfect, and the lack of fit's F-value of 1.67 and P-value (P0.0001) had no impact on the pure error. The P value of lack of fit was 0.8750 (> 0.05) and the P value of probability was greater than or equal to 0.05, both of which demonstrated the high degree of precision and consistency of the investigative results. Moreover, the additional impacts of glucose content, yeast extract concentration, M-pH, and fermentation time were linear and quadratic, and significant (P 0.0001). All of these statistical data prove that the mode is significant and appropriate and the obtained fermentation conditions can be applied in large tank bioreactors for the commercial production of Pestalopine.

Composition of Pestalopine

Exopolysaccharide synthesized by endophytic fungi *P. chamaeropsis* CEL6 was precipitated by applying chilled ethanol and crude EPS was then dialysed, and purified by gel filtration chromatography with Sepharose-6B column. Two carbohydrate-rich fractions were obtained and the major fraction (designated as pestalopine) with higher sugar concentration was eluted between 37–51 tubes (Fig. 2a). The absence of protein was confirmed by colorimetric tests. The glucan profile of the pestalopine was majorly comprised of β-glucan – 37.74 ± 1.92 g/100g of EPS and total glucan, α-glucan were 41.07 ± 2.97 g/100g, and 03.33 ± 0.63 g/100g of EPS respectively. Pestalopine is composed of glucose (retention time-46.2), arabinose (RT-25.3), fucose (RT-35.7), rhamnose (RT-31.5), galactose (RT-59.2) in a 10:1:2:2:4 ratio with a molecular weight of ~ 3.29 x 10^5 Da (Fig. 2c). Each repeating unit of pestalopine contains ten glucose units, two fucose and rhamnose units, one arabinose, and four galactose units. So, in total ninety-eight repeating units are present in the sample.

FT-IR analysis of the EPS sample revealed the occurrence of strong absorption peaks at particular wavelengths of 3531.8, 2929.5, 2319.3, 1691.8, 1492.3, 1049.2, 871.5 which represents C-H, O-H, C-O (Fig. 2b) asymmetric stretching respectively that indicates the basic characteristics and purity of the carbohydrate moiety.

The ^{1}H NMR spectrum of the Pestalopine gave protons within chemical shifts (δH) between 3.40 to 4.0 indicating that the compound possesses –CH and $–CH_2$ functional groups, which resonate with the oxygen atom of the –OH group. The occurrence of anomeric protons at δH 3.44, 3.58, 3.70, 3.75, and 3.45 (Fig. 2d) regions revealed that the compound is a polysaccharide (Singha et al., 2021).

Antioxidative action of Pestalopine

Pestalopine exhibited an IC_{50} value of 101.43 ± 4.89, 17.19 ± 5.01, 143.12 ± 5.37, 149.06 ± 5.87 μg mL^{-1} against DPPH, ABTS, FRAP, and H_2O_2 free radical generator respectively. Total antioxidant capacity was reported to be 98.69 ± 1.07 μg mL^{-1}, i.e., 1 mg of pestalopine and 98.69 ± 1.07 μg mL^{-1} of ascorbic acid exhibit the equal value of antioxidant action. GSSG and MDA levels in Pestalopine-treated peritoneal macrophage cells decreased in a concentration-dependent manner (Fig. 3a, e). 500 μg mL^{-1} dosage maximally inhibited the events of lipid peroxidation and reduced the amount of oxidised glutathione levels. Glutathione peroxidase activity and function of superoxide dismutase of the pestalopine-treated cells were way improved than the untreated peritoneal macrophage cells (Fig. 3b, d). Also, the reduced glutathione values improved upon treatment with 500 μg mL^{-1} pestalopine in comparison to the control set (Fig. 3c).

Recovery of TAA-induced hepatic damage by Pestalopine treatment

A dosage of 500 mg/kg of body weight of Pestalopine administration (oral) exhibits the most fruitful outcomes. There was a significant reduction in the levels of bile pigment- total bilirubin and direct bilirubin, hepatic enzymes- alkaline phosphatase, gamma-glutamyl transferase, aspartate aminotransferase, alanine transaminase in the treated rats in comparison to control (only TAA treated sets). The amount of total albumin recovered up to normal ranges in the pestalopine-treated groups. Also, the levels of triglycerides were found to be balanced in the treated (III^{rd} and IV^{th}) groups (Table 5). The effect of 500 mg/kg of Pestalopine treatment yields more or less similar results with the normal-untreated (group-I) and silymarin (group-V) treated one (Table 3). Liver and spleen index of the III^{rd} and IV^{th} groups (300 and 500 mg/kg of body weight of Pestalopine treatment respectively) of rats improved than the group-II and more or less closely resemble group-I and group-V; control and silymarin treated respectively (Table 6).

Liver tissues of TAA-treated rats were examined and there was necrosis located on the hepatocytes and hemorrhagic locations or congestions and binucleation were found whereas Pestalopine-treated rats have a relatively healthy liver almost similar to control sets (Fig. 4a-c). The hepatocytes were arranged in cords and were clearly visible in the case of Group IV (Fig. 4d). Cross section of hepatic lobules bears no significant lyses in blood cells, and there were no instances of infiltration of lymphocytes, neutrophils, or macrophages (Fig. 4c-d). The Group-IV rats exhibit healthy hepatic characters in relation to the control set (Fig. 4a-d).

Pestalopine improves the gut microflora

The enumeration of total anaerobes, total aerobes, *Lactobacillus* sp., *C. perfringens*, enterobacteria, and *E. coli* were reported in terms of log10CFU/g wet of the fecal sample of the treated and untreated rats.

Initially, in 1–5 days, there was no valid difference in bacterial load in the case of Pestalopine-treated and un-treated rats but after 10 days of treatment, some variations were noted. On the 30th day of treatment, there was a prominent difference in microbial load between the treated and untreated (control) sets (Table 7). There was more or less similar microbial load in the untreated set before and after the experiment. A sharp increase in the population of total anaerobes, and *Lactobacillus* are detected on the 30th day in Pestalopine-treated rats whereas the *C. perfringens* population is reduced. Among the treated sets a dosage of 300 mg EPS/kg of body weight is the most effective one in terms of a significant reduction in the population of *C. perfringens*, and an increase in the number of Lactobacillus, total anaerobes. The population of *E. coli*, total aerobes, and other enterobacteria remained unchanged in all the treated and control set throughout the experimental tenure.

Discussion

Optimisation of the Pestalopine production

Endophytic fungi have been a constant source of potent bioactive compounds with multidomain utility in pharmaceuticals and agriculture (Chen et al., 2016). Secondary metabolites from endophytes are being utilised as an alternative to contemporary antimicrobials and antioxidative. Especially exopolysaccharides are immensely effective in this field (Sun et al., 2020). For the wide pharmaceutical application of these agents, EPSs are needed to be produced in maximised amounts. So, different optimisation techniques are needed to adopt for successful implication.

The fermentation period is the first criterion to be assured for an efficient production strategy and in the present investigation maximum Pestalopine production has been noted after seven days of incubation. Though there was an increase in the growth of the endophytic fungus *P. chamaeropsis* CEL6 (confirmed by an increase in biomass) after seven days, but Pestalopine production decreased. Similar types of incubation period profiles for EPS production were also noticed in the findings of Lee et al. (2007), Nehad and El-Shamy (2010), Mahapatra and Banerjee (2013, 2016), Santra and Banerjee (2022, 2023).

The pH of the fermentation medium is always a factor in microbial metabolite production which influences enzymatic activity and some metabolic channels may open at specific pH whereas other gets blocked (Mahapatra and Banerjee, 2013). The synthesis of Pestalopine by *P. chamaeropsis* CEL6 was assessed at various initial medium pH values ranging from 4 to 8. At pH 7.0, the isolate produced the optimum biomass and Pestalopine (16.90 ± 0.02 g/l EPS). Alkaline pH (pH 7.2-8.0) resulted in a reduction in both biomass synthesis and Pestalopine production (Table 1). Similar types of initial medium pH have been designated as optima for exopolysaccharide production for different fungal species (Roukas and Biliaderis 1995, Banerjee et al., 2009, NehadandEl-Shamy, 2010, Nour El-Dein, 2004, Mahapatra and Banerjee, 2013, 2016, Santra and Banerjee, 2022, 2023).

Most fungi favour carbohydrates as their primary source of energy, which was associated with both metabolite synthesis and organism growth (Kim et al., 2003, Yang and He, 2006). Glucose had a superior

impact on biomass and Pestalopine production than other carbohydrates used in this study (18.93 ± .08 g/l). Glucose was selected as the first choice of supplementary carbon source for further optimisation studies as it was cost-effective in comparison to other costly supplements fructose, maltose, rhamnose, and raffinose. For the synthesis of Pestalopine, nitrogen is likewise regarded as a key nutrient (Banerjee and Mahapatra, 2009). When yeast extract was used as an extra nitrogen source, *P. chamaeropsis* CEL6 produced the most biomass and Pestalopine (19.89 ± 0.10 g/l EPS). This organic nitrogen supply optimum might be viewed as a desirable physiological trait given that fungi usually required extended time periods for good submerged growth, exposing them to contamination issues (Kim et al., 2005). Following selection, various glucose concentrations were examined to determine their impact on Pestalopine and biomass production. Within a range of glucose concentrations (2–8 g%), it was evident that simultaneous increases in biomass and Pestalopine production occurred. Above this range, however, Pestalopine and biomass production declined (Table 1). Maximum Pestalopine (18.93 ± 0.08 g/l EPS) and biomass production were observed at 12 g% glucose content.

The effects of various nitrogen sources especially yeast extract concentrations were also investigated. It was noted that as yeast extract concentration increased, Pestalopine yield likewise increased—but only until a certain point, after which both growth and Pestalopine output dropped (Table 1). When 1.75 g% yeast extract concentration was subsequently added to the growth medium, *P. chamaeropsis* CEL6 produced the highest levels of Pestalopine (19.89 ± 0.10 g/l EPS) and biomass. A similar type of effect of additional nitrogen sources has been demonstrated by several other works while working on different types of fungal products (Choi et al., 2007, Yang and He, 2008).

Mineral salts and metal ions influence microbial metabolisms (Hwang et al., 2003, Yang and He, 2008). Here NaCl had the most positive impact on the generation of biomass and Pestalopine (26.78 ± 0.20 g/l EPS) out of all the extra salts evaluated (Table 1). Although the precise mechanism is unclear, it's possible that this ionic salt enhanced cell membrane permeability, which in turn affected how much Pestalopine was excreted (Banerjee et al., 2009). The impact of several supplementary phosphate sources was evaluated on Pestalopine production. The production of Pestalopine and biomass by *P. chamaeropsis* CEL6 was observed to be unaffected by additional phosphate sources.

In the flask culture, an oxygen gradient existed between the fungal growth medium and the headspace gas (Wonglumsom et al., 2000). Oxygen was delivered to the liquid phase because the system always favoured equilibrium. Although a cotton plug in a culture flask did not prevent air penetration, it could limit the flow of free air from the external environment to the internal area of the Erlenmeyer flask. Thus, the headspace air volume may have an impact on the oxygen concentration in a liquid media. Additionally, the surface area may be used to influence oxygen transport in liquid media. Oxygen diffusion in liquid media was caused by an increase in the surface area. Another key element that controls the amount of oxygen in liquids is medium depth. Oxygen diffusion frequently reduced as medium depth increased. We employed four different culture medium contents (25 ml, 50 ml, 75 ml, and 100 ml) in 250 ml Erlenmeyer flasks to examine how oxygen affected the synthesis of Pestalopine and biomass. It is reasonable to suppose that lower medium volume corresponds to higher dissolved oxygen

and higher medium volume corresponds to lower dissolved oxygen when considering the surface area, headspace volume, and medium depth (Table 2). The outcome demonstrated that *P. chamaeropsis* CEL6 produced the highest amount of Pestalopine (28.91 ± 0.33b g/l EPS) when cultivated in 50 ml of medium (Table 2). Growth and EPS production both fell off significantly as medium volume fell below or above 50 ml. Therefore, it was evident that *P. chamaeropsis* CEL6 favoured a somewhat low quantity of dissolved oxygen compared to endophytic *Fusarium solani* SD5, *Pestalotiopsis* sp. BC55 for the formation of EPS (Mahapatra and Banerjee, 2013). We selected various physical conditions and nutrients that were beneficial in raising the yield of Pestalopine by *P. chamaeropsis* CEL6 with the help of the OVAT optimisation procedure. A maximum output of 28.91 ± 0.33 g/l EPS was achieved.

Pestalopine modulates gut micro-flora

Probiotics can improve human health by stimulating the growth of good bacteria, limiting the growth of harmful ones, lowering serum cholesterol levels, reducing cell/tissue inflammation, modulating immune cells, and acting as antioxidants. Exopolysaccharides, organic acids, and bacteriocins are a few examples of the metabolites produced by microbes that can directly induce these effects. The gut is considered the second brain and gut health is mostly dependent on the microbial population it harbors. The intestinal microbiota has several advantageous benefits for its host. Colonic and intestinal microflora produced short-chain fatty acids by fermenting non-digestible dietary waste and these acids increased the absorption of calcium, magnesium, and iron ions and controlled the growth and differentiation of epithelial cells in the large and small bowels. Vitamin production was aided by colonic microorganisms, which also served as an essential line of defense against the colonisation of dangerous exogenous germs (Guarner & Malagelada, 2003). They were therefore extremely important in preventing pathogen invasion of tissues.

Prior literature has shown that healthy intestinal physiological circumstances are characterised by an increase in Lactobacillus and a decrease in *C. perfringens*. Up to 200 mg/kg of body weight in Pestalopine feed, depending on the coliform (*E. coli*) counts, there was a slight increase in the count as measured after one month. It was also noted that the starting count of other enterobacteria (non-coliforms) and the count after the same treatment were nearly identical. Other than *E. coli*, enterobacteria were often pathogens or opportunistic pathogens. In all of the tested doses, there were no substantial variations in the counts of *E. coli* and other enterobacteria. These results additionally implied that the gastrointestinal tract was in good health (Deng et al., 2007; Lidbeck et al., 1991). Treatments using Pestalopine higher than 200 mg/kg body weight did not significantly modify the microbial count compared to that dose. This could be a result of the intestinal flora controlling their own growth or the intestinal milieu favoring microbe proliferation up to a point where it is beneficial to the host. Every group that received Pestalopine treatment had a discernible improvement in gastrointestinal microbiota as compared to the control sets. The present findings showed that Pestalopine had a positive impact on intestinal health by selectively promoting, limiting, and lowering the proliferation of a small number of bacteria in the colon which has an immense influence on the host's health.

Antioxidative and hepatoprotective action of Pestalopine

Pestalopine exhibited strong dose-dependent invitro antioxidative activity against different free radical generators, especially against DPPH. It is a cationic free radical scavenged by two different techniques-HAT (hydrogen atom transfer) and SET (single electron transfer) mechanism (Craft et al., 2012). So, it could be predicted that violet-coloured methanolic DPPH accepted hydrogen from Pestalopine and transformed it into colourless DPPH. Pestalopine actually acts as a hydrogen donor. The promising IC_{50} value of the exopolysaccharide opens up new alternatives over synthetic antioxidants. Popular synthetic antioxidants Butylated hydroxytoluene (BHT) and butylated hydroxanisole (BHA) possess carcinogenic and hepatotoxic effects. They could be replaced by these types of natural products (Hettiarachchy et al., 1996).

Other than free radical scavenging assay, Pestalopine also exhibited in-vitro antioxidative activity in the case of treated macrophage cells. Free radicals are generated randomly by normal cellular biochemical activities in almost every living cell of our body. Mostly lipid peroxidation is the major cause of free radicals' generation which leads to the production and accumulation of several types of aldehydes (Barrera et al., 2008). A high amount of PUFA (Polyunsaturated fatty acids) is present in the plasma membrane of peritoneal macrophages which undergoes rapid peroxidation during normal cellular processes (Kar Mahapatra et al., 2010). Pestalopine-treated macrophage cells exhibited a dose-dependent response and a lower MDA value has been reported. So, it is acting as an antioxidant and is preventing the formation of peroxy radicals which is directly reflected in its MDA contents.

Another common toxic free radical is a superoxide anion which has an unpaired electron in its oxygen atom. These are generally generated in the cells by auto-oxidation or by enzymatic actions and trigger the formulation of numerous components like singlet oxygen, hydrogen peroxide, and hydroxyl radicals (Halliwell, 1992). Superoxide dismutase is an enzymatic antioxidant involved in the removal of these superoxides from cells and thus protects the cells from oxidative stress. Macrophages through performing their regular phagocytosis generate ample amounts of superoxide ions (Bannister et al., 1987). Pestalopine significantly accelerated the SOD activity in treated peritoneal macrophages dose-dependently.

Glutathione is known to be a cysteine-rich tripeptide with an active thiol group and takes part in the removal of toxic free radicals i.e., superoxide, singlet oxygen, and hydroxyl radicals, along with stabilisation of membrane lipid peroxidation. In Pestalopine treated macrophage cells, the reduced glutathione levels increased randomly probably due to the rapid activity of glutathione reductase. This enzyme catalyses oxidised glutathione (GSSG) to reduced one (GSH). Actually, different free radicals oxidise glutathione and lead to the formation of glutathione disulfide which is indicative of major oxidative stress in the cell. Pestalopine gradually decreases the GSSG levels in peritoneal macrophage populations. Another enzymatic parameter GPx (Glutathione peroxidase) which is a glutathione-dependent biocatalyst found in the cell cytoplasm and converts peroxides to different alcohols and decontaminated ROS (reactive oxygen species) is induced upon Pestalopine treatment. All the present

outcomes confirm the beneficial impact of the studied EPS- Pestalopine on the antioxidant enzyme activities of peritoneal macrophages in multiple ways.

In the present investigation, Pestalopine is also reported to be hepatoprotective in nature and promotes the recovery of Male Wistar Rats from TAA-induced toxicity. TAA is a potent hepatotoxin and causes necrosis of liver lobules, the proliferation of biliary ducts, fibrosis, and finally cirrhosis of hepatic lobes (Seki et al., 2009). TAA cause the upregulation of genes or transcription factors (IGFBPrP1, TGF-β1, and Smad3) which are responsible for fibrosis (Ye and Lu, 2006, Ding et al., 2010). Hepatoprotective drugs block these TGF-β1/ Smad3 signal pathways and there is a deactivation of serum AST and ALT in treated rats (Pietrangelo et al., 2007, Liang et al., 2009). These are indicative of recovery from hepatic fibrosis. Pestalopine-feed rats have normal liver tissues, prominent hepatic lobules, and hepatic sinusoids in comparison to the non-treated ones which are characterized by interrupted liver tissues, and numerous connective tissues. A prominent recovery of liver function test profiles in Pestalopine treated rats is indicative of the fact that Pestalopine downregulated the expressions of TGFβ/Smads pathway-related FN, IGFBPrP1, Smad3, TGFβ1 proteins in hepatic tissues. This was the probable mechanism of fibrosis resistance and there was a drop in the apoptotic index of hepatocytes (Liang et al., 2009, Li and Friedman, 1999).

Conclusion

The pharmaceutical industries are currently dealing with a severe shortage of potent antioxidants, probiotics, and hepato-protectants. There are plant-based formulations and microbial consortiums, but still, there is a significant need for new and affordable combinations. In the current work, several fermentation conditions were used to optimise the production of glucose-rich heteropolysaccharides, such as Pestalopine. Pestalopine has demonstrated probiotic efficacies and is a powerful in-vitro and in-vivo antioxidant agent. *Lactobacillus* sp. populations increased, which in turn prevented the growth of the putrefying bacterium *Clostridium perfringens*, and the gut microbiota was positively altered. In comparison to untreated rats, the Pestalopine-treated ones showed improved liver function test parameters and recovered from thioacetamide-induced hepatic damage. The findings confirm that oral administration of Pestalopine effectively enhances gut health and has hepatoprotective effects in vivo (in male Wistar rats). Pestalopine is a good option for commercial exploitation due to its higher production and capacity to repair liver damage and alter the population of gut bacteria. The findings of this research could encourage the adoption of novel substitute substances such as probiotics, antioxidants, and hepatoprotective drugs like Pestalopine.

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Tables

Table 1
Effect of different physical conditions and chemical supplements, on biomass and exopolysaccharide production by endophytic fungi *P. chamaeropsis* CEL6 in submerged fermentation conditions

Parameters tested	Effectors	The concentration of the effectors (g L^{-1})	Biomass (g L^{-1})	EPS (g L^{-1})
Fermentation time (h)	3 days	-	6.20 ± 0.18a	9.12 ± 0.02a
	5 days	-	6.90 ± 0.23a	11.29 ± 0.06b
	7 days	-	**7.92 ± 0.07b**	**14.94 ± 0.05c**
	9 days	-	8.76 ± 0.11c	13.33 ± 0.03d
Fermentation temperature (°C)	22	-	8.82 ± 0.17a	14.01 ± 0.04a
	24	-	8.99 ± 0.10a	15.91 ± 0.09b
	26	-	9.91 ± 0.14b	16.98 ± 0.07c
	28	-	10.06 ± 0.18b	16.05 ± 0.09b
Initial medium pH	6.6	-	10.07 ± 0.11a	16.01 ± 0.07a
	6.8	-	10.81 ± 0.21b	17.71 ± 0.05b
	7	-	10.99 ± 0.14b	**16.90 ± 0.02c**
	7.2	-	11.11 ± 0.17c	16.03 ± 0.04c
Additional carbon sources	Fructose	-	11.01 ± 0.13a	17.17 ± 0.09a
	Glucose	-	12.99 ± 0.10b	**18.93 ± 0.08b**
	Maltose	-	11.09 ± 0.11a	17.32 ± 0.03a
	Starch	-	11.68 ± 0.19a	17.90 ± 0.04a
	Rhamnose	-	12.27 ± 0.24b	18.34 ± 0.02c

Parameters tested	Effectors	The concentration of the effectors (g L^{-1})	Biomass (g L^{-1})	EPS (g L^{-1})
	Raffinose	-	11.29 ± 0.19a	18.57 ± 0.02c
	Glycerol	-	11.18 ± 0.16c	18.78 ± 0.03c
Additional nitrogen sources	Peptone	0.4	12.21 ± 0.14a	18.51 ± 0.09a
	$(NH_4)_2SO_4$	0.4	12.39 ± 0.11a	18.94 ± 0.08a
	NH_4NO_3	0.4	12.11 ± 0.18a	19.41 ± 0.04b
	Urea	0.4	13.01 ± 0.13a	18.08 ± 0.05c
	NH_4Cl	0.4	13.31 ± 0.14b	18.90 ± 0.07a
	Glycine	0.4	13.62 ± 0.18b	19.00 ± 0.09d
	Yeast Extract	0.4	13.79 ± 0.17b	**19.89 ± 0.10e**
Glucose concentration	Glucose	8	13.81 ± 0.20a	19.9 ± 0.19a
		10	13.27 ± 0.17a	20.08 ± 0.11b
		12	13.94 ± 0.12a	**22.87 ± 0.07c**
		14	13.53 ± 0.14a	20.09 ± 0.05a
Yeast extract concentration	Yeast extract	1.5	13.29 ± 0.17a	22.15 ± 0.14a
		1.75	13.33 ± 0.18a	**24.08 ± 0.17b**
		2	13.71 ± 0.14a	21.96 ± 0.16a
		2.25	13.49 ± 0.16a	20.72 ± 0.11c
Different metal ions	$MgCl_2$	0.1	11.9 ± 0.14a	24.52 ± 0.21a

Parameters tested	Effectors	The concentration of the effectors (g L^{-1})	Biomass (g L^{-1})	EPS (g L^{-1})
	$FeCl_3$	0.1	12.71 ± 0.19b	23.17 ± 0.14b
	KCl	0.1	12.74 ± 0.21b	23.45 ± 0.17b
	NaCl	0.1	12.89 ± 0.24b	**26.78 ± 0.20c**
	NaH_2PO_4	0.1	11.78 ± 0.27a	22.17 ± 0.18d
	K_2HPO_4	0.1	11.47 ± 0.11a	22.25 ± 0.17d
	KH_2PO_4	0.1	10.11 ± 0.19c	23.75 ± 0.10b

One-way ANOVA (Tukey's Multiple Comparison test) was performed to check the potential statistical differences in the case of the biomass (g L^{-1}) and EPS production (g L^{-1}) in different fermentation conditions (incubation time, temperature, the concentration of sugar and nitrogen sources, etc.). There were valid statistical differences in most of the cases ($P < 0.05$), the different letters a, b, and c indicate a significant difference, and the same letter at two positions indicates no statistical differences.

Table 2
Effect of medium volume, headspace volume, and medium depth on the dissolved oxygen level in the fermentation medium and their effect on EPS and biomass production.

Medium volume (mL)	The total volume of the flask (mL)	Headspace volume (mL)	Medium depth (cm)	Surface area (cm)	Biomass (g L^{-1})	EPS (g L^{-1})
25	320	295	1	2.5	11.98 ± 0.19a	26.54 ± 0.31a
50	320	270	1.5	2.58	13.21 ± 0.24b	28.91 ± 0.33b
75	320	245	2	2.53	12.2 ± 0.14a	26.81 ± 0.29a
100	320	220	2.5	2.41	12.36 ± 0.14a	27.12 ± 0.271a

One-way ANOVA (Tukey's Multiple Comparison tests) was performed to check the potential statistical differences between the data (column-wise) of biomass (g L^{-1}), and EPS production (g L^{-1}) in different medium volumes (mL). There was a valid statistical difference in most of the cases. Different letters (a, b, and c) indicate valid statistical differences ($P < 0.05$).

Table 3
Experimental design and results of the Box–Behnken design for optimization of the exopolysaccharide Pestalopine production by *Pestalotiopsis chamaeropsis* CEL6.

Run	Independent variables				Response (Pestalopine yield g L^{-1})	
	X_1: GC[a] (g L^{-1})	X_2: YEC[b] (g L^{-1})	X_3: MpH[c]	X_4: FT[d] (day)	Measured	Predicted
1	-1 (10)	-1 (1.0)	0 (7)	0 (7)	30.226	30.204
2	1 (14)	-1	0	0	30.122	30.114
3	-1	1 (2.50)	0	0	30.931	30.924
4	1	1	0	0	30.244	30.252
5	0 (12)	0 (1.75)	-1 (6.5)	0	30.027	30.030
6	0	0	1 (7.5)	-1 (5)	30.934	30.913
7	0	0	-1	-1	30.548	30.554
8	0	0	1	1 (9)	30.365	30.347
9	-1	0	0	1	30.177	30.203
10	1	0	0	-1	30.622	30.635
11	-1	0	0	-1	30.474	30.496
12	1	0	0	1	30.292	30.301
13	0	-1	-1	1	30.252	30.266
14	0	1	-1	0	30.731	30.748
15	0	-1	1	0	30.139	30.157
16	0	1	1	0	30.512	30.533
17	-1	0	-1	0	30.175	30.155
18	1	0	-1	0	30.847	30.825
19	-1	0	1	0	30.044	30.044
20	1	0	1	0	30.615	30.612
21	0	-1	0	0	30.441	30.438
22	0	1	0	-1	30.455	30.434
23	0	-1	0	-1	30.487	30.485

Run	Independent variables				Response (Pestalopine yield g L^{-1})	
	X_1: GC[a] (g L^{-1})	X_2: YEC[b] (g L^{-1})	X_3: MpH[c]	X_4: FT[d] (day)	Measured	Predicted
24	0	1	0	1	30.366	30.346
25	0	0	0	1	30.945	30.947
26	0	0	0	0	30.935	30.947
27	0	0	0	0	30.966	30.947
28	0	0	0	0	30.955	30.947
29	0	0	0	0	30.938	30.947

[a]Glucose concentration, [b]Yeast extract concentration, [c]medium pH, [d]Fermentation time.

Table 4
ANOVA for response surface quadratic regression model of EPS (Pestalopine) production.

Source	Sum of squares	df	Mean of Square	F-Value	P-Value
Model	2.714536	14	0.193890	3961.38	< 0.001
X_1	0.151261	1	0.112614	3190.09	< 0.001
X_2	0.250824	1	0.258240	2437.06	< 0.001
X_3	0.002585	1	0.002852	1193.10	< 0.001
X_4	0.223043	1	0.223043	4157.89	< 0.001
X_1^2	0.556257	1	0.562513	11717.37	< 0.001
X_2^2	0.588277	1	0.582771	11314.23	< 0.001
X_3^2	0.432484	1	0.424849	10147.49	< 0.001
X_4^2	0.975939	1	0.959394	10246.86	< 0.001
X_1X_2	0.013663	1	0.013660	1287.95	< 0.001
X_1X_3	0.000034	1	0.000043	908.14	< 0.001
X_1X_4	0.006641	1	0.006641	1003.29	< 0.001
X_2X_3	0.000091	1	0.000089	1167.00	< 0.001
X_2X_4	0.055461	1	0.055546	1291.96	< 0.001
X_3X_4	0.000367	1	0.000635	1180.01	< 0.001
Residual	0.004786	14	0.000396		
Lack-of-Fit	0.002393	10	0.000337	1.67	0.8750
Pure Error	0.002067	4	0.000516		
Cor total	2.574321	28			
R-squared	0.998982				
Adj R-squared	0.997462				
Pred R- squared	0.994210				
Adeq Precision	77.67311				

Table 5
Effect of Pestalopine treatment on the hepatic enzyme profiles and other necessary biochemical parameters of hepato-fibrosis induced (by TAA) male Wistar rats.

Treatment	Group I- control	Group II- only TAA treated	Group III- TAA + EPS treated – 300mg/kg of body weight	Group IV- TAA + EPS treated- 500mg/kg of body weight	Group V- TAA + Silymarin treated 25 mg/kg of body weight
AST (UL^{-1})	25.81 ± 1.19[a]	59.09 ± 2.74[b]	43.79 ± 1.96[c]	26.97 ± 1.28[a]	26.07 ± 1.14[a]
ALT (UL^{-1})	21.04 ± 1.06[a]	84.91 ± 3.09[b]	50.02 ± 2.87[c]	24.73 ± 1.97[a]	25.31 ± 1.94[a]
Alkaline phosphatase (UL^{-1})	75.16 ± 3.92[a]	243.07 ± 8.14[b]	159.93 ± 5.24[c]	82.74 ± 1.48[a]	90.07 ± 1.95[d]
GGT (UL^{-1})	0.73 ± 0.01[a]	4.96 ± 1.04[b]	3.49 ± 0.75[c]	1.32 ± 0.03[a]	1.45 ± 0.01[a, d]
Triglycerides (mg dL^{-1})	82.07 ± 2.47[a]	111.32 ± 3.91[b]	90.14 ± 2.73[c]	77.59 ± 2.07[d]	84.14 ± 2.97[c]
Albumin (mg dL^{-1})	3.41 ± 0.04[a]	2.81 ± 0.05[b]	3.01 ± 0.02[b]	3.39 ± 0.03[a]	3.19 ± 0.01[a]
Direct Bilirubin (mg dL^{-1})	0.04 ± 0.003[a]	0.29 ± 0.01[b]	0.17 ± 0.01[c]	0.05 ± 0.002[a]	0.09 ± 0.002[a]
Total Bilirubin (mg dL^{-1})	0.27 ± 0.03[a]	0.43 ± 0.05[b]	0.29 ± 0.04[c]	0.14 ± 0.01[d]	0.13 ± 0.01[d]

Values on the table are the means ± Standard error (SE) of the three replicates. One-way ANOVA (Tukey's Multiple Comparison tests) was performed to check the potential statistical differences in the case of the control and different EPS-treated groups. The different letters a, b, c, d, and e indicates significant difference ($P < 0.05$), and the same letter at two positions indicate no valid statistical differences.

Table 6
Effect of Pestalopine treatment on liver and spleen indices in hepatic fibrosis-induced rats.

Treatment groups	Liver index	Spleen index
Group I-control	0.021 ± 0.0008[a]	0.019 ± 0.0009[a]
Group II-TAA treated	0.039 ± 0.0017[b]	0.043 ± 0.0014[b]
Group III- EPS treated – 300mg/kg of body weight	0.028 ± 0.0013[c]	0.031 ± 0.0019[c]
Group IV- EPS treated-500mg/kg of body weight	0.019 ± 0.0007[d]	0.020 ± 0.0004[d]
Group V- Silymarin treated 25 mg/kg of body weight	0.024 ± 0.0005[e]	0.023 ± 0.0009[e]

Values in the table are the mean of three replicates ± standard error and Tukey's multiple comparison tests confirm that there are valid statistical differences (different letters a-e indicate difference, and the same letter indicates no differences) between the data set in comparison to control ($p < 0.05$).

Table 7
Quantification of different types of bacterial populations obtained from faecal matter of the Pestalopine treated (after 30 days) male Wistar rats.

Treatment groups (EPS-mg/kg body weight)	Bacterial enumeration (log_{10} CFU/g wet faecal sample)					
	Total aerobic microbes	Total anaerobic microbes	*Lactobacillus* sp.	*Escherichia coli*	Other enterobacteria	*Clostridium perfringens*
Control	7.65 ± 0.011	12.18 ± 0.002 [a]	3.91 ± 0.013[a]	6.73 ± 0.010	4.47 ± 0.027	5.11 ± 0.019[a]
100	7.67 ± 0.013	12.51 ± 0.020 [b]	4.10 ± 0.011[b]	6.77 ± 0.011	4.49 ± 0.023	4.84 ± 0.013[b]
200	7.73 ± 0.010	12.71 ± 0.029 [c]	4.70 ± 0.007[c]	6.79 ± 0.009	4.41 ± 0.029	4.01 ± 0.017[c]
300	7.76 ± 0.012	13.46 ± 0.007 [d]	5.11 ± 0.006[d]	6.75 ± 0.010	4.46 ± 0.021	3.79 ± 0.019[d]
500	7.77 ± 0.013	13.26 ± 0.009 [e]	4.99 ± 0.003[e]	6.76 ± 0.011	4.40 ± 0.026	3.91 ± 0.017[e]

Values on the table are the means ± Standard error (SE) of the three replicates. One-way ANOVA (Tukey's Multiple Comparison tests) was performed to check the potential statistical differences in the case of the control and different EPS-treated groups. The different letters a, b, c, d, and e indicates significant difference ($P < 0.05$), and the same letter at two positions indicate no valid statistical differences.

Figures

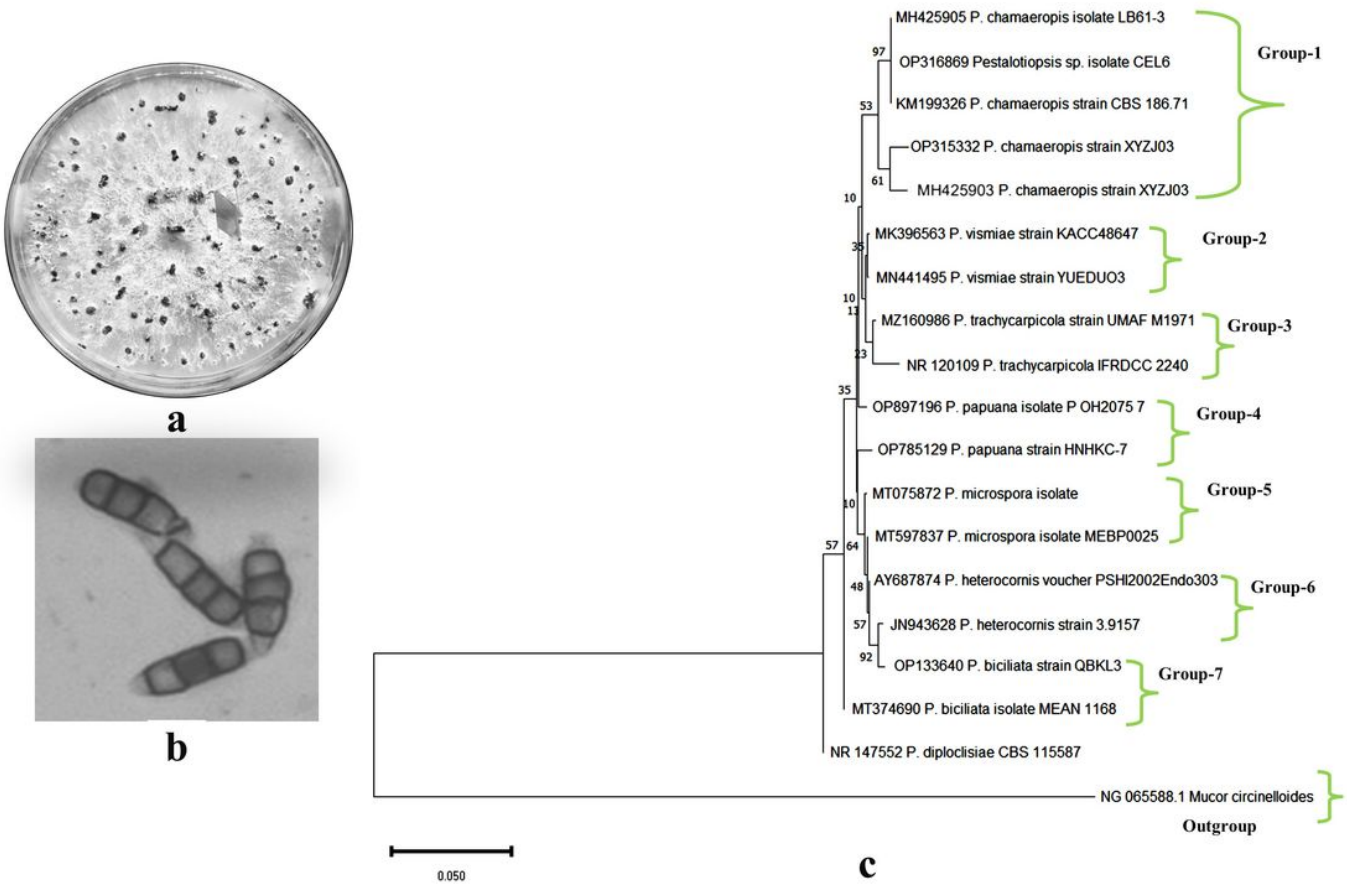

Figure 1

a- Colony morphology, and b- microscopic views of conidia of the isolated endophytic fungi, c- Evolutionary relationships of *P. chamaeropsis*CEL6 and other related fungi.

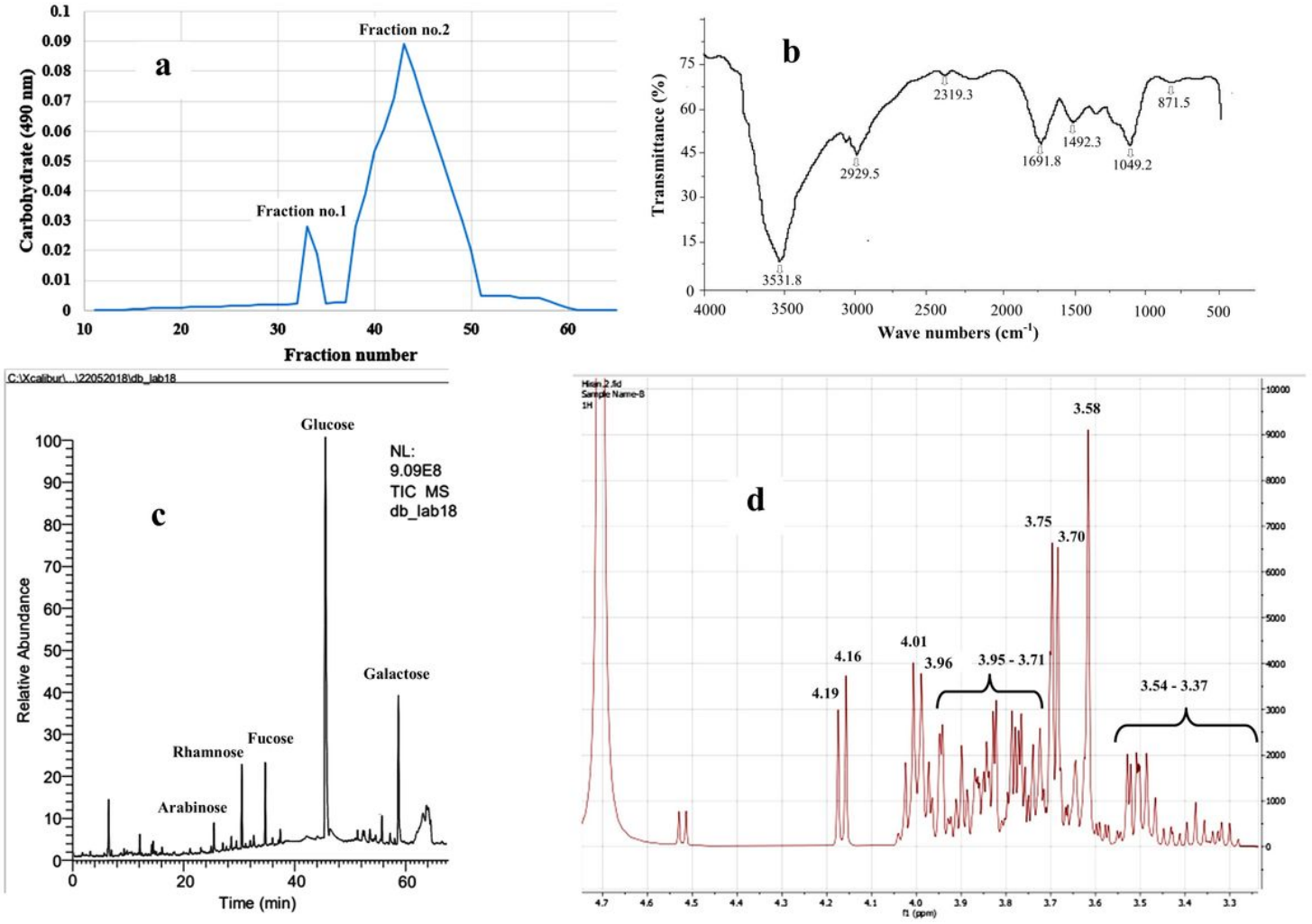

Figure 2

Enumeration of structural characters of Pestalopine a- Estimation of molecular weight, b- FT-IR spectra, and c- GC-MS chromatogram of the Pestalopine showing different monosaccharides glucose, galactose, fucose, rhamnose, and arabinose.

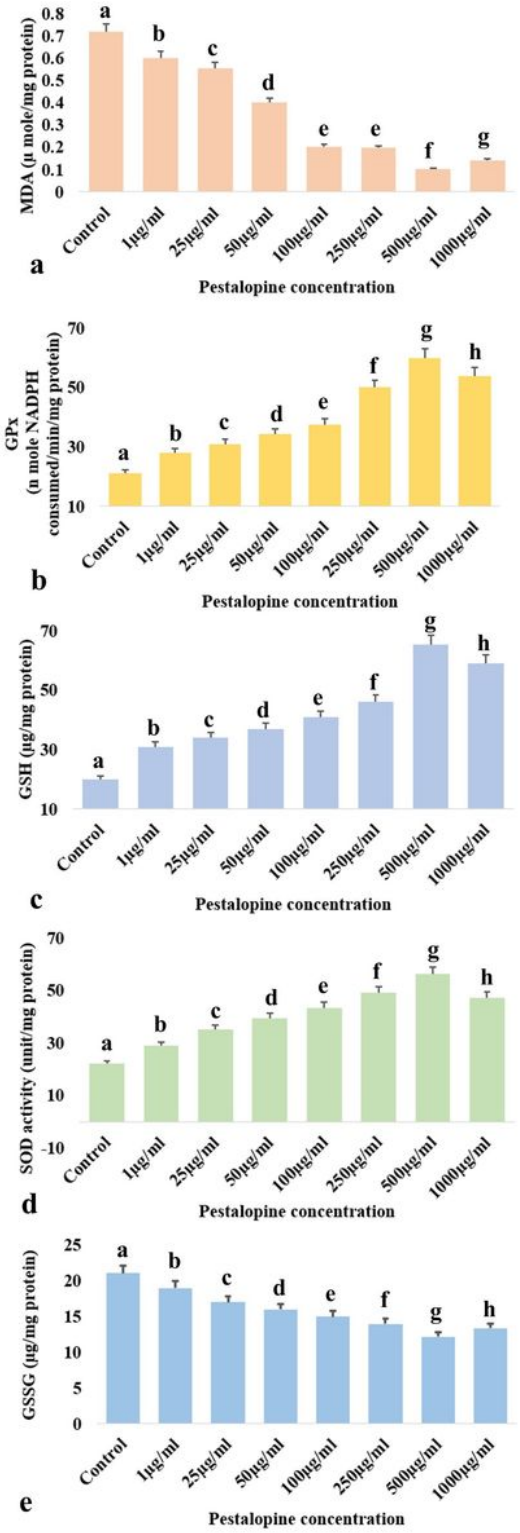

Figure 3

a- Levels of malondialdehyde (MDA) contents, b- glutathione peroxidase activity, c- reduced glutathione (GSH) and d- superoxide dismutase (SOD) activity, e- oxidised glutathione (GSSG) contents in control and different concentrations of Pestalopine treated peritoneal macrophages. Values in the graphs are the means ± Standard error (SE) of the three replicates. One-way ANOVA (Tukey's Multiple Comparison tests) was performed to check the potential statistical differences in the case of the control and different

Pestalopine treated groups. The different letters a, b, c, d, e, and f indicates significant difference (P<0.05), and the same letter at two positions indicate no valid statistical differences.

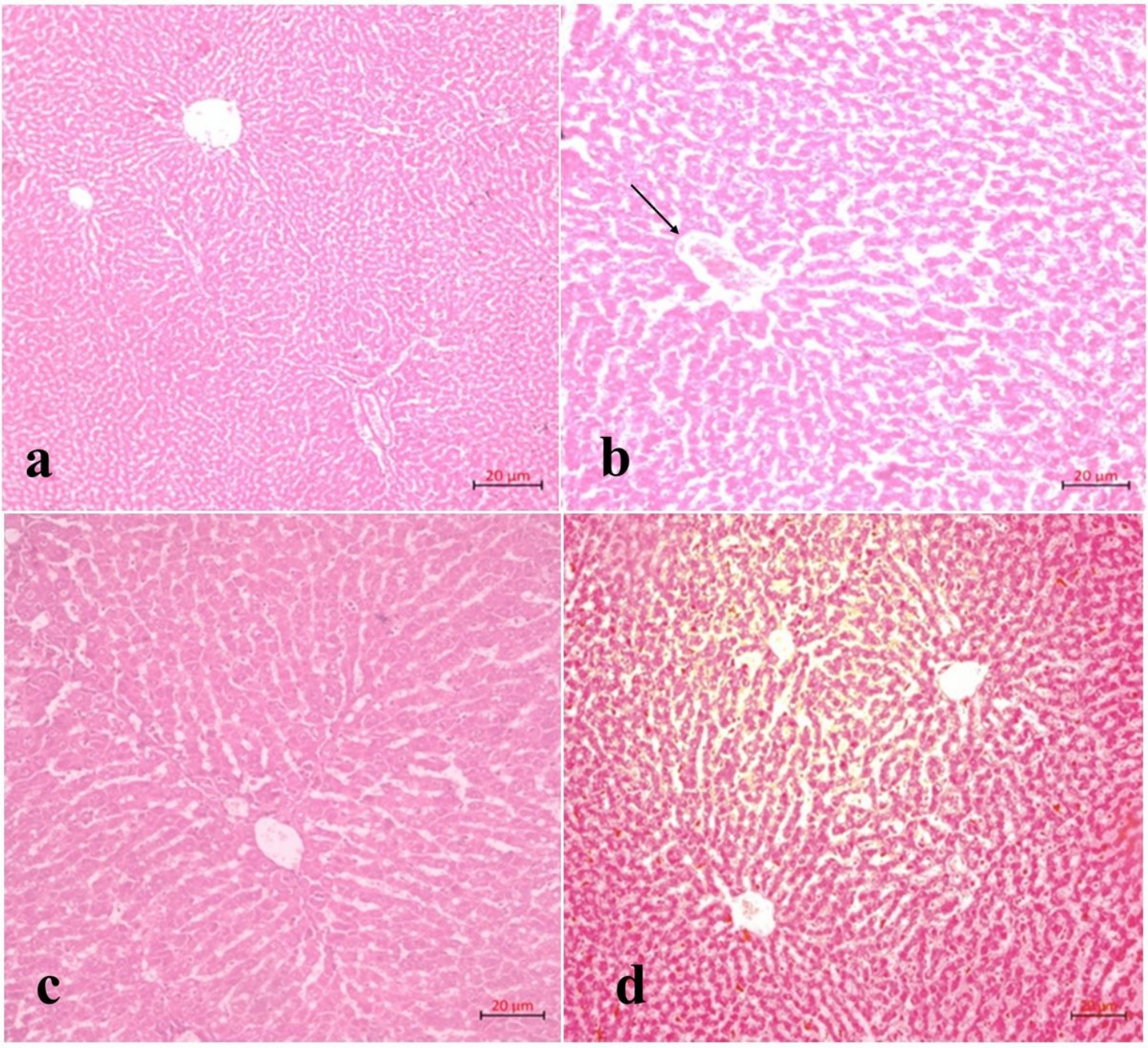

Figure 4

Liver sections of TAA-treated and Pestalopine-treated rats a- saline control (Group-I), b- TAA treatment (Group-II), c- treated with 300 mg/kg (body weight) EPS (Group-III), d- treated with 500 mg/kg (body weight) Pestalopine (Group-IV). The arrow indicates dilation of the central vein, necrotic lesion, and disintegration of hepatic structures. EPS treatment shows a partial recovery of hepatic cells. (20×, H&E stained).